Bacterial Antibiotic Resistance and Horizontal Gene Transfer in Slurries and Slurry Amended Agricultural Soils

A thesis submitted by

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DECLARATION

I hereby declare that the work described in this thesis was conducted by myself, under the supervision of Prof. Elizabeth Wellington, University of Warwick and Professor Peter Hawkey at the University of Birmingham; with the exception of those instances where the contribution of others has been specifically acknowledged. None of the information contained herein has been used in any previous application for a degree. All sources of information have been specifically acknowledged by means of reference.

Kathryne Byrne-Bailey

ABSTRACT

A two-year investigation was performed to assess the environmental impact of veterinary medicines released into the environment through the application of pig slurries to agricultural soils. Concern has been growing about the use of antibiotics in livestock husbandry and the possible selection and horizontal transfer of antibiotic resistance genes in bacteria.

The fate and mobility of sulphachloropyridazine, oxytetracycline and tylosin was measured, when added to soils via slurry. Plate counts and bacterial isolations were performed to calculate the numbers of antibiotic resistant culturable bacteria present in the soils before and after the application of slurry, from tylosin-fed pigs amended with sulphachloropyridazine and oxytetracycline. Soil cores were collected in years 1 and 2 after slurry application at days 1, 21, 90, 120 and 289 or 240 in year 2. The year 1, day 289 soil cores were also used as the pre-application samples for year 2. In total 583 bacterial isolates were collected from all slurries, soil time points and drainflow samples from non-selective Iso-Sensitest agar plates and selective plates containing a range of concentrations of sulphachloropyridazine, oxytetracycline and tylosin.

The 583 bacterial isolates were screened by PCR for the sulphonamide resistance genes; *sulI*, *sulII* and *sul3* and the *intI1* and *intI2* genes for the Class 1 and 2 integrons. PCR screening enabled an assessment of the prevalence and distribution of these mobile genetic elements and their potential and degree of horizontal gene transfer in the slurry and soil environments of this study, where the integrons were localised to a conjugative plasmid. PCR positive for the *intI1* gene were 5.83 % of bacterial isolates and 9.7 % were positive for the *intI2* gene. Of the *intI1* positive isolates, 57.56 % were collected from selective plates containing 50 μg/ml tylosin, suggesting a link between tylosin

resistance and Class 1 integrons. The *intI1* and *intI2* positive isolates were identified by 16S rRNA and a number of new bacterial genera encoding integrons were described. including a number of *Psychrobacter* spp. These integrase genes were also identified in number of putative Gram positive organisms including *Bacillus* sp. and *Arthrobacter* sp., providing evidence of horizontal gene transfer events from the *Enterobacteriacae* hosts commonly demonstrated to carry Class 1 and 2 integrons. Furthermore, the transfer of a plasmid with high similarity to that from *Corynebacterium glutamicum*, encoding a novel Class 1 integron, was demonstrated from an *Arthrobacter* sp. to *Pseudomonas putida* and *Escherichia coli* recipients.

Despite clinical studies confirming the *intI1* and *sulI* genes are commonly located together on a Class 1 integrons, this environmental study demonstrates no link between the two genes in cultured bacterial isolates from both antibiotic amended slurries and slurry amended soils.

The data presented in this study demonstrated there maybe a pool of antibiotic resistant bacteria, in the soil and pig slurry environments, which was detected both in the presence and absence of a selective pressure. Of the total number of isolates collected in this study, 31.05 % were PCR positive for one of the three known alternative DHPS genes conferring sulphonamide resistance. Of these 181 *sul* gene containing bacterial isolates (a number of which were identified by 16S rRNA), 12 encoded all three genes; *sulI*, *sulII* and *sul3*. These 12 isolates were characterised in detail, including the potential for transfer of the sulphonamide resistance genes. These 12 isolates belonged to the genera of *Acinetobacter*, *Psychrobacter* and *Bacillus*. No correlation was demonstrated between *sul* carriage and MIC levels for sulphachloropyridazine within

the 12 *sulI*, *II* and *3* positive isolates or within *Acinetobacter* sp. which were PCR positive for none, one or more *sul* genes.

Data is presented for the long-term survival of enteric bacteria in the soil environment as a result of release through slurry applications to the soils. A phenomenon was investigated in which MIC values were reduced through bacterial removal from soils and laboratory repeated subculture but increased on return back into soil microcosms.

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Abbreviations

1,P	Year 1, pre-application
16SrDNA	16S ribosomal DNA gene
16S rRNA	16S ribosomal RNA gene
ABC	ATP-binding cassettes
Amp	ampicillin
ANOVA	analysis of variance analysis
App.	Appendix
ARDRA	amplified rDNA restriction analysis
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
C-6	carbon atom number six
°C	degrees centigrade
Ca^{2+}	calcium cation
CCCP	carbonyl cyanide-*m*-chlorophenyl hydrazone
CDC	US Centre for Disease Control and Prevention
cfu	colony forming units
Cm	chloramphenicol
CS	conserved sequence
CTC	chlorotetracycline
$CuSO_4$	copper sulphate
DGGE	denaturing gradient gel electrophoresis
DHFR	dihydrofolate reductase
DHFS	dihydrofolate synthase
DHPS	dihydropterate synthase
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
ssDNA	single stranded DNA
DNase	deoxyribonuclease
dNTP	equimolar mixture of all 4 deoxynucleotide triphosphates
dw	distilled water
EDTA	ethylene diamine tetra-acetic acid
EEC	European Economic Community
ELISA	Enzyme-Linked Immunosorbent Assay
EMEA	European Agency for the Evaluation of Medicinal Products
EtBr	ethidum bromide
EU	European Union

FACS	Fluorescence-activated cell sorting
FDA	U.S. Food and Drug Administration
FeII	iron oxidised state
Gfp	green fluorescent protein
GMO	genetically manipulated organism
GTP	guanosine triphosphate
dH_2O	distilled water
H_2O	water
H_2O_2	hydrogen peroxide
h/hrs	hours of study
Ha	hectare
HCl	hydrochloric acid
HGT	horizontal gene transfer
HPLC	high pressure liquid chromatography
IR	inverted repeats
ICU	intensive care unit
IS	insertion sequence
Kan	kanamycin
Kb	kilo base
K_d	sorption coefficient
K_{OC}	organic carbon normalised sorption coefficient
LB	Lauria-Bertani
Leu^-	unable to metabolise leucine
MATE	multi-drug and toxic compound extrusion
MD	multiple drug
MF	major facilitator
Mg^{2+}	magnesium cation
$MgCl_2$	magnesium chloride
MIC	minimum inhibitory concentration
MIC(50)	minimum inhibitory concentration (50 % growth)
min	minutes
MINE	Minature Inverted-repeats Transposable elements
MLS	macrolide, lincosamide, streptogramin resistance
mRNA	messenger RNA
MRSA	methicillin resistant *Staphylcoccus aureus*
-ve	negative
NaCl	sodium chloride
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	dihydro-nicotinamide adenine dinucleotide phosphate
Nal	nalidixic acid
NCCL	U.S National Culture Collection
ND	not done
NEPA	US National Environmental Policy Act
NG	no growth
nm	nanometers
Nm	neomycin
NOAH	National Office of Animal Health (UK)

OC %	percentage of organic content
OD	optical density
OECD	Organisation for Economic Cooperation and Development
ORF	open reading frame
OTC	oxytetracycline
+ve	positive
PABA	ρ-amino-benzoic acid
PBS	phosphate buffered saline
PCA	plate count agar
PCR	polymerase chain reaction
PEG	polyethylene glycol
pers. comm.	personal communication
PF	pre-filter
PFLA	phospholipid fatty acid
PFGE	pulse field gel electrophoresis
ppm	parts per million
QAC	quaternary ammonium compounds
QRTPCR	quantitative reverse transcriptase PCR
R	resistant
RFU	relative fluorescence units
RFLP	restriction fragment length polymorphism
Rif	rifampicin
RISA	ribosomal intergenic spacer analysis
RIVM	Dutch National Institute of Public Health and the Environment
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
tRNA	transfer RNA
RND	Resistance-nodulation-division
RQ	resistance quotient (%)
RTPCR	reverse transcriptase PCR
Sm	streptomycin
SCP	sulphachloropyridazine
SD	standard deviation
sdw	sterile distilled water
sec	seconds
SMR	small multi-drug resistance
sp.	species one representative of
spp.	species, more than one representative of
SRL	*Shigella* Resistance Locus
Tet	tetracycline
t½	half-life
TAE	tris acetic acid EDTA
TBE	tris borate EDTA
TE	tris EDTA
TGGE	temperature gradient gel electrophoresis

Thi^-	unable to metabolise thiamine
Thr^-	unable to metabolise threonine
Tm	Trimethoprim
Tmax	maximum temperature
Tmin	minimum temperature
Tn	transposon
T-RFLP	terminal restriction fragment length polymorphism
Tris	tris-(hydroxymethyl)-methylamine
TY	tylosin
u	units of enzyme activity
UK	United Kingdom
US	United States
UV	ultra-violet
V	volts
w/v	weight per volume

CHAPTER 1:

INTRODUCTION

Chapter 1: INTRODUCTION

1.1 Brief Project Overview

The intensive use and misuse of antibiotics in human and veterinary medicine, as well as in animal production, has resulted in wide spread bacterial resistance (DuPont and Steele, 1987; Wegener, 2003). This elevated bacterial resistance has consequently increased awareness of the overuse and inappropriate chemotherapeutic practices in human medicines, improved research into the areas of monitoring and knowledge, as well as legislative measures world-wide to curb the use of antibiotics for prophylactic use in animal husbandry and in situations where those chemicals could enter the environment. Increased monitoring and understanding has led to concerns about a number of agricultural practises, namely the prophylactic use of antibiotics in animal husbandry and the application of slurry, from these animals, onto arable and livestock fields (DuPont and Steele, 1987).

Due to the raised awareness and concerns an EU directive 81/852/EEC was convened to investigate the environmental risk of certain veterinary medicines released into the environment through the spreading of manure, slurry and sludge. Cranfield University was involved in co-ordinating and participating in a European Framework V project to address this directive. The specific aims of their project were to study degradation, sorption and leaching of study compounds, assess the distribution of the compounds at field scale, as well as assessing their effects on soil fauna and flora. This study, performed at The University of Warwick, aimed to assess the effects of the study compounds on the soil bacterial population. Through risk assessment, including extensive literature reviews, to appraise persistence and toxicity in the environment, the

study compounds were oxytetracycline (OTC), sulphachloropyridazine (SCP) and tylosin (TY) (Boxall et al., 2003). These three compounds were antibiotics, from three different chemical groups, having different mechanisms of biological activity focusing on the first most consumed (of veterinary medicine, in the UK, in 2002); tetracycline (217 tonnes), the second most consumed; sulphonamides (88 tonnes) and the fifth; macrolides (56 tonnes). This equates to 48 %, 19 % and 12 % of total veterinary therapeutic usage in 2002 (NOAH, 2002).

During this study (which started in 1998), national and international guidelines and regulatory rules were established covering the usage of antimicrobial agents as growth promoters in animal husbandry (EMEA, 1997, 1999). In 2006 there were only four chemical compounds still licensed for use as prophylactic agents, none of which had any structural similarities with antimicrobial drugs used for human and veterinary medicine, although, in intensive agriculture, there is a fine distinction between chemotherapeutic and prophylactic use due to intensive rearing methods and close proximity of animals (Martel et al., 2001).

1.2 Antibiotics

Antibiotics are a diverse group of chemicals, naturally and synthetically produced which can have bacteriostatic and bactericidal activities. The term, coined by Selman Waksman, originally described only those formulations derived from living organisms, in contradistinction to "chemotherapeutic agents", which were purely synthetic (Waksman, 1941). Based on their functions and chemical structures antibiotics may be divided into a number of groups. The study compounds representing members of three groups; the sulphonamides (SCP), a macrolide; tylosin, and OTC which belongs to the antibiotic group; the polyketides.

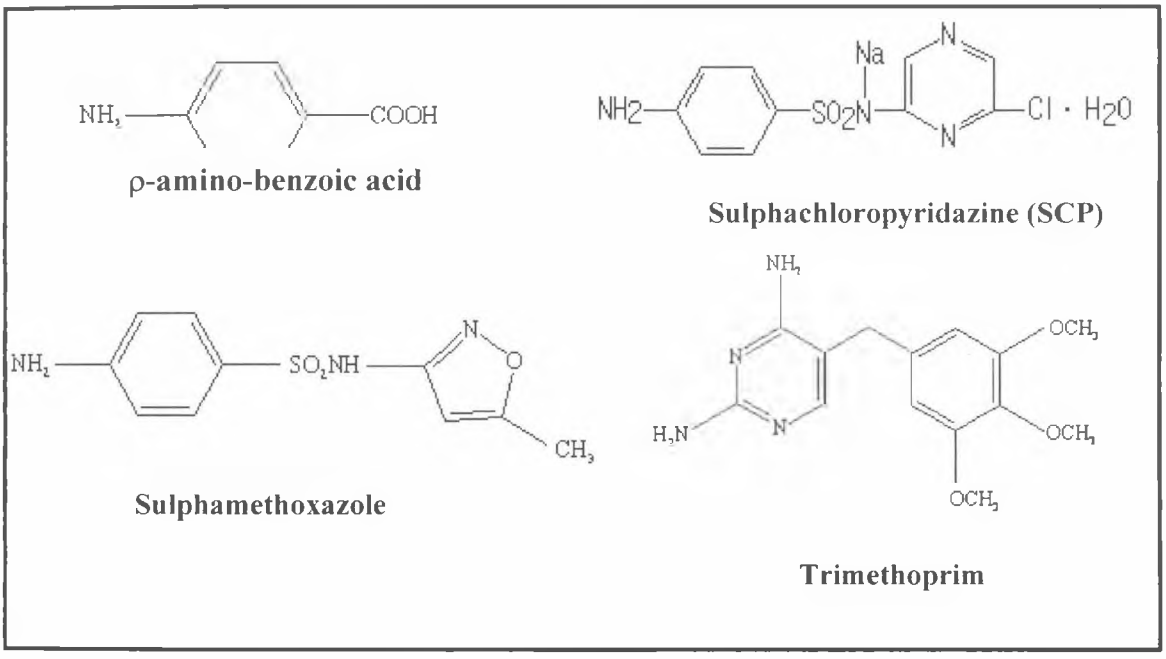
For several years the determining of the environmental fate and effects has been a legal requirement, in the US, for the licensing of human drugs. This process began with the Food and Drug Administration (FDA) issuing a National Environmental Policy Act (NEPA) in 1985 for human pharmaceuticals and in March 1987, the FDA published its' Environmental Technical Handbook for environmental assessment. Finally in 1995, the FDA issued guidance for industry requiring the submission of an environmental assessment in human drug applications, the rules did not go as far as covering veterinary drugs (Halling-Sørensen et al., 1998).

In Europe legislation for pharmaceuticals was initiated at the beginning of the 1990's, distinguishing between medical substances which do and do not contain genetically modified organisms (GMOs) and included an environmental dimension, meaning all new draft EU laws would be assessed for potential environmental impact. The two groups were further divided into veterinary and human medicinal products (Halling-Sørensen et al., 1998). Following the ban of all food animal growth promoting antibiotics by Sweden in 1986, the EU banned avoparcin in 1997 and bacitracin, spiramycin, tylosin and virginiamycin in 1999 (Casewell et al., 2003).

1.2.1 Sulphachloropyridazine

The sulphonamides are synthetic antibiotics, first produced in 1932 and put into clinical use in 1935 (Sköld, 2000). Sulphonamides are the fifth most widely used group of veterinary antibiotics in the world and the second in the UK (Ungemach, 2000). These synthetic compounds are structural analogues of ρ-amino-benzoic acid (PABA) and thus compete for binding to dihydropteroate synthase (DHPS), a catalytic enzyme in the folic acid biosynthesis pathway, inhibiting the formation of folate and ultimately

Fig. 1.1 Chemical formulas for ρ-aminobenzoic acid, sulphachloropyridazine, sulphamethoxazole and trimethoprim.

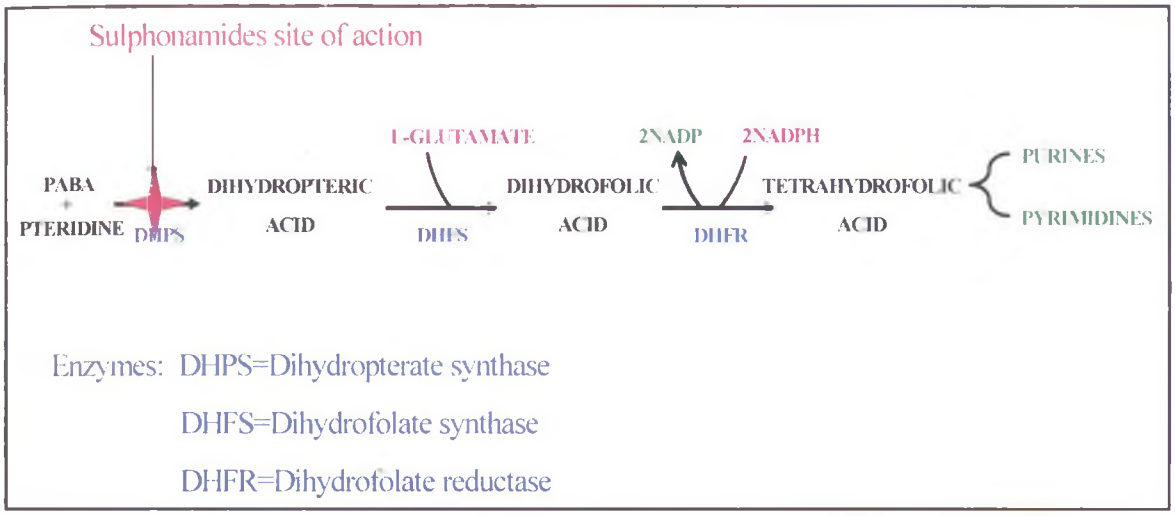


These chemical formulas demonstrate the structural similarities related to competitive inhibition of PABA, and the structure of trimethoprim which is commonly used in combination with sulphamethoxazole (Sköld, 2000).

the cells synthesis of purines and pyrimidines (Figs. 1.1 and 1.2) (Sköld, 2000). There are a number of structurally different sulphonamides, including the medium long-acting sulphamethoxazole, sulphadiazine and SCP which is a nitrogen substituted derivative of sulphanilamide (Fig. 1.1).

Sulphonamides are bacteriostatic, taking a number of generations for the folate pool in the bacteria to decrease. They are commonly used in combination with trimethoprim, as Co-trimoxazole. Co-trimoxazole (sulphamethoxazole and trimethoprim in a 5:1 weight

Fig. 1.2 The Folate synthesis pathway is inhibited by sulphonamide antibiotics



The sulphonamide class of antibiotics are structural analogues of PABA and therefore compete for binding with the DHPS enzyme, preventing production of folic acid and purines and pyrimidines (Sköld, 2000).

to weight ratio) has been used in treatments of human bacterial infections due to its synergistic activity (Bushby and Hitchings, 1968). Both drugs effect the same pathway, trimethoprim inhibits dihydrofolate reductase (DHFR), which catalyses the formation of tetrahydrofolate from dihydrofolate and although the steps of inhibition follow one another this is not thought to be the reason for synergy (Fig. 1.2) (Bushby, 1975).

Regardless of the synergistic effect, the drug combination has proved effective among pathogenic bacteria due to their different bacterial spectrums and resistance profile (Huovinen et al., 1995).

Sulphonamides and trimethoprim both have broad antibacterial spectrums, effective against Gram negative rods including members of the Enterobacteriacae family, although *Proteus mirabilis* and *Enterobacter* spp. are less susceptible (Burman, 1986).

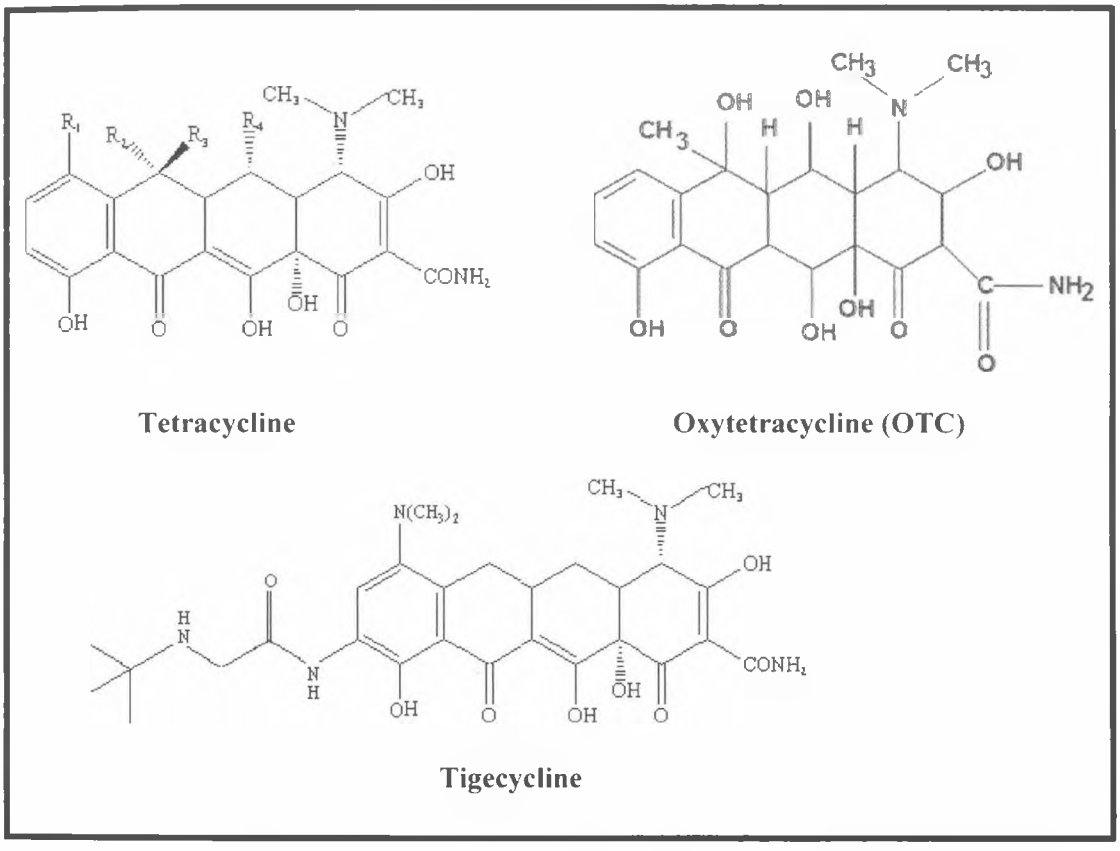
The importance of sulphonamides has decreased over a number of decades as a result of both increasing resistance and the emergence of newer, less toxic alternatives (Sköld, 2000).

After sulphonamide treatment, animals excrete an unaltered parent compound or non-active acetic acid conjugates, causing concern that large volumes of biological active sulphonamides are entering the environment and damaging the indigenous flora and fauna, hence their inclusion in this study (Langhammer, 1989).

1.2.2 Oxytetracycline

The tetracycline group of broad spectrum antibiotics are polyketide compounds based around four benzene rings (Fig. 1.3) (Chopra and Roberts, 2001). The first of which, tetracycline, was developed for use in 1955 (Roberts, 1996). Naturally, these polyketides are produced by *Streptomyces rimosus*, its biosynthesis being driven by an operon containing a ketosynthase, ketoreductase/cyclase/aromatase and a C-6 hydroxylase (Hranueli et al., 1999). New generations of semi-synthetic tetracyclines such as tigecycline, were developed for their effectiveness against methicillin resistant *Staphylococcus aureus* (Petersen et al., 1999). Tetracyclines mode of action is to inhibit protein synthesis by the prevention of amino acyl-tRNA entering the acceptor sites in the 30S ribosome, where one molecule per ribosome is sufficient for inhibition (Roberts, 1996). The binding is reversible hence tetracyclines' bacteriostatic activity. The chemical properties of tetracyclines mean this antibiotic group is capable of binding to soil and slurry matrices, persisting in the environment as a result, hence its inclusion as a study compound (Hamscher et al., 2002). This persistence will be discussed in more detail in Chapter 3.

Fig. 1.3 Chemical structures of tetracycline, tigecycline and oxytetracycline.



Tetracycline was the first of the group to be discovered, whereas tigecycline is a newly introduced synthetic member, but all are based around four benzene rings (Chopra and Roberts, 2001).

Tetracyclines are broad-spectrum antibiotics, effective against a wide variety of bacteria including; *Brucella*, *Chlamydia*, *Rickettsia, Vibrio cholera, Bacillus anthracis*, *Helicobacter pylori, Yersinia pestis, H. influenzae, S. pneumoniae, Mycoplasma pneumoniae, Neisseria gonorrhoeae*, and many others especially in patients allergic to β-lactams and macrolides (Roberts, 1996). However, their use, as with the sulphonamides, has decreased due to widespread resistance development in the disease causative organisms.

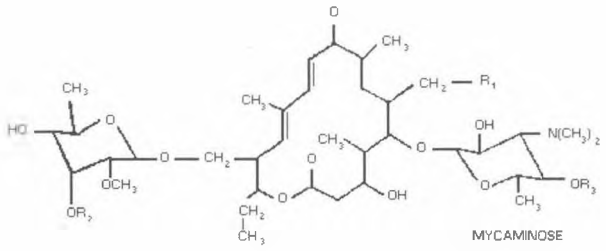
1.2.3 Tylosin

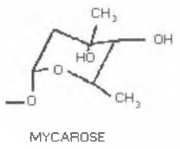
Tylosin belongs to the macrolide group of antibiotics, which are defined by their lactone structure with rings of more than 10 C-atoms. Tylosin consists of a mixture of tylosin A, B, C and D, where A is the major component of the four (approximately 90 % and not less than 80 %) and the most widely studied of the four composed of a 16-membered macrolide ring (Fig. 1.4) (Loke et al., 2000). Its mode of action is to block translation during protein synthesis by binding reversibly to the 50S ribosomal subunit, thereby preventing transfer of the peptidyl-tRNA from the acceptor site to the promoter site (Gaynor and Mankin, 2003). The macrolides are also known to block elongation by preventing peptidyltransferase from forming peptide bonds between amino acids (Douthwalte et al., 1995).

Tylosin is produced naturally by a number of *Streptomyces* species, in which its biosynthetic pathway has recently been elucidated (Fouces, 1999). The biosynthetic gene cluster for tylosin was found in the tylosin producer, *Streptomyces fradiae,* and contains 43 genes, 4 of which are resistance genes (Fig. 1.5) (Fouces et al., 1999; Stratigopoulos et al., 2004). The 43 genes, regulatory, structural as well as resistance, form an operon of 85 Kb in length which takes up 1 % of *S. fradiae*'s genome (Cundliffe et al., 2001).

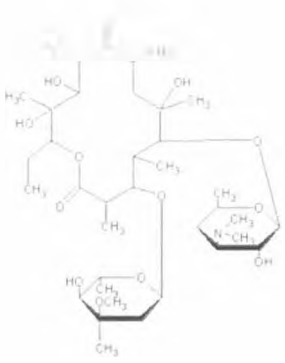
Tylosin is also a bacteriostatic antibiotic, now only licensed for use in the treatment of infection in animals, it is effective against Gram positive bacteria, some Gram negative bacteria (excluding coliforms), *Mycoplasma* spp., β-haemolytic *Streptococcal* spp, a number of spirochetes and is commonly used in pigs to treat *Treponema hyodysenteriae*, pig dysentery, prevented previously by tylosin's prophylactic use as a feed additive which also enhanced food utilisation (Casewell et al., 2003).

Fig. 1.4 Chemical structure of tylosin compounds and erythromycin, macrolide antibiotics.





Tylosin mixture of compounds

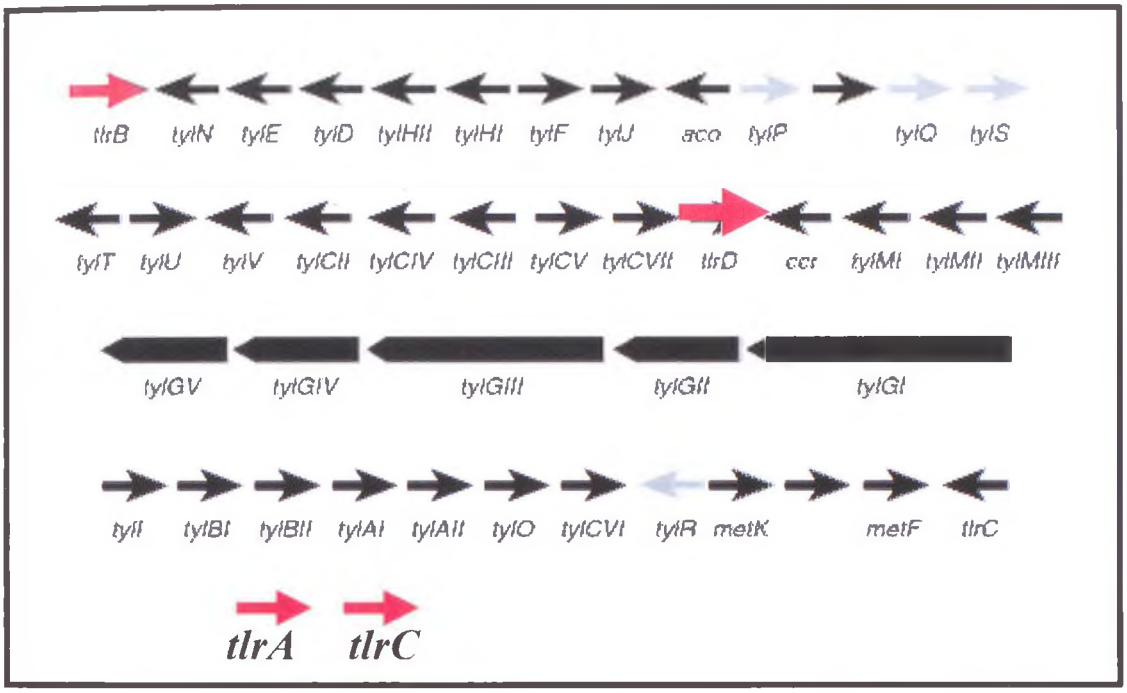


Erythromycin

COMPOUND	R1	R2	R3
TYLOSIN (A)	-CHO	$-CH_2$	MYCAROSE
DESMYCOSIN (B)	-CHO	$-CH_2$	-H
MACROSIN (C)	-CHO	-H	MYCAROSE
RELOMYCIN (D)	$-CH_2OH$	$-CH_2$	MYCAROSE
DIHYDRODESMYCOSIN (A)	$-CH_2OH$	$-CH_2$	-H

Tylosin's constituent compounds displaying a 16-membered ring compared to the structure of erythromycin (and a large majority of the other macrolides members) has a 14-membered ring (Retsema and Fu, 2001).

Fig. 1.5 Tylosin biosynthetic gene cluster from *Streptomyces fradiae*



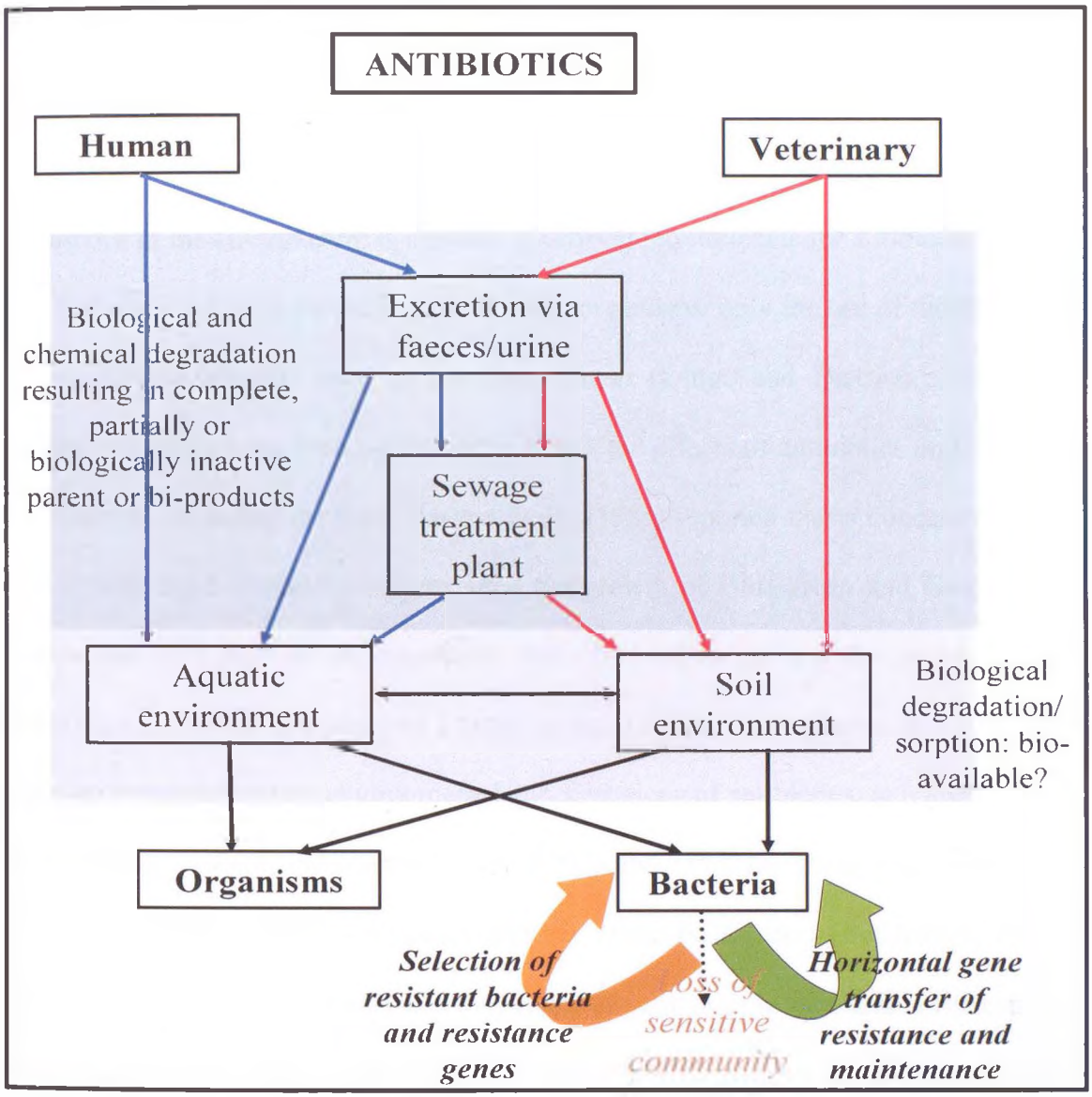
The tylosin biosynthetic cluster contains 4 resistance genes; *tlrA (ermSF), tlrB (rlmA), tlrC, tlrD (ermN)* (arrows in red). Names designated in brackets renaming by Roberts et al., (1999). Grey arrows are regulatory genes and black arrows are structural genes. Adapted from Stratigopoulos et al., (2004).

1.2.4 Environmental impact of antibiotics

Antibiotics can enter the environment via solid or liquid passage which can include direct application via accidental spillage or indirectly, for example, by hospital waste (Kummerer, 2001), dumping in landfill sites (Holme et al., 1995), water contamination by human/animal treated and untreated waste if not biodegraded and application of untreated sewage onto agricultural land (Rooklidge, 2004). Fig. 1.6 highlights these entrance routes, of antibiotics, to the environment and their fates and effects dependent on these routes, many of which are now being studied, especially since many of them, including SCP, OTC and tylosin, are largely released as unaltered parent compounds, or as active by-products of degradation by unknown pathways (Rang and Dale, 1991). Due to the tonnage of antibiotics used, there is an increasing risk that substantial quantities will end up in the environment. Calculations have indicated that 70-80 % of chemicals used in fish farms end up in the environment, with substantial concentrations being reported in the sediments underneath these farms (Samuelsen et al., 1992).

Antibiotics have been detected in a diverse number of environments; sulphonamides were detected in waste from the pharmaceutical industry in a landfill site by Holme et al., (1995), as well as in eggs where the drug was administered in drinking water to the animals (Roudaut and Garnier, 2002) and in rivers, reservoirs and ground-waters (Battaglin et al., 2000; Richardson and Bowron, 1985). OTC was reported in sediments underneath fish farms (Samuelsen et al., 1992) and soils where manures have been applied, as has tylosin (De Liguoro et al., 2003; Halling-Sørensen et al., 2005). Tylosin was also recorded in dust on a pig fattening farm, highlighting the possible passage of both the antibiotic and resistant bacteria directly into the lungs of humans due to pollution in the environment (Hamscher et al., 2003).

Fig. 1.6 Potential release routes, fates and effects of both veterinary and human pharmaceuticals in the environment.



To follow the fate and effects of antibiotics, they can be sub-divided into human and veterinary products, and their release followed depending on whether it is directly or indirectly released through sewage treatment works or ecosystems. Its fate and effects depend on a number of processes, displayed above. The effects on the bacteria also can be observed; selection of resistant pheno/genotypes, transfer of resistant genes but also the death and loss of sensitive strains.

The fate of a number of the drugs has been studied, with findings indicating that biodegradation, sorption to soils, hydrolysis and photo-degradation represent the main sinks for the antibiotics and their bi-products (Halling-Sørensen et al., 1998). Sorption and biodegradation of antibiotics will be discussed in more detail in Chapter 3.

Assessment of the effects of antibiotics where they are detected and are found to persistence in the environment is needed. Licensing requirements for antibiotic toxicity does not require tests to be performed on microorganisms, only the use of model plants or crustaceans/copepods such as *Daphnia magna* (Lange and Dietrich, 2002). A number of studies have been performed to assess the effects of antibiotics on a number of organisms including bacteria; Harrass et al., (1985) reported that a concentration of 0.09 to 0.86 mg/l streptomycin prevented the growth of Blue-green and Green algal species and 2.03 mg/l of metronidazole was observed to prevent the growth of the Green alga *Chlorella* in a study by Lanzky et al., (1997). Plant growth has also been observed to be effected by environmental concentrations of antibiotics; at a level of 160 ppm in cattle manure, chlorotetracycline (CTC) and OTC (at lower concentrations), brought about the death of *Phaseolus vulgaris* (pinto bean plant) (Batchelder, 1981). Many more studies have investigated the effects of antibiotics in the model organism *D. magna* and *Artemia satina*. The toxicity of sulphadimethoxine, aminosidine, bacitracin, erythromycin and lincomycin have all been reported in these organisms and are of particular relevance as three of these four antibiotics have been used in the past as prophylactic agents on intensive farms (Brambilla et al., 1994; Dojimi de Delupis et al., 1992). The above studies concluded that antibiotics have a broader effect on organisms other than microbial when they are present in sufficient quantities in the environment, through a number of routes. It is commonly agreed that the extent of usage, subsequent

concentrations and bioavailability of antibiotic substances in the environment are closely related to the development of antibiotic resistance due to the selective pressure they exert on the bacterial population and will be discussed in more detail below.

1.2.5 Bioavailability of antibiotics in the environment

The bioavailability of antibiotics is subject to a number of processes dependent on a number of factors linked to either the antibiotics' chemical structure or the soil matrix. The chemical structure of an antibiotic will dictate its sorption or transport through soils, its sorption coefficient based on this structure and experimental evidence indicative of its persistence and binding properties (see Chapter 3 for more details) (Ingerslev and Halling-Sørensen, 2000). If an antibiotic such as OTC or tylosin has been observed to persist in a soil environment, the question arises as to whether these chemicals remain biologically active under these conditions and therefore may have an effect on the surrounding micro-flora (Hansen and Sørensen, 2001; Ingerslev and Halling-Sørensen, 2000). Bioavailability is difficult to measure in soils but the development of bio-sensors may allow accurate quantification of concentrations of antibiotics which remain active in the soil environment (Hansen and Sørensen, 2001). Chander et al., (2005) reported that tetracycline and tylosin both retained their antimicrobial properties in respect to *Salmonella* spp. seeded in clay and loamy sand soils, with higher activities observed in the clay soils due to an increased binding of the antibiotics and therefore higher concentrations persisting indicating bioavailability. A whole cell bio-sensor *Escherichia coli* strain has been used to study bioavailable CTC and the active amounts excreted in pig faeces, observing that despite decreasing measurable concentrations of the antibiotic over time due to sorption, its activity remained unchanged, although bioavailability will only occur in the absence or partial

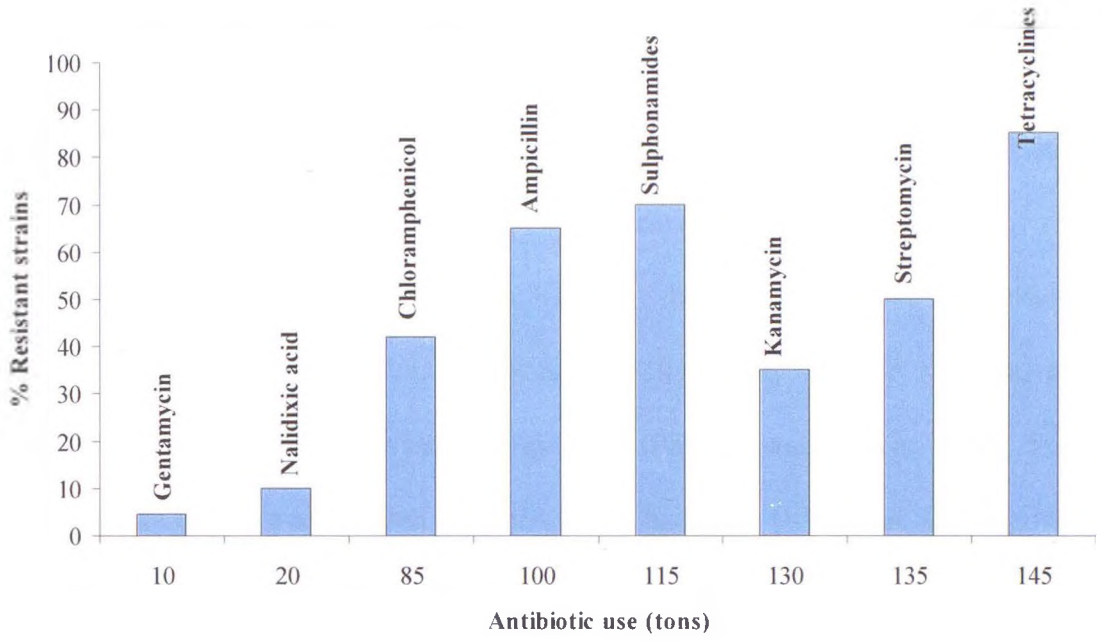
bio-degradation of the antibiotic in question (Hansen et al., 2002; Ingerslev and Halling-Sørensen, 2001). In contrast, Sumano et al., (2004) reported that in the case of enrofloxacin, with increased binding with increased hardness of waters, there was decreased bioavailability. These studies therefore concluded that bioavailability of antibiotics may vary based on the chemical structure of the antibiotics and nature of the environment.

1.3 Antibiotic resistance

It is now commonly agreed that the extent of usage, subsequent concentrations and bioavailability of antibiotic substances in the environment are closely related to the development of antibiotic resistance and there are increasing data to support this dogma. The diagram below was drawn using data from the Centre for Disease Control and Prevention (CDC) in the US and correlates the emergence of antibiotic resistance in bacteria with usage of these agents (Fig. 1.7) (Nwosu, 2001).

The concern over using antimicrobial agents began as early as the 1960s, when penicillin and tetracycline were banned as growth promoters due to the increasing resistance found in *Salmonella* isolated from young calves (Swann, 1969). To date, there are reports of bacterial resistance to every antibiotic introduced by man, whether synthetic or natural, despite the development of new generations of drugs. This bacterial resistance coupled with spiralling production and drug development costs has led to a down turn in new antimicrobials, although there are still companies which see monetary and social benefits in continuing research in the area of antimicrobial drugs (Bush, 2004; Projan and Shlaes, 2004).

Fig. 1.7 **Relationship between antibiotic use and increase in resistant bacteria.**



The data represented displays a strong correlation between the quantity of antibiotic used and the percentage of resistant strains, except where the number of resistant strains were higher for kanamycin and streptomycin at a lower usage than ampicillin and sulphonamides. This differential ability of antibiotics to induce resistance may be related to the ease of transfer of resistance genes under increased antibiotic selective pressure. Adapted from Nwosu et al., (2001).

1.3.1 Antibiotic resistance mechanisms

There are a number of different mechanisms attributed to bacterial resistance of antibiotics, including specific mechanisms which differ for each antimicrobial group and non-specific, conferring resistance to a large multitude of drugs. Bacterial antibiotic resistance can be intrinsic or acquired. Intrinsic resistance is commonly chromosomal and includes mechanisms such as cell wall permeability, efflux (specific

and multi-drug), avoidance via bio-film formation, anaerobic growth and antibiotic inactivation.

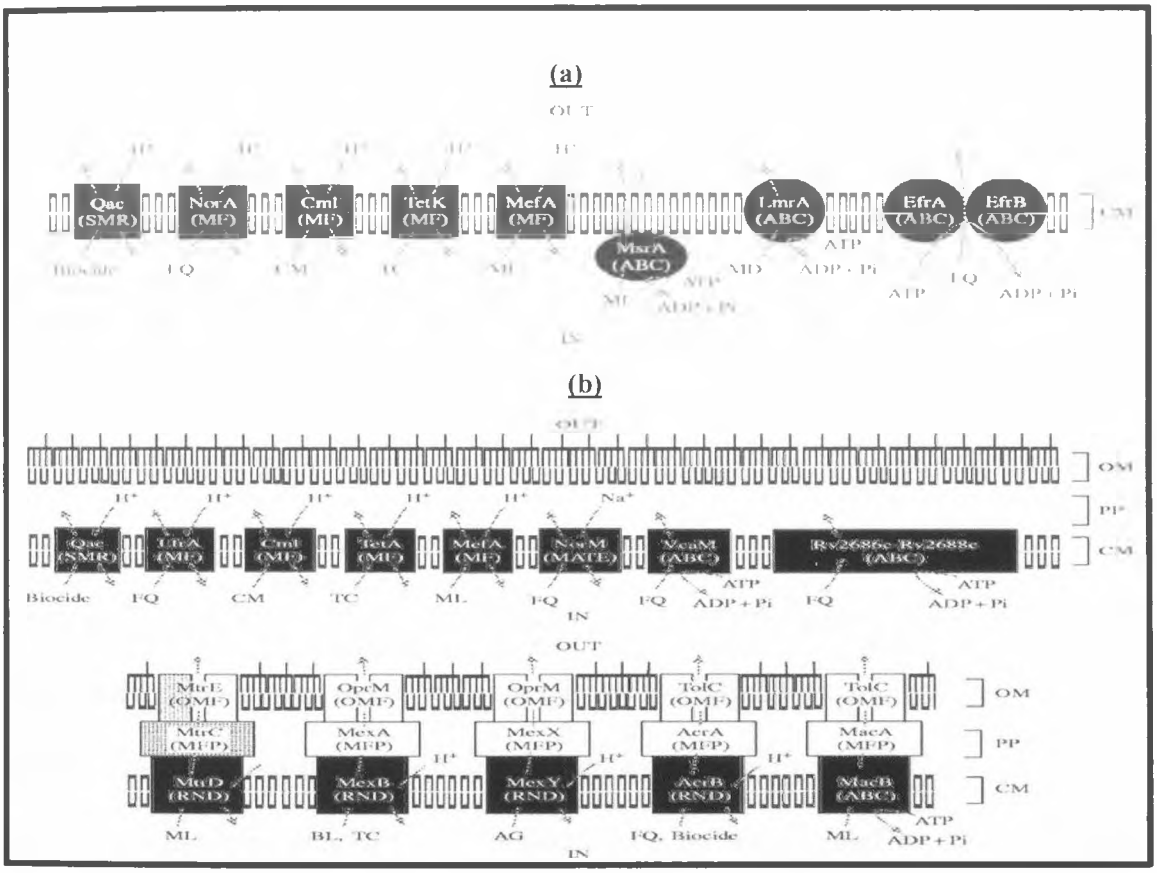
Bacterial efflux pumps confer resistance to their hosts by the extrusion of antibiotics from the cell through channels in the membrane (Van Bambeke et al., 2000). Bacterial efflux systems are generally grouped into five classes; the major facilitator (MF) superfamily, the ATP-binding cassettes (ABC), the resistance-nodulation-division (RND), the small multi-drug (MD) resistance (SMR) family and the MD and toxic compound extrusion (MATE) system (Fig. 1.8) (Paulsen and Lewis, 2001; Poole, 2004b, 2005). Efflux pumps such as NorA (MF) and Lmr (ABC) are Gram positive specific providing resistance against fluoroquinolones and MD respectively, or Gram negative-specific such as TetA (MF) for tetracyclines and AcrB (RND) a MD transporter, whereas the MefA (MF) efflux pump for macrolides and chloramphenicol can be found in either (Fig. 1.8) (Butaye et al., 2003; Poole, 2005).

Bacterial resistance can also be acquired from extracellular DNA, giving rise to mechanisms such as efflux, as described above, enzyme inactivation/modification of the antibiotic and modification or alternative target sites.

1.3.2 Antibiotic resistance mechanisms for sulphonamides

Resistance to the sulphonamide group of antibiotics emerged soon after their clinical introduction in 1935. Resistance to sulphonamides is mediated by 3 mechanisms: efflux pumps, mutations or recombination changes in the chromosomal DHPS gene (*folP*) and acquired mechanisms which encode for alternative DHPS enzymes with an altered binding site and a lower affinity for the antibiotic group (Swedberg et al., 1993).

Fig. 1.8 Efflux pumps in (a) Gram positive bacterial cell wall and (b) the outer membrane and inner membrane of a Gram negative cell.



Examples of MD-resistance membrane transporters in Gram positive and Gram negative bacteria. The NorA and Lmr proteins are highlighted on the Gram positive membrane (a) and the TetA (outer) and AcrB (inner) pumps can be observed on the Gram negative membranes (b). The MefA pumps can be observed in both membranes. Taken from Poole, (2005).

Köhler et al., (1996) demonstrated that the MD resistance efflux system encoded for by the *mexABoprM* genes was responsible for intrinsic resistance to sulphonamides, as well as trimethoprim, tetracyclines and macrolides in *Pseudomonas aeruginosa.*

Clinical isolates of *E. coli* (Dallas et al., 1992), *S. aureus* (Hampele et al., 1997), *H. influenzae* (Enne et al., 2002), *Mycobacterium leprae* (Williams et al., 2000) and *Campylobacter jejuni* (Gibreel and Sköld, 1999), among others, have been found with single base mutations in their chromosomal *folP* gene. Sulphonamide resistance in *S. pneumoniae* was found to be conferred by a 6 bp repeat of 2 amino acids in the *folP* gene altering the tertiary structure of the DHPS enzyme (Padayachee and Klugman, 1999). HGT has been implicated in the acquisition and exchange of *folP* gene mutations within *Neisseria* spp. conferring sulphonamide resistance, for example; a 6 bp insert in the *folP* gene in *N. meningitidis*, results in a 10 % difference in the sequence from susceptible strains and a resistance to $\geq$0.5 mM sulphonamides (Fermer et al., 1995). This insertion is thought to have occurred due to a recombination event rather than a mutation and although sulphonamides are no longer used to treat meningitis caused by *Neisseria* spp., resistance is still prevalent in clinical strains due to this genetic alteration (Huovinen et al., 1995).

Acquired resistance mechanisms confers a greater protection to sulphonamides than chromosomal mutations but this may in part be counteracted by a fitness cost (Gibreel and Sköld, 1999). Chromosomal mutations in *S. pneumonia* were reported to have a fitness cost on the organism (Haasum et al., 2001). This was also the case in a study by Enne et al., (2004) in which a plasmid with acquired sulphonamide resistance exerted a fitness cost upon its host, therefore conflicting with Gibreel and Sköld's predictions.

There have been 3 identified acquired genes which encode for alternative DHPS enzymes; *sulI*, *sulII* and *sul3* (Perreten and Boerlin, 2003; Rädström and Swedberg, 1988; Sundström et al., 1991). The *sulI* and *sulII* genes from *E. coli* share 57 % DNA identity, and their origins remain unknown as their sequences are distinct from all the known chromosomal DHPS genes (Rädström and Swedberg, 1988). The *sulI* gene was identified on the transposon, Tn*21*, in a highly conserved region designated a Class 1 integron (Sundström et al., 1991). The *sulII* gene is frequently found on plasmids in Gram negative bacteria and was first described on the IncQ plasmid RSF1010 (Rädström and Swedberg, 1988). The third acquired gene, *sul3*, was identified in sulphonamide resistant *E. coli* isolated from pigs in Switzerland (Perreten and Boerlin, 2003). The gene was encoded on a 54 Kb conjugative plasmid, pVP440, flanked by insertion sequences, IS*15*Δ/26. The name sul3 has been inappropriately used for a DHPS gene found in *M. fortuitum* and *Corynebacterium striatum* (GenBank accession no. AJ294721), in these cases the protein has high homology to that of the *sulI* gene product, with the exception of four additional amino acids at its N terminus (Huovinen et al., 1995; Martin et al., 1990).

1.3.3 Antibiotic resistance mechanisms to tetracyclines

Three characterised resistance mechanisms are employed by bacteria to fight the tetracycline group of antibiotics; efflux, ribosomal protection and enzyme inactivation (Chopra and Roberts, 2001). At the last major review of tetracycline resistance genes, 37 had been identified to encode proteins for the three above mechanisms (Roberts, 2005) and 1 gene, *tet*(U), did not appear to be related to the other proteins encoding an unknown mechanism (Ridenhour et al., 1996). The tetracycline efflux proteins are the most well studies of the resistance mechanisms and belong to the MFS (Paulsen et al.,

1996). The genes encoding efflux pumps, 23 at the last count, have been identified in Gram negative and Gram positive bacteria, and include *tet(A)-tet(D)* and *tet(G)-tet(H)*. The Gram negative efflux genes are commonly associated with large plasmids, mostly conjugative, encoding a host of other antibiotic resistance and heavy metal resistance genes (Chopra and Roberts, 2001). For example, pCCK3259, the *tetL* encoding plasmid from *Mannheimia glucosida* (Kehrenberg et al., 2005) which bore similarity to the *tet(B)* carrying tetracycline resistance plasmid, pHS-tet from *Haemophilus parasuis* (Lancashire et al., 2005).

Ribosomal protection proteins have been found to be encoded by 11 different genes to date (Roberts, 2005). These cytoplasmic proteins protect the cell from the antibiotic, in the case of by Tet(M), this is possibly either by its actions on an elongation factor protein, EF-G (Dantley et al., 1998) or blocks/aiding dissociation of tetracycline binding to the ribosome in a GTP dependent manner (Burdett, 1996).

As apposed to ribosomal protection, only 3 genes encoding for enzymatic protection against tetracycline have been identified (Roberts, 2005). All 3 genes, *tet*(X) identified on a transposon in *Bacteroides* strains (Speer et al., 1991), *tet*(34) from *V. cholerae* (Nonaka and Suzuki, 2002) and tet(37) (Diaz-Torres et al., 2003) encode for an oxidoreductase which confers resistance by modifying tetracycline to an inactive form in the presence of oxygen and NADPH (Speer et al., 1991). The Tet(34) protein encodes for oxytetracycline resistance (Nonaka and Suzuki, 2002), as do the *otr(A)* (Doyle et al., 1991), *otr(B)* (McMurry and Levy, 1998) and *otr(C)* genes (Roberts, 2005), of which B and C encode for efflux proteins and A for ribosomal protection.

1.3.4 Antibiotic resistance mechanisms to tylosin

Tylosin resistance is not necessarily determined by resistance genes conferring protection to the macrolide family of antibiotics, particularly those conferring resistance to the 14 and 15-membered rings. The biosynthetic gene cluster for tylosin was characterised in the tylosin producer, *S. fradiae,* and contains 43 genes, 4 of which are resistance genes (Fig. 1.5) (Fouces et al., 1999; Stratigopoulos et al., 2004). The four resistance genes have not been found outside of the producer but share homology to the Erm family of methlytransferases, hence their re-naming in 1999 (Roberts et al., 1999). The producer resistance genes, *rlmA* and *ermN* methylate the amino acids G748 and A2058 (numbering according to the *E. coli* system) of the 23S rRNA, in synergy, to give resistance from tylosin (Liu and Douthwaite, 2002). Mutations in the 23S rRNA commonly confer resistance to macrolides but only the A2062C mutation in spirochetes has been reported to confer resistance to tylosin (Prapasarakul et al., 2003).

A further number of non-cluster methlytransferases have been observed to confer tylosin resistance, for example; *ermB* and *ermX* identified in *Arcanobacteria pyogenes* (Jost et al., 2003; Jost et al., 2004). The *ermC* gene has been observed to confer tylosin resistance in *S. aureus* strains (Lodder et al., 1997), the *ermT* gene was reported in *Lactobacillus reuteri* tylosin resistant strains (Whitehead and Cotta, 2001) and the *ermAMR* gene operon was recorded to provide 5-fold inducible tylosin resistance identified in *Enterococcus faecalis* (Oh et al., 1998).

Efflux pumps have also been reported to confer resistance to tylosin. One such mechanism, encoded by the *CmeABC* genes, was observed to act in synergy with the 23SrRNA mutation A2075G in *Camplylobacter coli* drastically increasing the MIC for tylosin and erythromycin (Cagliero et al., 2005). Resistance has also been reported to

be conferred by enzyme modification genes; *msrC* in *E. faecium* to give an MIC of 16 μg/ml (Singh et al., 2001), and *mphA, B, K* and *C* in *E. coli* which inactivate tylosin molecules by phosphorylation (Roberts et al., 1999).

1.3.5 Origins of antibiotic resistance

The origins of antibiotic resistance are still unclear; one possibility due to the prevalence of antimicrobial producers in natural (e.g. soil) environments is the evolution of resistance genes originating from these organisms. A second possibility being the development of drug-specific efflux mechanisms arising from earlier, as yet undefined, non-drug exporters via mutation and selective pressure (Sheridan and Chopra, 1991). There is much debate in the literature regarding the natural function of MD efflux systems, with evidence for induction of these systems by drugs exported providing support for proposed roles in protection against these drugs. It is also possible, that the action of these substances on their targets, ribosomes for example, induces expression of the efflux system as a result of accumulation of cellular products, whose export is performed by this efflux system (Grkovic et al., 2002; Jeannot et al., 2005). Furthermore, there are studies which have observed that antimicrobial export may not be the intended function of exporters, for example, *Pseudomonas aeruginosa* encodes genes for 11 or more RND pumps, of which seven transport many of the same antimicrobials, each appearing to be independently regulated by linked genes but not (with one exception) in response to antibiotics, implying a distinct function independent of antimicrobial efflux (Poole, 2004a).

1.4 Dissemination and mobilisation of antibiotic resistance

1.4.1 Horizontal gene transfer

The disseminations and mobilisation of antibiotic resistance occurs by release of DNA encoding antibiotic resistance determinates through host natural release or cell death and recipient uptake by transformation in naturally competent bacteria (estimated to occur in 1 % of bacterial species) (Lorenz and Wackernagel, 1994), through cell to cell transfer via conjugation using pili or via bacteriophage transduction (Nielsen and Townsend, 2004). The process of cell to cell transfer of DNA via conjugation has been designated Horizontal Gene Transfer (HGT) and was first proposed in the 1950's, prior to the discovery of plasmids in the 1960's (Bennett, 1999). Sequence analysis and comparison of whole genomes has revealed that significant portions consist of horizontally acquired genes (Jain et al., 1999). The uptake of DNA by transformation, conjugation or transduction also requires its integration and in the case of plasmids; replication and self maintenance of the DNA in the new recipient. Mutation and HGT continually give rise to new bacterial genotypes, infrequently such bacterial genotypes become established and spread in the larger population through either selective pressures or random genetic drift (Hall and Collis, 1995).

The transfer, uptake and stabilisation of foreign DNA acquired by bacteria are limited by a number of factors, hence decreased transfer rates. These include the stability of DNA in the environment (Romanowski et al., 1992), limits on competent recipients and on the host range of the transfer and maintenance mechanisms of mobile genetic elements (Anthony et al., 1994; Grohmann et al., 2003), recipient restriction enzyme activity, and limited ability for homologous recombination of foreign DNA (Palmen and Hellingwerf, 1997). For homologous recombination, depending on the system, evidence

has been collected observing that the foreign DNA must contain regions of between 25 to 200 bp in length with at least 25 to 30 % similarity to the recipient's genome (Palmen and Hellingwerf, 1997).

Studies have resolved the basic biological mechanisms involved in HGT, but there is a lack of understanding when it comes to the environmental variables involved. There is a need to develop a quantitative and qualitative understanding of ongoing gene transfer processes occurring in the environment (Thomas and Nielsen, 2005). The importance of environmental conditions on DNA preservation and degradation has been demonstrated in studies where ancient DNA, in conserved specimens that are estimated to be several million years old, have been detected and experiments have revealed that DNA is not immediately degraded upon entering a soil environment, persisting for hours to days, as measured in natural transformation assays (Hofreiter et al., 2001; Nielsen et al., 1997; Romanowski et al., 1992).

The environment itself has been reported to effect transfer rates in the environment, studies observe different transfer rates in different soils, Schwaner et al., (2001) recorded higher transfer rates in rhizospheres than in bulk soils in investigations of conjugal transfer for a RP4 plasmid between *P. fluorescens* AS12 and *Serratia plymuthica* RF7 and one study has observed that HGT occurred and created new genotypes in a number of species without the selective pressure of antibiotics in the 'pre-antibiotic' era (Hughes and Datta, 1983; Lebaron et al., 1997). The application of manures to soils has also been observed to increase transfer rates in a study by Götz and Smalla (1997) into plasmid mobilisation and survival in *P. putida.* Bale et al., (1988) reported that in river systems, transfer rates were dependent on host to recipient ratios and not river water temperature, calculating rates of between 2.2×10^{-1} and 2.5×10^{-6}

per recipient for ratios of 489/1 and 0.0047/1. The process of HGT has been used in a number of studies to investigate the elements involved, isolating mobile elements and cassettes from the environment (Coleman and Holmes, 2005; Smalla et al., 2000b).

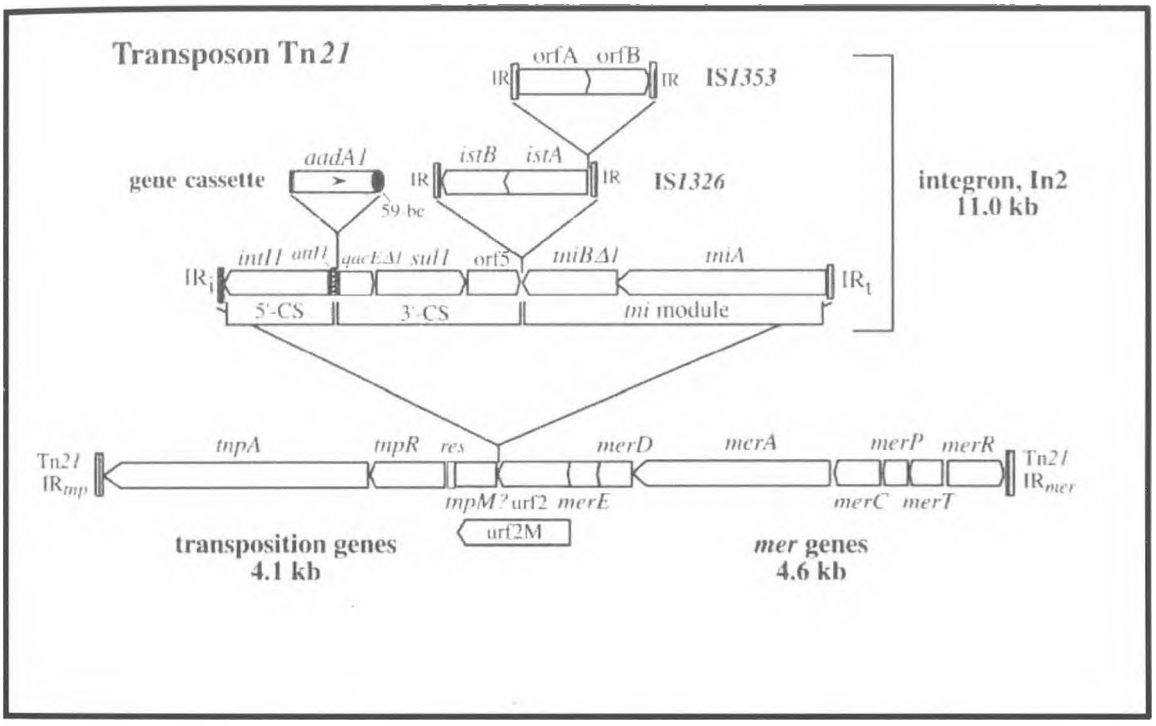
1.4.2 Mobile Genetic elements

HGT as well as involving the uptake of naked DNA has been reported to involve self-transmissible elements, mobile genetic elements such as; integrons and super integrons, transposons, plasmids, gene cassettes and bacteriophage, representing flexibility in the bacterial gene pool and represents a large potential for genetic exchange and variability within the bacterial communities (Hall and Collis, 1995). These mobile elements are commonly preserved in the bacterial genome due to their selective advantages, for example antibiotic resistance, but have been observed to persist in the absence of a selective pressure (Enne et al., 2001).

1.4.2.1 Transposons

Transposons were first discovered in maize by Barbara McClintock in the 1940's, whose work on which earned her the Nobel prize in 1983 (Comfort, 2001). There are three classes of transposons; retrotransposons (class I), replicative transposons (class II) and class III, the Minature Inverted-repeats Transposable Elements (MINEs). Only the class II transposons are commonly found in bacteria and are the only class to be discussed here.

Fig. 1.9 Example of a transposon: Tn*21* Structure



Tn*21* consists of a transposition (*tnp*) region, a Class I integron, and the *mer* operon. Vertical bars indicate flanking IR of the transposon and IS. The *tnp* region consists of; transposase (*tnpA*), a resolvase (*tnpR*), the putative transposition regulator (*tnpM*), and the resolution site (*res*). The integron encodes; an integrase gene (*intI1*), an *attI1* insertion site, *aadA1* gene cassette, a *qacE*Δ1 gene, *sul1* and an ORF (orf5) of unknown function. The arrow indicates the direction of transcription. The 3′-CS includes two IS (IS*1353* is inserted into IS*1326*). The mercury resistance (*mer*) operon consists of the regulatory genes *merR* and *merD* and the structural genes *merT*, *merP*, *merC*, and *merA*. There are two unknown reading frames, urf1 (also called *merE*) and urf2, downstream of *merD*. urf2M is a hypothetical gene which may have existed before integron insertion. Taken from Liebert et al., (1999).

Transposons are segments of DNA which can insert into bacterial genomes, consisting of; one or more transposase and resolvase proteins, inverted repeats (IR) at both ends usually of 15 to 40 bp in length and IS elements with homology to the target sequence (Fig. 1.9) (Liebert et al., 1999). Transposition of a transposon, for example Tn*21*, is carried out by the transposase, TnpA and involves specific recognition of and binding to the IR of Tn*21* by TnpA, which then mediates joining of the donor and recipient replicons (Fig. 1.9) (Grinsted et al., 1990; Liebert et al., 1999; Plasterk, 1995). This co-integrate intermediate contains a copy of Tn*21* and is resolved by the action of the *tnpR* gene product, a resolvase which acts at a specific site (*res*) adjacent to the *tnpR* gene (Rogowsky et al., 1985). The resolvase catalyzes site-specific recombination between super-coiled DNA and two directly repeated copies of the transposon DNA (Stark et al., 1989).

As displayed in Fig 1.9, transposons can carry genes with functions other than self-transfer, such as the *mer* operon which encodes mercury resistance, and can carry other mobile genetic elements such as integrons, thus enhancing their selective advantage in a bacterial population (Barrineau et al., 1984; Bennett, 1999; Liebert et al., 1999). Transposons can also be carried on plasmids, allowing an even wider dissemination (Nakaya et al., 1960).

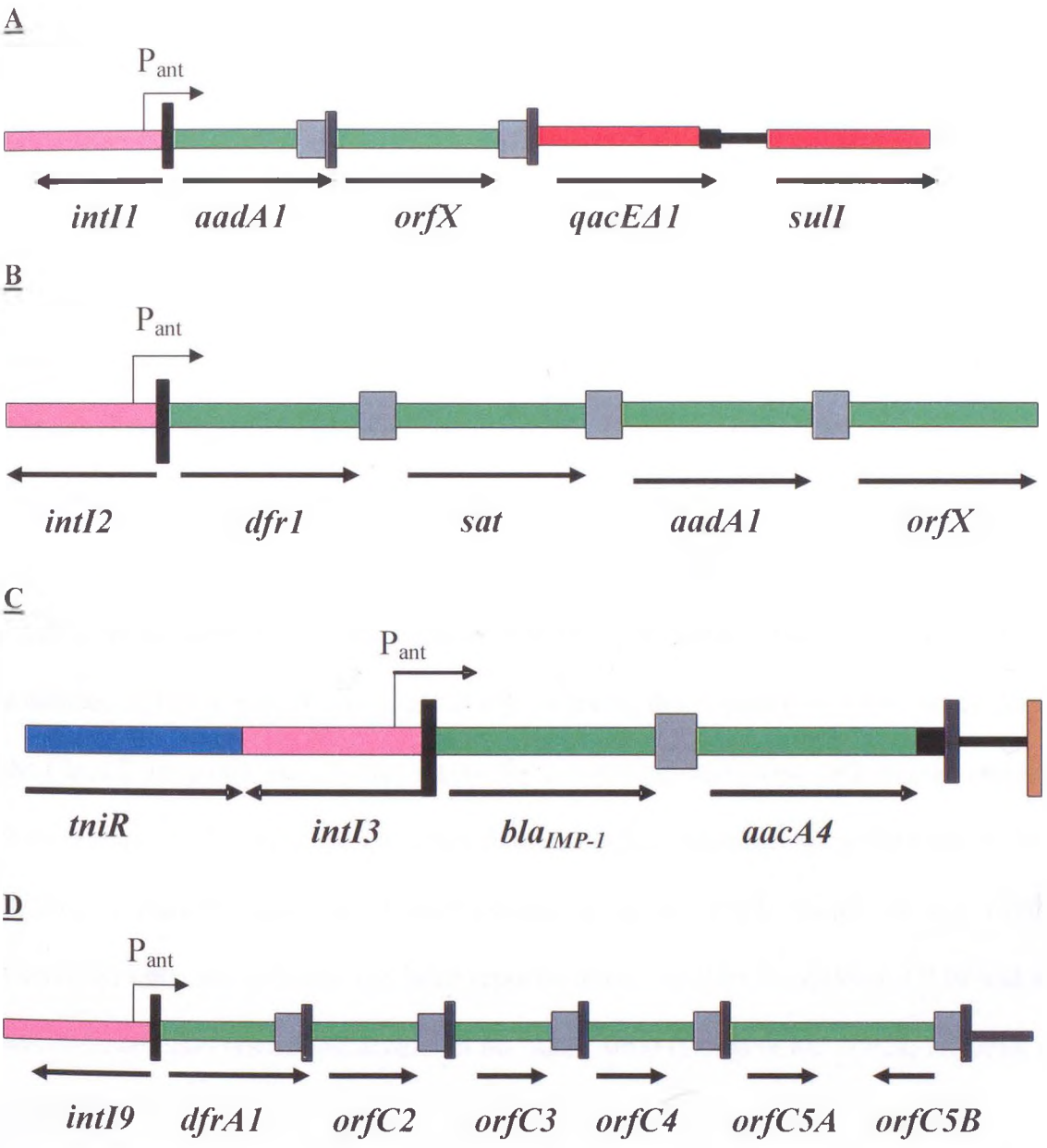
Transposons are widespread in both the environment and the clinical setting worldwide and across the bacterial genera of Gram positive and Gram negative bacteria, disseminating with them a wide range of antibiotic resistance genes, such as the metallo-β-lactamases in Gram negative bacteria (Osborn et al., 1997; Walsh, 2005).

1.4.2.2 Integrons

Integrons are mobile genetic elements that potentially have the ability to move if flanked by IR or present on secondary mobile elements such as transposons, as discussed above (Liebert et al., 1999). Integrons also contain gene cassettes that can be mobilised to other integrons or to secondary sites in the bacterial genome. The present day definition of an integron was coined by Hall and Collis (1995). An integron structure at the 5' end includes the gene for a site-specific recombinase, belonging to the integrase (Int) and tyrosine recombinase super-family, and a site-specific recombination site (*attI*) that is a receptor for gene cassettes, allowing their integration (Fig. 1.10) (Hall and Collis, 1995; Nunes-Duby et al., 1998).

Gene cassettes are themselves mobile elements consisting of one coding sequence, flanked on their 3' end by a 59 bp element which can be of variable length (Bennett, 1999). The 59 bp element contains imperfect IR with two 7 bp core regions, integration takes place between the 59 bp element and the *attI1* site due to the enzymatic action of the integrase (Hall and Collis, 1995). Up to at least 10 cassettes may be present in an integron leading to multiple resistance and co-selection (Fluit and Schmitz, 1999). The majority of gene cassettes are antibiotic resistance determinates conferring resistance to all known antibiotics including the β-lactamases, aminoglycosides, trimethoprim and chloramphenicol, with new cassettes continually being described (Stokes and Hall, 1989; White et al., 2001). Most gene cassettes encode for resistance to antibiotics which have been in use for a long time, but cassettes also exist on integrons to newer antibiotics such as extended-spectrum-β-lactamases (Poirel et al., 2000). Numerous different combinations of gene cassettes have been reported as integration occurs at random and useful new combinations arise which can

Fig. 1.10 Examples of integron structures; (A) Class 1 integron from Tn*21* (Bennett, 1999), (B) Class 2 integron from Tn*7* (Hansson et al., 2002), (C) Class 3 integron (Arakawa et al., 1995) and (D) Class 9 integron (Hochhut et al., 2001).



The grey boxes represent *attc* sites including a 59 bp element, *attI* sites are indicated by black rectangles, IR by brown rectangles. The blue rectangles: Tn genes, pink: integrase genes, red: integron backbone genes and green: gene cassettes.

then be selected for by environmental conditions (Bennett, 1999).

Expression of the integron relies on its promoter, P_{ANT}, often encoding P1 and/or P2, although some gene cassette have been described which encode their own promoter sequence (Collis and Hall, 1995; Stokes and Hall, 1991). Expression levels will vary depending on the promoter sequences and the transcript levels, heightened with proximity to the promoter (Collis and Hall, 1995).

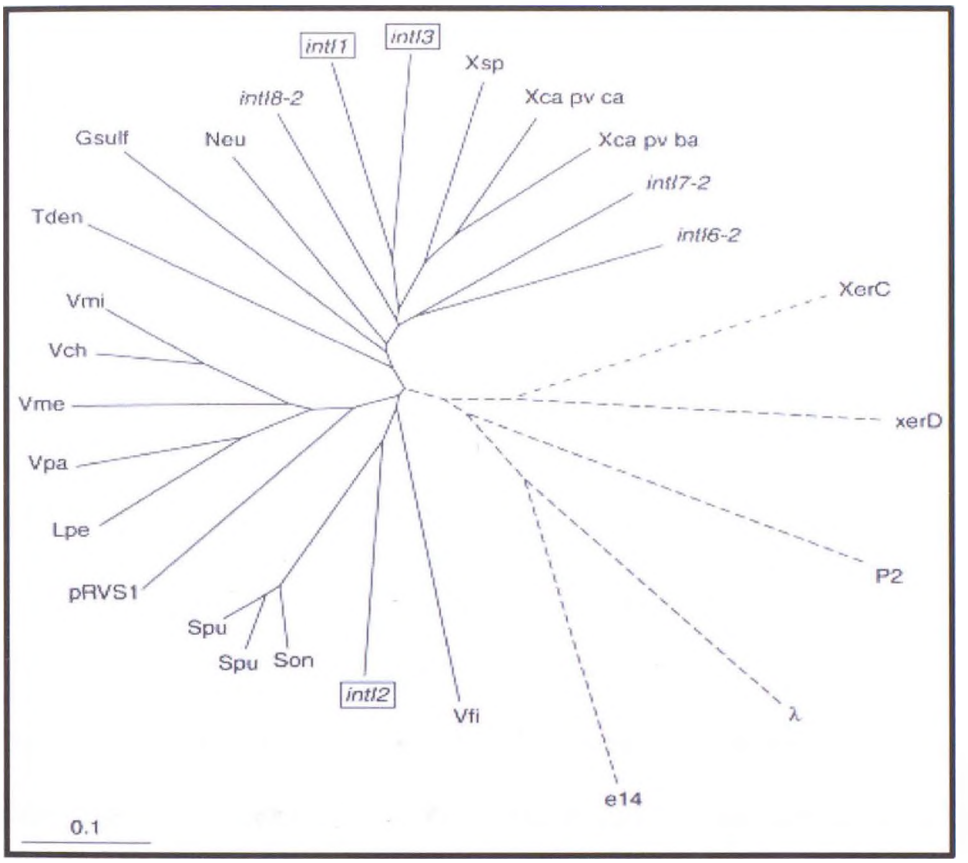
To date six distinct classes of antibiotic resistance encoding integrons have been described each with its own integrase, although large numbers of new integrases have been sequenced so the number of integron classes could rise steeply when these are characterised (Fig. 1.10) (Arakawa et al., 1995; Bennett, 1999; Hansson et al., 2002; Hochhut et al., 2001; Nield et al., 2001). The number assigned to each class corresponds to the *intI* gene and its corresponding IntI integrase (Fig. 1.10). Class 1 and Class 2 are the most well characterised and the most frequent, Class 4 has been found in a number of *Vibrio* spp. (Clark et al., 2000). Class 3, the structure of which is similar to the Class 2 integrons, encodes resistance for a metallo-β-lactamase carbapenem and has been found in *S. marcescens*, *Pseudomonas* spp., *Alcaligenes xylosoxidans* and *Klebsiella pneumoniae* (Fig. 1.10c) (Arakawa et al., 1995; Senda et al., 1996). Currently, only one example has been reported for Class 9 in *V. cholerae* O139 and an unnumbered class (GenBank accession no. AJ277063) (Collis et al., 2002a; Hochhut et al., 2001).

The majority of known integrons belong to the Class 1 family, Tn*21* in origin which are associated with the *sulI* gene, encoding for sulphonamide resistance (Fig. 1.10a) (Martinez and de la Cruz, 1990; Sundström et al., 1988). Class 1 integrons are widespread in *Enterobacteriacae* (White et al., 2001), but have also been reported in

many other Gram negative genera including *Acinetobacter* (Nordmann and Poirel, 2002), *Campylobacter* (Gibreel and Sköld, 1999), *Pseudomonas* (Schnabel and Jones, 1999) and *Vibrio* (Dalsgaard et al., 1999). There have been a number of reports of their detection in Gram positive bacteria; on a tetracycline resistance plasmid in *Corynebacterium glutamicum* (Tauch et al., 2002), the chromosome of *Mycobacterium smegmatis* and a resistance plasmid in *E. faecalis*, although in this study an *aadA* gene (encoding aminoglycoside resistance) was characterised and no integrase was sequenced but inferred due to the presence of a 59 bp element (Clark et al., 1999; Martin et al., 1990). A study into integrons, in poultry litter found over 85 % of positive isolates were from the Gram positive genera *Staphylococcus* and *Corynebacterium* (Nandi et al., 2004).

Class 2 integrons are found within non-replicative Tn*7* transposons (Fig. 1.10b) (Hansson et al., 2002). Tn*7* inserts into unique sites in bacterial chromosomes from which it is transferred to other bacterial cells and onto conjugative plasmids (Wolkow et al., 1996). The *intI2* gene sequence contains a termination codon which experimental data has demonstrated not to prevent gene cassette insertion into class 2 integrons but cells may initiate integration by either encoding a further integrase down stream, using a different integrase such as IntI1 encoded on another integron in the cell or by the use of an unknown mechanism to change the termination codon into a sense codon (Hansson et al., 2002). Class 2 integrons have been found in *Acinetobacter* (Gonzalez et al., 1998), *Shigella* (McIver et al., 2002) and *Salmonella* (Orman et al., 2002), among others.

Fig. 1.11 Phylogenetic relationship derived from integron gene sequences, *intI* among the Proteobacteria. Taken from Rowe-Magnus et al., (2001).



Unrooted dendrogram based on known *intI* gene sequences generated using Phylip. Three integrases are boxed and super-integrons are shown by organism abbreviation: Vch, *Vibrio cholerae*; Vmi, *V. mimicus*; Vme, *V. metschnikovii*; Vpa, *V .parahaemolyticus*; Vfi, *V. fischeri*; Lpe, *Listonella pelgagia*; Son, *Shewanella oneidensis*; Spu, *S. putrefaciens*; Xca pv ca, *Xanthomonas campestris* pathovar *campestris*; Xca pv ba, *X. campestris* pathovar *badrii;* Xsp, *X.* species; Neu, *Nitrosomonas europaea*. Sources of *intI6-2*, *intI7-2*, *intI8-2* and the *intI* genes of the plasmid pRVS1 (GenBank accession no. AJ277063) are unknown. Classic

representatives of the tyrosine recombinase family (dashed lines) are integrases of phage λ, P2, e 14 (an *E .coli* lambdoid phage) and the XerC and XerD recombinases of *E.coli*. The scale bar represents 10% divergence at the nucleotide level (Rowe-Magnus et al., 2001).

The Class 4 integrons, re-designated super-integrons, can contain arrays of 179 gene cassettes, only a few of which encode for antibiotic resistance (Clark et al., 2000; Rowe-Magnus et al., 2001). The super-integrons are species specific and have been reported in the chromosomes of several diverse species belonging to the Proteobacteria (Fluit and Schmitz, 1999), including *V. cholerae* (Clark et al., 2000), pseudomonads (Vaisvila et al., 2001) and *Geobacter sulfurreducens*, as well as *T. denticola*, a spirochaete (Roberts et al., 1996).

The origin of gene cassettes is unclear, a model does exist for the evolution of diverse and separate classes of integrons but it does not account for the lack distribution for some of the classes and the insertion of a number of gene cassettes with reverse orientations and those with their own promoters (Fluit and Schmitz, 1999; Recchia and Hall, 1995; Rowe-Magnus and Mazel, 2002). It is evident from sequence data for Class 1 and 2 integrons that a number of gene cassettes can transfer between different classes, for example the *aadA1* and *dfrA1* gene cassettes (Fig. 1.10) (Collis and Hall, 1995). There is evidence to suggest that integrons may have evolved from the larger chromosomal super-integrons in that all integron/integrases group together phylogenetically forming a special clade within the tyrosine recombinase family (Fig. 1.11) (Rowe-Magnus et al., 2001).

1.4.2.3 Plasmids

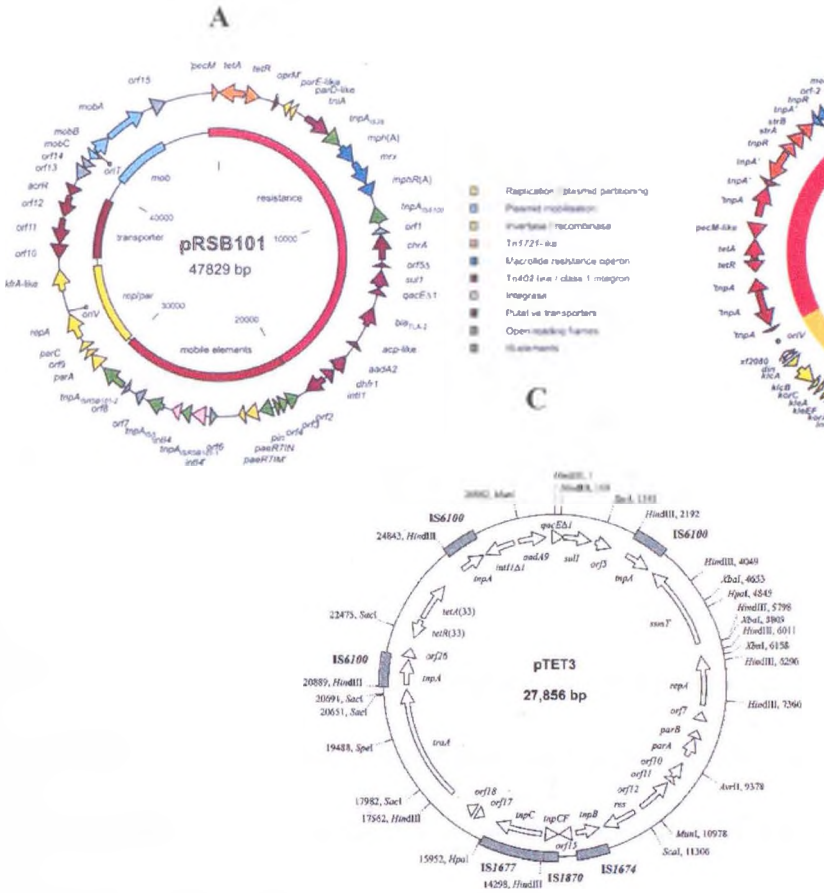
Plasmids are circular, double stranded, mobile DNA elements. They have the ability to either integrate onto a recipient chromosome or to self-replicate in the recipient due to the encoding of specific genes within a replication region on the plasmid DNA (Fig. 1.12). Naturally occurring plasmids commonly encode ancillary genes for antibiotic resistance (R-plasmids) (Schluter et al., 2003), heavy metal resistance (Tauch et al., 2002), virulence (Sirard et al., 2000), toxin production (Shukla and Sharma, 2005) and metabolic genes for the degradation of a whole host of chemicals (Eaton, 2001; Igloi and Brandsch, 2003). Genes required for replication and transfer include the *tra* genes (transfer), *rep* (replication) and an *ori*, for example, *oriT* the origin of transfer for rolling-replication (Kramer et al., 1997). Resistance plasmids such as those belonging to the IncP and IncQ groups, both highly promiscuous plasmids in a large number of bacterial genera including the group of *Enterobacteriacae,* their host range determined by their ability to form a productive junction with the surfaces of various cell types (Frey and Bagdasarian, 1989; Rawlings and Tietze, 2001; Sakai and Komano, 1996). IncP, for example, can transfer to both Gram negative bacteria and Gram positive bacteria, although the transfer efficiency of IncP plasmids can be dramatically effected by the identity of the donor, Bingle et al., (2003) observed that the plasmid RK2 transfer between *E. coli* strains or from *E. coli* to *P. putida* was lower than transfer between *P. putida* strains, its original host (Adamczyk and Jagura-Burdzy, 2003; Margesin and Schinner, 1997).

Many plasmids are not self-transmissible, but can nevertheless be mobilized from one bacterium to another in the presence of a self-transmissible plasmid, normally involving specific interactions of mobilisation proteins encoded by the plasmid and assembly at

the transfer origin to form a relaxosome, with the mating pair apparatus formed by the conjugative plasmid (Lanka and Wilkins, 1995). The broad host range and successful dissemination of IncQ plasmids like RSF1010, as well as retaining the potential for spread due to mobilisation, also encode three replication proteins; RepA, RepB and RepC, thereby self-encoding the necessary machinery for replication, whereas IncP plasmids carry just one *rep* gene and require the recruitment of the recipient helicase for replication, slowing this process and therefore copy numbers are lower (Adamczyk and Jagura-Burdzy, 2003; Frey and Bagdasarian, 1989; Thomas and Nielsen, 2005).

Plasmids belonging to the IncP and IncQ groups, have been found in diverse environments, including slurries, soils, waste water treatment plants, as well as the clinical environment (Götz et al., 1996; Normark and Normark, 2002; Schlüter et al., 2003). Examples of IncP and IncQ, isolated from the environment are, pRSB101 and pB10, respectively, both characterised from a waste water treatment site with Gram negative bacteria host ranges (Fig. 1.12) (Schlüter et al., 2003; Szczepanowski et al., 2004). Both encode multiple antibiotic resistance determinants and other mobile elements such as transposons and integrons. The pRSB101 plasmid, the smaller of the two at 47 829 bp in length, encodes 8 resistance determinates for spectinomycin, streptomycin, trimethoprim, nalidixic acid, roxythromycin, sulphonamides, cephalosporins, norfloxacin and erythromycin, with a number of these determinates carried on a Class I integron within a Tn*403*-like transposon (Fig. 1.12a) (Szczepanowski et al., 2004). The pB10 plasmids, is 65 508 bp in length, encoding amoxicillin, streptomycin, sulphonamide, tetracycline as well as mercury resistance, carried on three different transposons and a Class I integron (Fig. 1.12b) (Schlüter et al., 2003). The R-plasmid, pTet3, is an example of a narrow host range element, isolated

Fig. 1.12 Environmental plasmids with broad and narrow host ranges encoding antibiotic resistance determinates (A) pRSB101 and (B) pB10 and (c) pTet3.



(A) pRSB101. Arrows indicate the direction of transcription of coding regions are. Black circles mark the oriV and oriT. The backbone of the plasmid consists of a replication/partitioning module (*rep/par* – yellow) and a mobilization module (*mob* – blue). The 'genetic load' of the plasmid is composed of a 20 Kb resistance region (magenta), a region containing different mobile genetic elements (red) and a region encoding a tripartite MDR transporter (brown). The colour code for the different gene categories present on pRSB101 is given on the right (Szczepanowski et al., 2004). (B) pB10. The different functional modules of the plasmid backbone are presented in different colours: conjugative transfer genes, green; genes for regulation of plasmid functions and stable inheritance, yellow; replication, grey. Class 1 integron (light yellow), a Tn*501*-like mercury-resistance (*mer*) transposon (dark blue), streptomycin resistance transposon (orange), IS*1071* (brown) and a tetracycline-resistance transposon (red). Genes of unknown function and origin are drawn in white (Schlüter et al., 2003). (C) R-plasmid pTET3 (Tauch et al., 2002). Arrows of ORFs, some of which encode *tet(33)*, *tet(R)*, *aadA9* and *sulI* genes for antibiotic resistance. The position of IS are shown by boxes.

only from the Gram positive bacterium, *C. glutamicum* (Tauch et al., 2002). It is 27.8 Kb in total, with a resistance region carrying a transposon and Class I integron encoding resistance to tetracycline, aminoglycosides and sulphonamides. Other plasmids within *C. glutamicum* have been found to carry resistance determinates for streptomycin, spectinomycin and chloramphenicol, displaying a wide diversity of resistance genes which span the bacterial division of Gram negative and Gram positive bacterial cell walls (Katsumata et al., 1984; Na et al., 1994; Tauch et al., 2002).

1.4.2.4 Bacteriophage

Bacteriophages are viruses that infect, parasitize or kill bacteria, temperate phage have two life stages, lytic and lysogenic, within which transduction of phage DNA is integrated into the bacterial chromosome as a prophage and there is increasing evidence that phage have a significant impact on gene transfer in bacterial communities (Canchaya et al., 2003a). Phage-mediated gene transfer can be read from published bacterial genome sequences, for example; two-thirds of the sequenced low GC Gram positive bacteria and Gammaproteobacteria contained identifiable prophages, up to 16 % of the chromosomal DNA from *E. coli* 0157 strain Sakai was reported to be phage DNA, encoding 18 prophages (Canchaya et al., 2003b; Ohnishi et al., 2001).

Bacteriophage which encode their own integrase genes are capable of integrating into recipient bacterial chromosomes at att*x* sites without cofactors and imprecise excision in the lytic phase can take small flanking regions of the recipients DNA and deposit them in a new recipients DNA, this has been observed in the genomes of low GC content Gram positive bacteria, containing regions of high GC content (Desiere et al., 2001; Ferretti et al., 2001). In cases where whole genes are transferred, these have been observed to encode for virulence factors such as bacterial toxins; the cholera toxin from

V. cholerae and the shiga-like toxin from enterohaemorrhagic *E. coli* have both been identified on prophages (Baba et al., 2002; Boyd and Brussow, 2002; Wagner et al., 2002). Phage integrase genes have also been observed in bacterial genomes on several occasions suggesting that these genes are of selective value to the bacterial host (Hacker and Kaper, 2002).

One example of HGT on a large scale due to phage is the genomic comparisons of *S. enterica* serovars Typhimurium and Typhi, where a number of the larger gaps in the alignment of the two chromosomes were explained by prophage insertion (Deng et al., 2003; Figueroa-Bossi et al., 2001). Figueroa-Bassi et al., (2001) conducted a number of animal experiments with *Salmonella* deletion mutants which demonstrated that prophages were not just selfish DNA that littered the bacterial chromosome, but contributors of numerous virulence factors and pathogenicity determinants in *Salmonella*.

1.5 Soil bacterial community

The bacterial community in soils is difficult to study in situ, although increasingly knowledge is becoming available with the advent of new molecular techniques which determine diversity in situ (LaMontagne et al., 2002; Muyzer et al., 1993). More recently, taxonomic structure has been related to function using techniques such as microarray and metagenomics in isolates and soils (Rhee et al., 2004; Stahl, 2004; Torsvik and Ovreas, 2002). It is estimated that only 0.1 % to 10 % at the most of microorganisms have been cultured and characterised, leaving the soil environment to a large extent uncharted (Amann et al., 1995). The stumbling block for characterising the soil bacterial community is the diversity, complexity and variability dependent upon a large number of factors including soil type and chemical composition, ecosystem and

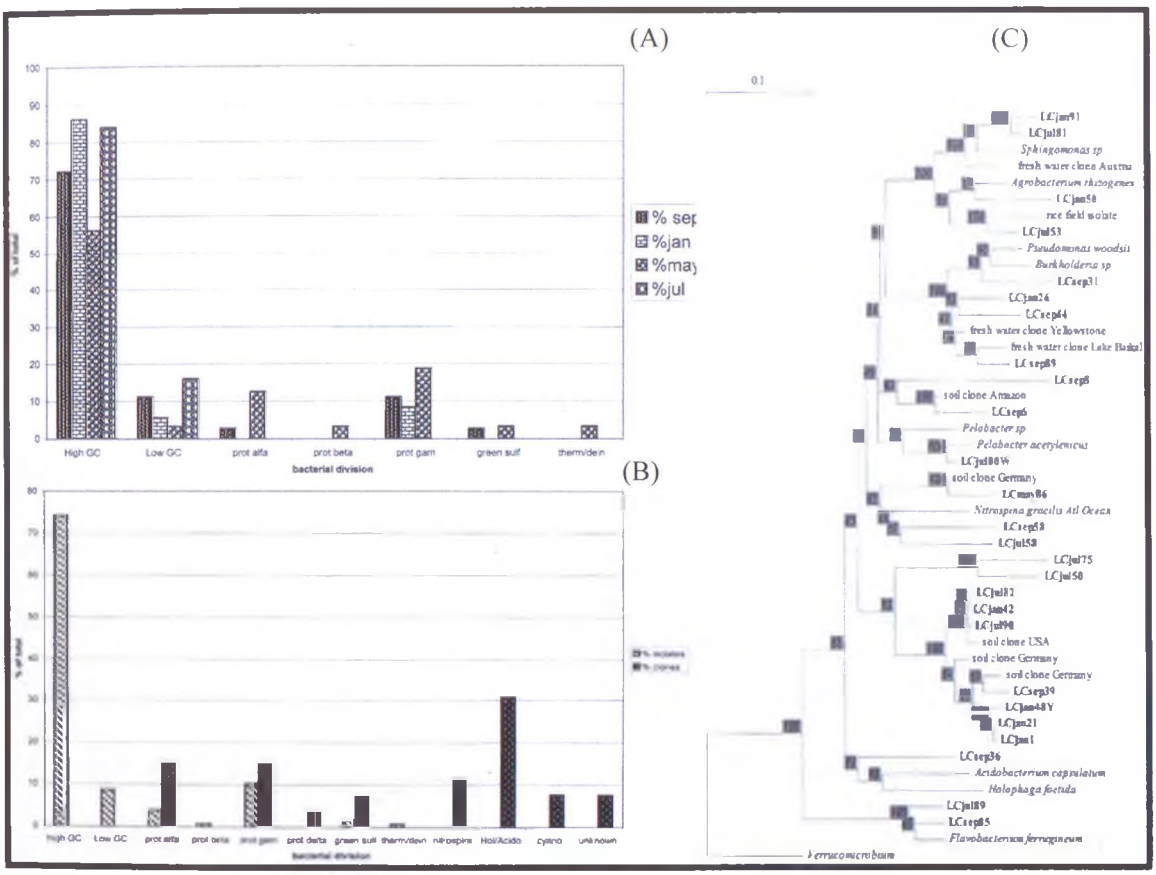
biological organisation; the bacterial species present, their abundance and evenness (Torsvik and Ovreas, 2002).

A number of high resolution methods have been developed for characterisation of microbial communities that includes both the culturable and unculturable microorganisms. Most of these methods are based on the analysis of 16S rRNA genes, enabling total community DNA studies from the environment, a number of which have also been coupled with functional gene analysis and measures of microbial activity (De Fede et al., 2001; Stokes et al., 2001b; Torsvik et al., 1998). These methods have uncovered part of the microbial diversity in soil, yielding phylogenetic lineages (De Fede et al., 2001). PCR-based fingerprinting techniques have allowed an increasingly detailed insight into community structures, utilising phospholipid fatty acid (PLFA) analysis, denaturing and temperature gradient electrophoresis (DGGE) (TGGE), amplified rDNA restriction analysis (ARDRA), terminal restriction fragment length polymorphism (T-RFLP), ribosomal intergenic spacer analysis (RISA) and random amplified polymorphic DNA (RAPD) to provide information (Grosskopf et al., 1998; Torsvik and Ovreas, 2002). These fingerprinting techniques are limited by PCR, DNA extraction and electrophoretic problems but used in combination or with modern software systems have provided high resolutions of bacterial communities (Kropf et al., 2004; Ranjard et al., 1998).

Soil is a complex system of micro-habitats with different chemical gradients and discontinuous environmental conditions. Microorganisms adapt to these different micro-habitats and live together in consortia, interacting with each other and with other parts of the soil biota. A number of investigations have emphasized the impact of soil structure and spatial isolation on microbial diversity and community structure; Sessitsch

et al., (2001) analysed the spatial distribution of bacteria at different micro-habitat levels and observed that in soils subjected to different fertilization treatments, more than 80 % of the bacteria were located in micro-pores of soil aggregates (2–20 μm). These micro-habitats, as defined by particle size, were further observed to provide the best conditions for microbial growth with respect to water and substrate availability, gas diffusion and protection against predation, whereas factors such as bulk pH and the type and amount of organic compound had a lesser impact (Sessitsch et al., 2001). Other factors influencing microbial abundance and diversity include the type and amount of available organic material and seasonal variations; Smit et al., (2001) reported that soils with a higher concentration of available nutrients had higher numbers of Alpha and Gammaproteobacteria, potentially a selection of high growth rate bacteria and displayed seasonal variation, in a study of agricultural soils from Lovinkhoeve, The Netherlands (Fig. 1.13). Smit et al., (2001) concluded that the ratio between the number of Proteobacteria and *Acidobacterium* (slower growth rate bacteria) was indicative of the nutritional status of soils (Fig. 1.13). The study by Smit et al., (2001) also displayed the difference in phylogenetic trees obtained from unculturable versus culturable methods for assessing the microbial communities in soils, the diversity being higher in the former (Fig. 1.13).

Fig. 1.13 Lovinkhoeve soils microbial community distribution (A) culturable isolates take in different months of the year over various bacterial phyla, (B) unculturable 16S rDNA sequences and isolates, (C) neighbor-joining tree



(A) Distribution of cultured soil isolates from September, January, May, and July separated into bacterial phyla; prot alfa, Alphaproteobacteria; green sulf, green sulphur bacteria; therm/dein, *Thermococcus/Deinococcus*; Hol/Acido, *Holophaga/Acidobacterium*; cyano, Cyanobacteria, (B) culturable and unculturable 16S rRNA gene sequences (C) neighbor-joining tree representing the phylogenetic relationship of the most abundant sequences to various closely related clone and isolate sequences obtained from Blast searches (clones detected in this study are given in boldface). The scale indicates genetic distance (Smit et al., 2001).

Soils from a wide range of ecosystems have been assessed for their microbial communities, differing in the presence of a number of specific bacterial phyla which define the soil type, in general the percentage distributions of a defined number of phyla changes in relation to the soil type and environmental disturbances, the changes in phyla possibly linked to a change in biochemical activities in the soils (Nagy et al., 2005). A study of a Scottish indigenous soil community where pollution with 2,4-dichlorophenoxyacetic acid had occurred, was reported to show a shift towards the Betaproteobacteria, especially those in which the degradation pathway of this pollutant was commonly identified (Chinalia and Killham, 2006). Table 1.1 summaries four studies of microbial community analysis based on molecular techniques, the agricultural soil lacked the diversity of the desert/arid soils, with increased numbers of members of the Proteobacteria indicative of animal contamination possibly through fertilization (Buckley and Schmidt, 2003; Kuske et al., 1997; Nagy et al., 2005).

A number of studies have also highlighted the effects of manure application to agricultural land; Ibekwe et al., (2003) reporting a community shift towards dominating bacteria originating in the gastrointestinal tract of animals and a biochemical shift towards ammonium-oxidising bacteria, those of the *Bacillus*, *Clostridium*, *Mycoplasma*, Proteobacteria phyla; and Cotta et al., (2003) also identified *Clostridium* and bacteria in predominating Gram positive, low GC, obligate anaerobes in microbial communities in swine faeces, manure storage pits and soils with freshly applied slurries. Onan and LaPara, (2003) investigated the soil bacterial community in 6 agricultural soils which had received different subtherapeutic levels of antibiotics.

Table 1.1 Prevalence comparison of bacterial phylogroups detected and identified from four molecular based studies, in three desert and one agricultural soil.

Sonoran soils (percent of DNA amplified and identified)[a]	Colorado Plataeu soils (percent of DNA amplified and identified)[a]	Arid soils (percent of clones)[b]	Agricultural soils (percent rRNA present in soil)[c]
Cyanobacteria (54.8)	Cyanobacteria (38.4)	Acidobacteria-like (51.1)	Proteobacteria (27)
Actinobacteria (15.1)	Proteobacteria (16.3)	Proteobacteria (15.5)	Actinobacteria (11.1)
Proteobacteria (13.8)	Actinobacteria (11.8)	Flexibacteria and relatives (13.3)	Planctomycetes (7.2)
Acidobacteria (11.1)	Bacteriodetes (10.6)	Actinobacteria (6.7)	Acidobacteria (3.5)
Bacteriodetes (0.9)	Firmicutes/Bacilli (5.2)	Planctomycetes (4.5)	Verrucomicrobia (1.9)
Chloroflexi (0.7)	Thermomicrobiales (2.9)	Unknown (8.9)	Bacteriodetes (0.4)
Others (0.9)	Acidobacteria (2.5)		
Unknown (2.7)	Unknown (12.6)		

[a] **Nagy et al., (2005)**

[b] **Kuske et al., (2005; 1997)**

[c] **Buckley and Schmidt, (2003)**

They observed a difference in the number of tylosin resistant culturable bacteria enumerated in the soils, the highest in soils with tylosin-fed swine manure applied (69 %) and also reported the dominance of Alpha and Beta-tylosin resistant proteobacteria in soils with a subtherapeutic level of antibiotic usage compared to *Streptomyces*-like tylosin resistant bacteria (high GC content) in soils where no usage has occurred. Müller et al., (2002) studied the transient presence of tylosin on soil microbial communities, revealing small changes compared to a control soil.

1.6 Antibiotic resistance in the environment

1.6.1 Studies into antibiotic resistant bacteria in the environment

As with community studies, investigations into antibiotic resistance in the environment commonly focus on clinically relevant bacterial species, clinically significant antibiotic resistance determinates or focus on phenotypic resistance and do not investigate the molecular basis for the resistance. The release of antibiotics into the environment may cause the development of single, cross and multiple resistance, in bacteria over time (Wegener, 2003). Where slurry is applied to soils, both enteric animal and indigenous soil bacteria can be affected and both may serve as a reservoir of resistance. Bacterial resistance may develop or be transferred to the environment due to a number of mechanisms;

1. In response to continual sub-lethal doses of antibiotics in the feed (as growth promoters), manure/slurry and soil where repeated slurry spreading has occurred or naturally occurring resistant bacteria (Aarestrup et al., 2001; Onan and LaPara, 2003; Thiele-Bruhn, 2003);
2. Transfer of resistant bacteria from the gut flora (Buckley and Schmidt, 2003),
3. Horizontal gene transfers from pre-existing resistant bacteria either surviving enteric or indigenous bacteria (Smalla and Sobecky, 2002).

A continual sub-lethal dose of antibiotic provides a selective pressure for a stepwise mutation of genes whose products play a role in physiological cell metabolism. Schmitt et al., (2004) investigated the pollution-induced community tolerance of soil microbial communities caused by SCP and reported an increased of 10 % at a concentration of 7.3 mg/Kg dry weight of SCP. Westergäard et al., (2001) recorded the effects of tylosin on a soil microbial community over time, 3 weeks after application the tylosin no longer

remained in the soil and 2 weeks after application an increase in numeration of soil microorganisms was observed which then decreased to pre-application numbers 8 weeks after application, reported in bacteria, protozoa and the fungal biomass. Investigations have also observed that many soil bacteria have a natural tolerance towards antibiotics, in particular pseudomonads are often intrinsically resistant to antibiotics due to multiple export pumps and slime layers (Esiobu et al., 2002; Halling-Sørensen et al., 2003a).

The release of antibiotics into the environment may not be the only source generating antibiotic resistant bacteria. Two other possible sources include the natural production of antibiotics and selection in the gut of antibiotic-fed animals, where resistant bacteria may be directly introduced via faeces into soils. This introduction was observed in studies into pig faeces where CTC and a number of other antibiotics were used. Langlois et al., (1978) recorded the excretion of multiple resistant microorganisms after pigs received therapeutic antibiotic doses and van Den Bogaard et al., (2000) also reported a high prevalence of multiple resistant bacteria where antibiotics were used as growth promoters. A investigation by Sengeløv et al., (2003) concluded that there was a relationship between tetracycline resistance and soil fertilized by manure, in their study; a higher load of manure resulted in a higher level of resistance but this elevated resistance decreased over time to normal levels, indicating a temporary influence on resistance levels.

As well as the development of resistance and the transfer of resistant bacteria into the environment via faecal waste, resistance maybe transferred from resistant bacteria into non-resistant bacteria via horizontal means, such as plasmids, enhanced by the nutrient rich environment of manures and slurries (Nield et al., 2001; Rosser and Young, 1999;

Smalla et al., 2000a; Smalla and Sobecky, 2002; Wegener, 2003). Several studies have shown transfer of plasmids between introduced and indigenous strains of soil bacteria (DiGiovanni et al., 1996; Glew et al., 1993; Smit et al., 1998; Top et al., 1990). Götz and Smalla (1997) reported that the presence of pig manure increased transfer rates by mobilisation were 10 fold in IncQ plasmids, concluding that agricultural land amended with pig slurry is an environment that could favour horizontal transfer.

1.6.2 Antibiotic production in soils

Antibiotic production in soil-borne bacteria has been well documented in several cases of *Streptomyces*, although the precise roles of these secondary metabolites can only be hypothesised, for example natural bio-control agents and defence from competitors (Nowakthomson et al., 1994; Rothrock and Gottleib, 1984). The concentration of these naturally produced antibiotics has been difficult to quantify due to low concentrations, but is thought to have been sufficient for the selection of resistant bacteria in the environment (Lucrecia et al., 1987; Roughley et al., 1992; Thomashow et al., 1990). Bacteria which produce antibiotics, encode resistance genes within their genomes for self-protection, such as the *ermSF, rlmA, tlrC* and *ermN* genes from the tylosin biosynthetic gene cluster of *S. fradiae* (Roberts et al., 1999; Stratigopoulos et al., 2004). These gene clusters or resistance genes have not been detected outside of antibiotic producing strains, but HGT of these elements is thought to occur between producers (Egan et al., 1998; Wiener et al., 1998). The selective pressure on soil-borne bacteria may play a major role in the selection of resistant bacteria and the generation of reservoir of resistant bacteria in the environment (Wiener et al., 1998).

1.6.3 Maintenance of antibiotic resistant determinates

Previous studies have observed conflicting evidence for the collection and maintenance of antibiotic resistance genes. Andersson, (2003) discussed in a recent review, that investigations, both laboratory and epidemiological, have collectively observed that a decrease in antibiotic usage does not always lead to a decrease in bacterial resistance with several processes including compensation evolution, occurrence of cost-free resistances and co-selection between antibiotics leading to the long-term persistence of resistant bacteria and resistance genes. One such investigation studied sulphonamide resistance in *E. coli* from human sources, and concluded that despite a decrease in sulphonamide prescriptions, the prevalence of *sulI* and *sulII* increased across the years examined (Enne et al., 2001). In contrast, Danish researchers recorded a decrease in resistance to a number of antibiotics with a decrease in their tonnage use in veterinary medicines and growth promotion (Wegener, 2003). Park, (2003) reported the occurrence of empty or non-functional Class 1 integrons in coliform bacteria from aquatic environments in the absence of any antibiotic selective pressure. However, caution should be taken in the interpretation of these results, as it is unclear whether there may have been an undetectable environmental selective pressure. Aarestrup et al., (2001) followed the incidence of resistance genes and phenotypic resistance in *E. faecium* strains from pigs and broilers, to avilamycin, erythromycin, vancomycin, and virginiamycin, from 1995 to 2000 with a decrease in the study antibiotics usage due to the European bans. They reported a decrease in resistance in the years following withdrawal of the respective antibiotics, but only observed a decrease in resistance for vancomycin and erythromycin when a decrease in both

avoparcin and tylosin was limited due to a link in these resistance mechanisms in their isolates.

1.6.4 Fitness

The maintenance of antibiotic resistance genes in a recipients genome maybe directly effected by whether these genes confers a selective advantage to the cell, even in the absence of a selective pressure. Studies have reported that under some conditions and for some resistance genes, a fitness cost is borne due to their translation. One such study reported that two R-plasmids present in *E. coli* K12 strains imposed a fitness cost and Giraud et al., (2003) observed that fluoroquinolone resistance in *Salmonella* strains conferred a fitness cost as a result of growth defects and altered morphology in stationary phase (Dahlberg and Chao, 2003).

Increasing evidence obtained from laboratory and epidemiological studies, indicate that several processes will act to procure long-term persistence of resistant bacteria including; compensatory evolution that ameliorates the costs of resistance, the occurrence of cost-free resistances and genetic linkage between the non-selected and selective resistance genes (Andersson, 2003). Amino acid repeats were found to have evolved in the chromosomal *folP* gene coding for DHPS of *S. pneumonia,* decreasing the enzyme kinetics for sulphonamides with resulting resistance but at no or little fitness cost to the organism (Haasum et al., 2001). Genetic mutations in the ribosomal protein S12 were observed to compensate for the fitness cost conferred by streptomycin resistance in *S. typhimurium* due to an increase in ribosomal proofreading and increased protein synthesis (Maisnier-Patin et al., 2002). Plasmid p9123, encoding sulphonamide and streptomycin resistance conferred a 4 % fitness advantage upon its original clinical

host, gene linkage was suggested as the mechanism of amelioration but no molecular linkage was established (Enne et al., 2004).

1.7 Summary of Aims

As discussed above, there are concerns that the release and persistence of antibiotics into the environment, through direct and via co-selection, may enhance the prevalence of bacteria with antibiotic resistance and antibiotic resistance genes, aiding the dissemination of these genes in the soil and slurry bacterial populations through the process of horizontal gene transfer. There is a lack of microbial community studies which interrogate the whole bacterial population and investigate effects of antibiotics on both the phenotypes and genotypes of the bacterial members of a specific ecosystem. This project aimed to address these issues by examining soils and slurry samples from an organic farm with no history of antibiotic usage in the swine population and a study farm, designated Cranfield, which had a history of prolonged application of swine slurries from tylosin-fed animals onto agricultural land which was spiked, or not spiked (control), with SCP and OTC for experimental purposes over a two year period (Kay et al., 2004). The culturable bacterial population with and without antibiotic resistance to the study compounds (SCP, OTC and tylosin) were examined in relation to their measured concentrations in the samples over time.

The objectives of this study were therefore to:

1. Estimate numbers of culturable bacteria, resistant and sensitive to the study compounds, before and after the application of slurry to agricultural soils at a number of time points, also examining drain-flow and slurry samples;

2. Isolate culturable bacteria from all samples with varying antibiotic resistant phenotypes. Examining these both phenotypically and genotypically with respect to the study compounds;
3. Investigate HGT in these environments with respect to the study compounds and a number of mobile genetic elements, and to;
4. Investigate the effects of the soil environment on antibiotic resistance in culturable bacteria isolates in this study.

The project used traditional microbiology techniques, coupled with molecular, biochemical and biophysical techniques to reach its potential.

CHAPTER 2:

MATERIALS AND METHODS

Chapter 2: MATERIALS AND METHODS

2.1 SITE INTRODUCTION

The soil and slurry samples collected for this study were from two sites. The first site was from an investigation performed by Cranfield University at a farm in Leicestershire, UK and the second was an organic farm on the outskirts of Coventry (Blackwell et al., 2004; Boxall et al., 2002; Kay et al., 2005a; 2004; Kay et al., 2005b, c).

Site 1:

A field study was conducted in which antibiotics were examined from three distinct chemical classes; the macrolides, polyketides and the sulphonamides. The three antibiotics, sulphachloropyridazine (SCP), oxytetracycline (OTC) and tylosin, were investigated with respect to their sorption and transport properties in soils with a view to providing further information for risk assessment studies and prediction models. Sorption coefficient (K_d) values were calculated in soil, slurry and soil/slurry mixtures for each antibiotic and HPLC was used to measure the concentrations of the antibiotics in the soil cores (Blackwell et al., 2004; Boxall et al., 2002).

The study was conducted on a 1.55 ha arable field located on a working farm in the upper reaches of the River Soar catchment in Leicestershire, UK. The field had an under-drained clay soil with neutral pH (Table 2.1), which discharged at a single outfall into the receiving ditch, subsequently feeding into a small stream (Blackwell et al., 2004; Boxall et al., 2002; Kay et al., 2005a; Kay et al., 2004, 2005b, c). Drain-flow samples were collected at times of high rainfall events.

At the time of study, the Landrace pigs (of various ages) were treated continuously with tylosin at 100 g/tonne of feed, which was sufficient to feed 67 to 78 pigs for a one

week period. This equates to a weekly dose, per pig, of between 1.2 and 1.5 μg/kg of feed. In the cases of OTC and SCP, these were only added to slurry (not in the control slurries and soils) before spreading, at application rates of 0.9 and 1.2 kg/ha respectively, on to agricultural land using a broadcast spreader. The slurry was applied to the field at the same rate as normal agricultural practice (45,000 l/ha), final concentrations in the slurry were 18.85 mg/l for OTC and 25.58 mg/l for SCP. The predicted concentration of the tylosin parent molecule in the slurry was 117.94 mg/l, assuming no degradation had taken place during storage, decreasing to less than 1 ng/l after one months storage (Kay et al., 2004). The slurry had been stored for a maximum of three months before its application.

Soil cores were taken down to the depth of the drainage system at approximately 60 cm, collected at random from 10 different sites around the field, at 20 different time points per year. In this thesis study all experiments were performed on 3 soil cores, not composites and performed in triplicate. The time points used were year 1; pre-application (pre-app) and days 1 (1, 1), 21 (1, 21), 90, 120 and 289 after application. The year 1, day 289 samples were also used as the pre-application data for year 2. Time points in year 2 were taken at days 1 (2, 1), 21 (2, 21), 90, 120 and 240.

Both SCP and OTC were detected in soil cores 0-37 cm in depth, although there was some spatial variability which may have been due to the presence of visible large cracks forming in the soil during the summer months and macro-pores forming during the winter months (Figs. 2.1 a-d) (Kay et al., 2004). Tylosin was not detected in any of the soil cores. The detection limits in this soil type based on pre-concentrating 400 mls of sample were 0.35 μg/l for OTC and tylosin or 0.25 μg/l for SCP (Blackwell et al., 2004). The maximum measured concentrations of SCP in any soil sample were 365

µg/kg in year 1 and 212 µg/kg in year 2 (Fig. 2.1) (Kay et al., 2004). In both years, SCP was observed to have leached to a depth of 20 cm within 24 hrs of application, leading to a calculated dissipation of half the applied SCP in 29 days (Boxall et al., 2002). With some exceptions SCP was not detected in samples taken three months or more after slurry application (Kay et al., 2004). Maximum measured concentrations of OTC in any soil sample were 1,691 µg/kg in year 1 and 322 µg/kg in year 2 (Kay et al., 2005c). In comparison to SCP, OTC was found at a depth of 20 cm within hours of application and was detected in soil cores until the last days of the study at a depth of 30 cm (Kay et al., 2004).

Site 2

The organic farm, run according to Soil Association guidelines, had never used antibiotics prophylactically and had been organic for at least 25 years (Soil Association, 2001). The farm operated a rotation system of animals yearly, including cattle, swine, poultry and sheep. The swine were free-range Saddleback pigs of direct descendants from organically farmed animals, bought in, which were no older than 13 months. The soil was heavy clay which was not chemically analysed.

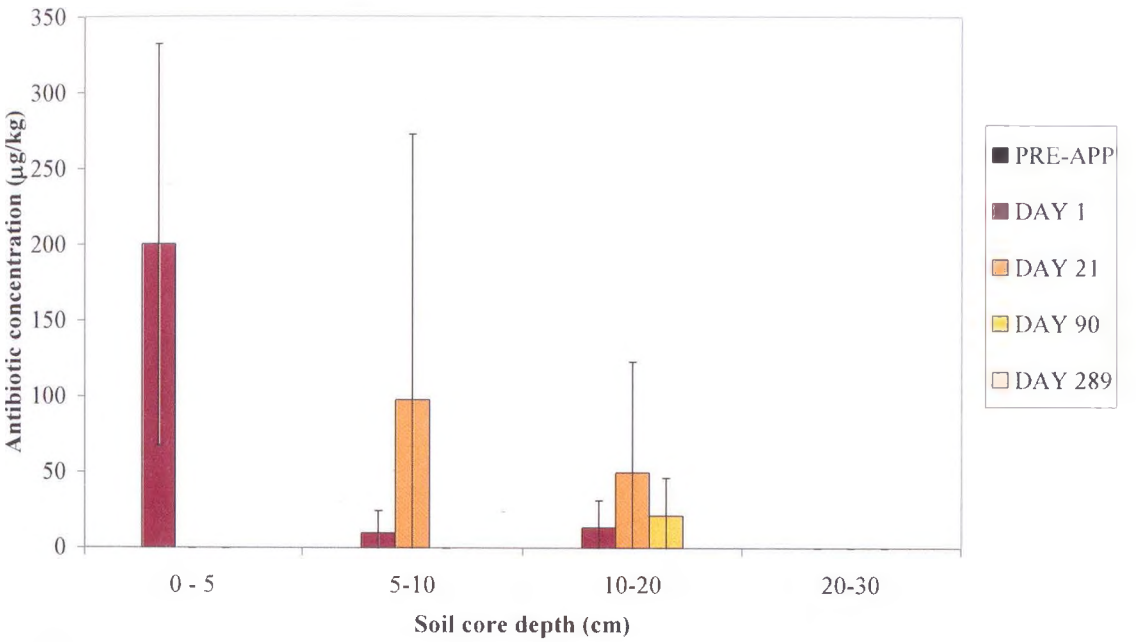
Table 2.1 Characterisation of the Cranfield study field soils to the depth of the drainage system.

	Top soil (0-37 cm)	Subsoil (37-65 cm)
Sand (63 µm-2 mm) %	42.63	34.17
Silt (2 µm-63 µm) %	32.26	27.68
Clay (<2 µm) %	25.11	38.15
pH (in $CaCl_2$)	6.8	7.3
Cation exchange capacity mEq/100g	22.4	25.2
Organic carbon %	2.2	0.7
Bulk capacity g/cm	1.3	1.56

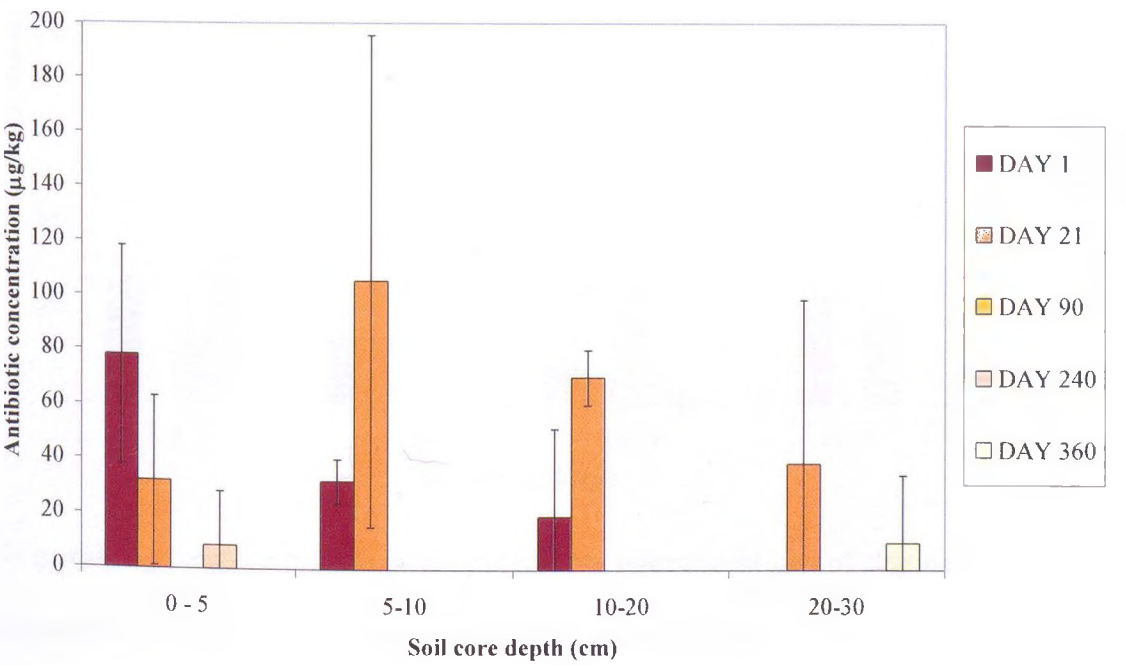
Taken from Kay et al., (2004)

Fig. 2.1 Antibiotic concentrations (µg/kg), as measured by HPLC, for sulphachloropridazine in years 1 (a) and 2 (b) of the Cranfield study and OTC in years 1 (c) and 2 (d) of the study (Kay et al., 2004).

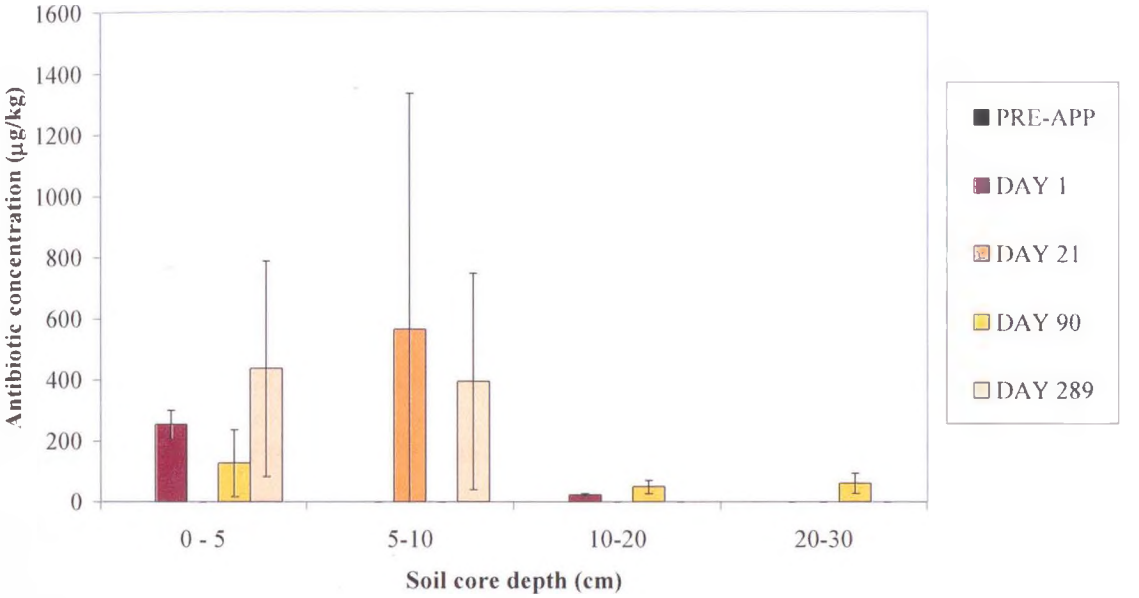
(a)



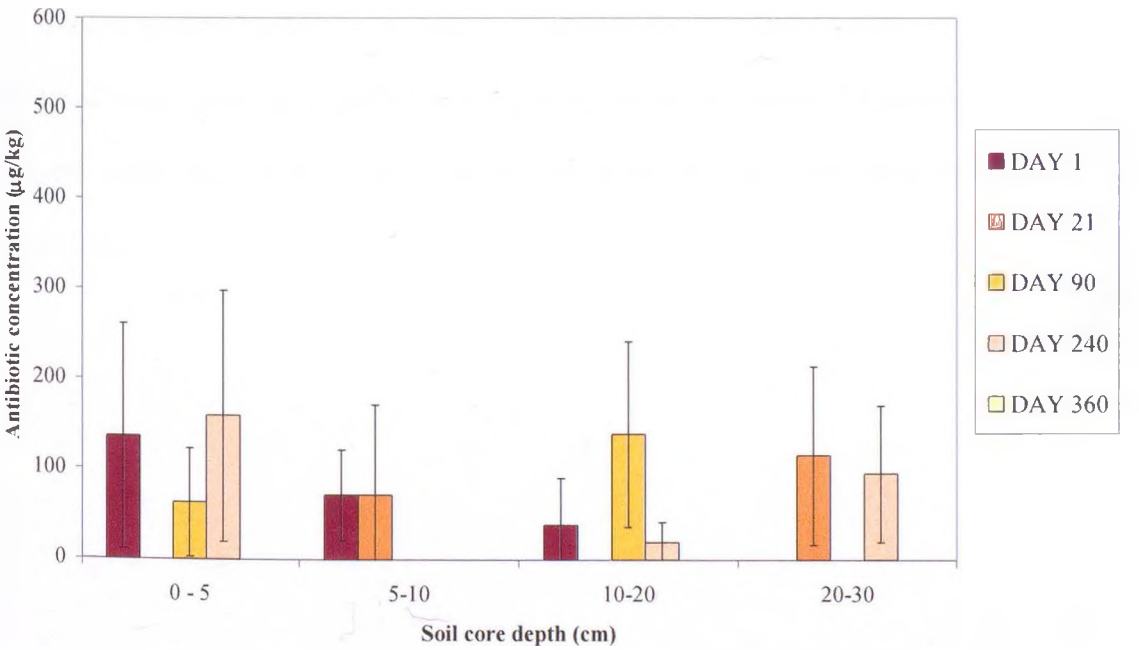
(b)



(c)



(d)



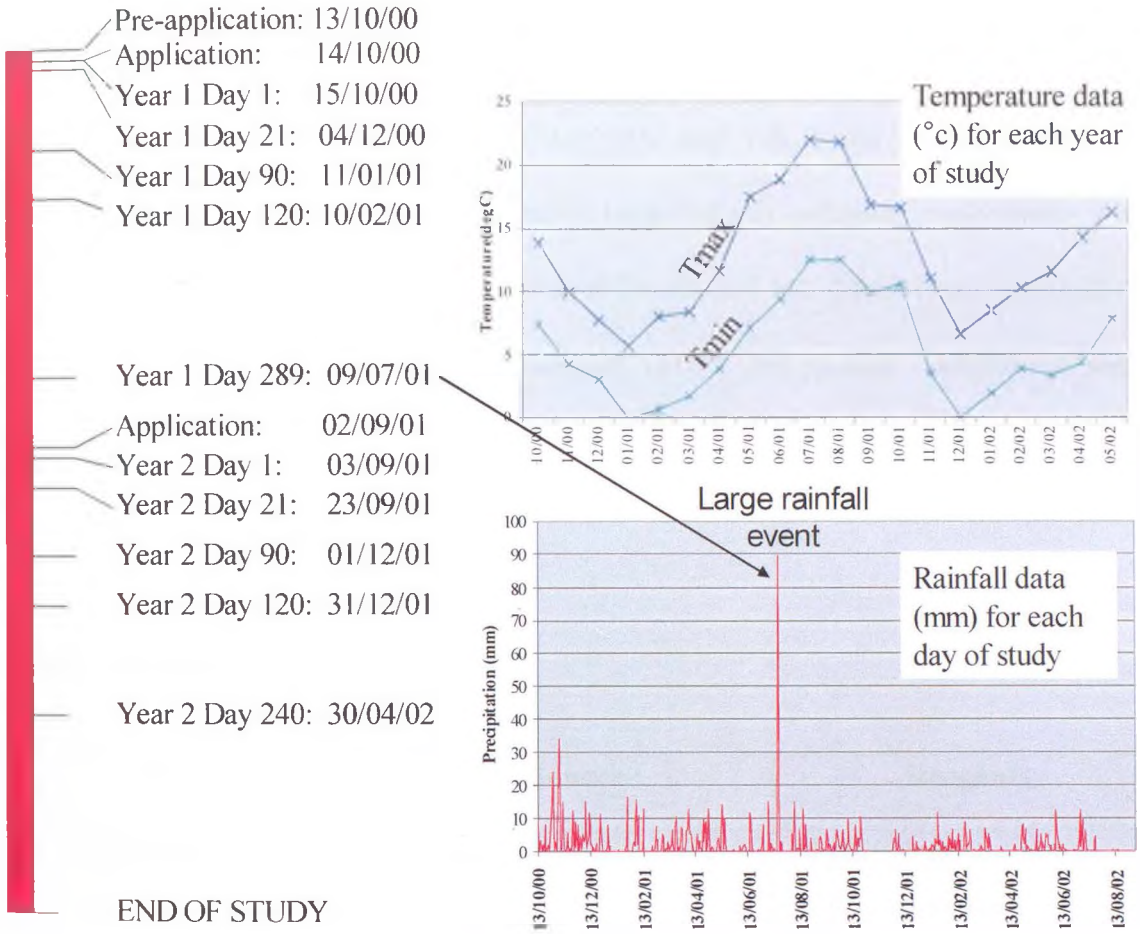
No graph is given for tylosin as no detectable concentrations of this antibiotic were present in either the drain-flow samples or the soil cores.

2.1.1 Soils and slurry samples

Soil core samples were obtained from the study at Cranfield University. The soil cores collected were; pre-application in year 1 (1.P) and days 1, 21, 90, 120 and 289 after application (Boxall et al., 2002). The study was repeated for a second year with year 1, day 289 acting as the second year's pre-application samples, and samples collected at days 1, 21, 90, 120 and 240 after application. The sampling days are displayed in Fig. 2.2 with accurate dates and corresponding rainfall and air temperature data that may have effected bacterial growth and subsequent viable counts. Drain-flow samples were collected at a number of rainfall events but only those at the highest rainfall events were examined in this study (Kay et al., 2005c).

Slurry samples were collected from the Cranfield study with (Cranfield slurry) and without (control slurry) the addition of OTC and SCP. These samples were frozen (-20 °C without glycerol prior to investigation in this study. The organic pig manure was collected fresh from an organic farm pig field on the outskirts of Coventry, UK. The samples were kept at 4 °C prior to investigation for no more than 48 hours after collection.

Figure 2.2 Study length and soil sample time points in relation to monthly air temperatures (°C) and daily precipitation data (mm).



2.2 MEDIA, REAGENTS AND SOLUTIONS

2.2.1 Media

All media were obtained from Oxoid and made up according to the manufacture's instructions with the exception of the LB agar and LB liquid media (Table 2.2) (Sambrook et al., 1989). Buffers, media, reagents and solutions were made with distilled water (dw), the pH was measured and corrected and then, where appropriate, the solutions were sterilised by autoclaving at 121 °C for 15 min. Antibiotics were made as described in Table 2.3.

Table 2.2 Media

Media	Source	Reagents
LB Agar/Broth	Sigma	10 g tryptone 5 g yeast extract 10 g NaCl Dissolve in 1 l of distilled water, for agar add to 2 %
Iso-Sensitest Agar/Broth	Oxoid	
Nutrient Agar/Broth	Oxoid	
Plate Count Agar (PCA)	Oxoid	

Table 2.3 Antibiotic stocks

Antibiotic	Stock concentration (mg/ml)	Solvent
Ampicillin	100	Water
Chloramphenicol	100	Water
Erythromycin	250	100 % DMSO
Kanamycin	100	Water
Oxytetracycline	100	70 % HCl
Nalidixic acid	100	Water
Neomycin	100	Water
Rifampicin	50	100 % DMSO
Streptomycin	50	Water
Sulphachloropyridazine	100	70 % HCl
Tetracycline	100	70 % HCl
Trimethoprim	100	Water
Tylosin	100	Water

All antibiotics were supplied by Sigma, were dissolved as above and filter sterilised through an Acrodisc PF filter (0.8-0.2 μm) (Gelman Scientific) before each experiment and used fresh. Final concentrations are stated with each application according to usage.

2.3 BACTERIAL ISOLATION PROCEDURES

2.3.1 CFU Counts

1 g of soil or 1 ml of pig slurry was resuspended in 9 mls of sterile distilled water (sdw) and serial dilutions made. From each dilution 50 µl was spread onto Iso-Sensitest agar containing antibiotic concentrations as recorded in Table 2.4, and 100 µg/ml cycloheximidine (Sigma). Plates were incubated overnight at 28 °C. Drain-flow samples were defrosted and the total 500 mls spun at 405 g for 15 minutes. The pellet was resuspended in 10 mls sdw and 1 ml was used as the other samples.

CFU were counted and single colonies were picked at random for streaking onto Iso-Sensitest agar plates and incubated at 28 °C overnight. Resistance quotients (RQ) were calculated: CFU on antibiotic containing plates/CFU on none antibiotic containing plate, for each antibiotic concentration and for each time point/sample. Plates were stored at 4 °C.

In total 583 bacterial isolates were collected from a range of selective and non-selective plates and soil and slurry sample (Table 2.5).

2.3.2 Storage Procedures

Isolates were grown overnight in Iso-Sensitest broth at 28 °C and an aliquot of each was kept in 55 % glycerol at both -20 °C and -80 °C. Plates containing isolates were kept at 4 °C.

Table 2.4 Antibiotic concentrations used in CFU count plates and bacterial isolations.

Antibiotic	Final concentration (µg/ml)
Oxytetracycline (OTC)	0.2
	1
	5
	10
	25
Sulphachloropyridazine (SCP)	5
	10
	25
	50
	100
Tylosin	0.2
	1
	5
	10
	25
	50

Table 2.5 Numbers of bacterial isolates collected from each soils and slurry sample and numbers taken from each antibiotic selective plate.

Antibiotic and concentration (µg/ml)	Number of bacterial isolates taken from each sample																		TOTAL
	Pig slurry (Cranfield)	Pig slurry (organic)	Pig slurry (control)	Control soil	DF 30/10/00	DF 22/02/01	DF 13/10/02	1.P	1.1	1.21	1.90	1.120	1.289	2.1	2.21	2.90	2.120	2.240	
None	24	0	4	15	0	0	0	6	2	4	4	1	5	0	0	0	4	6	*75*
TY 0.2	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	*3*
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	*3*
5	6	0	4	0	0	1	0	5	20	0	0	0	3	0	0	1	0	0	*40*
10	0	0	4	0	0	1	4	0	0	0	0	0	0	0	0	10	0	1	*18*
25	0	0	0	0	0	2	0	2	0	0	0	0	4	0	0	4	0	4	*16*
50	15	10	0	0	2	0	0	22	14	5	14	0	5	16	11	6	0	3	*123*
SCP 5	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	0	0	0	*3*
10	0	0	0	0	4	3	4	7	0	5	13	0	4	3	0	3	0	2	*48*
25	6	0	0	0	0	0	0	21	20	0	0	0	0	0	2	9	0	6	*64*
50	10	0	0	0	0	0	0	0	5	0	0	0	6	14	6	0	0	0	*41*
100	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	*10*
OTC 0.2	0	0	0	0	0	0	0	0	0	6	6	0	3	0	6	4	0	4	*29*
1	0	0	3	0	0	0	5	5	1	0	2	0	0	0	0	3	0	0	*21*
5	6	0	3	0	0	3	0	8	7	0	0	0	6	2	0	1	0	0	*36*
10	0	0	0	0	2	0	0	0	0	0	0	0	4	6	11	0	0	0	*23*
25	11	12	0	0	0	0	0	0	2	0	0	0	0	4	1	0	0	0	*30*
TOTAL	*78*	*32*	*18*	*15*	*8*	*10*	*13*	*79*	*71*	*20*	*39*	*1*	*41*	*45*	*39*	*41*	*4*	*29*	*583*

2.4 NUCLEIC ACID EXTRACTION

2.4.1 Chromosomal DNA extraction from bacterial isolates

Chromosomal DNA was extracted using a DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions with the following amendments; all isolates were treated as Gram positive bacteria therefore after cell harvesting, pellets were incubated for 2 hours at 37 °C in enzymatic lysis buffer (as detailed in the DNeasy tissue kit manual).

2.4.2 Plasmid DNA extraction

Plasmid DNA was extracted using a QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions.

2.4.3 DNA extraction from agarose gels

A QiaQuick gel extraction Kit (Qiagen) was used according to the manufacturer's instructions.

2.4.4 Restriction digests

Plasmid and chromosomal DNA was digested with restriction enzymes and run on 1 % PFGE gels to estimate the sizes. A reaction was set up with 15 µl DNA at 200µg/ml, 20 µl sdw, 1 U *Xba*I (Fermentas) or 1 U *Xba*I and 1 U *Sac*I (Fermentas). The reaction were incubated overnight at 37 °C and stopped by incubating at 65 °C for 15 mins.

2.5 PCR

2.5.1 Standard Protocol for PCR

The PCR conditions were as follows; 12.5 pmol forward primer, 12.5 pmol reverse primer, 0.2 mM of each dNTPs (Fermentas), 2 mM $MgCl_2$ (Fermentas), 1 x *Taq* buffer (Fermentas), 0.2 U *Taq* DNA polymerase (Fermentas), 100 ng DNA, made up to a final volume of 50 µl with sdw. Thermocycling was performed on a Hybaid PCR Express, amplification conditions were; 94 °C 5 min, 94 °C 1 min, variable annealing temperatures according to the primers used (Table 2.6) 1 min, 72 °C 1 min for 35 cycles

Table 2.6 Primers used in PCR and sequencing

Gene	Primer name	Primer sequence	Annealing temperature (°C)	Reference
Bacterial *16S rRNA*	PA	AGAGTTTGATCCTGGCTCAG	62	(Edwards et al., 1989)
	PH	AGGGAGGTGATCCAGCCGCA		
intI1	IntA	ATCATCGTCGTAACGTCGG	67	(Rosser and Young, 1999)
	IntB	GTCAAGGTTCTGGACCAGTTGC		
intI2	Int2F	CACGGATATGCGACAAAAAGGT	58.5	(White et al., 2001)
	Int2R	GTAGCAAACGAGTGACGAAATG		
sulI	SulIBF	CTTCGATGAGAGCCGGCGGC	63	(Sundstrom et al., 1988)
	SulIBR	GCAAGGCGGAAACCCGCGCC		
sulII	SulIIF	TCGTCAACATAACCTCGGACA	60	V. Enne
	SulIIR	GTTGCGTTTGATACCGGCAC		
sul3	Sul3F	GAGCAAGATTTTTGGAATCG	51	(Perreten and Boerlin, 2003)
	Sul3R	CATCTGCAGCTAACCTAGGGCTTTGGA		
Class 1 integron variable region	Cass1	ACAGGGCAAGCTTAGTAAAGCC	55	(Rosser and Young, 1999)
	Cass2	CTCGCTAGACCTTTTGGAAA		
	Cass4C361	CTGCGGGAGGATATTCTTGA	sequencing	This study
	Cass5C361	AATTGGGAGATATATCATGAAAGGCTG	sequencing	This study
	VarF	GGCATCCAAGCAGGAAG	55	(Levesque et al., 1995)
	VarR	AAGCAGACTTGACCTGA		
Class 2 integron variable region	Int2VarF	GACGGCATGCACGATTTGTA	58.5	(White et al., 2001)
	Int2VarR	GATGCCATCGCAAGTACGAG		
	Int22PG20	TTGCCTTGGTAGGTC	sequencing	This study
	Int23PG20	TTGCCTTGGTAGGTC	sequencing	This study
	Int2PG49	GGAGTGCCAAAGGTGAACAG	sequencing	This study
qacE	KazamaF1	GGGAATTCGCCCTACACAACAAATTGGGAGA	50	(Kazama et al., 1998a)
	KazamaR2	TACTCGAGTTAGTGGGCACTTGCTTTGG		
qacEΔ1	KazamaF1	GGGAATTCGCCCTACACAACAAATTGGGAGA	50	(Jensen et al., 1999; Kazama et al., 1998a)
	KazamaR1	GCTGCAGCTGCGGTACCACTGCCACAA		
ermB	ErmBF	CATTTAACGACGAAACTGGC	60	(Jensen et al., 1999)
	ErmBR	GGAACATCTGTGGTATGGCG		
ermX	ErmXF	GTTGCGCTCTAACCGCTAAGGC	55	(Jost et al., 2003)
	ErmXR	CCATGGGGACCACTGAGCCGTC		

ermC	ErmCF	AATCGTCAATTCCTGCATGT	55	(Strommenger et al., 2003)
	ErmCR	TAATCGTGGAATACGGGTTTG		
tet(M)	Tet(M)F	AGTGGAGCGATTACAGAA	55	(Strommenger et al., 2003)
	Tet(M)R	CATATGTCCTGGCGTGTCTA		
ermA	ErmAF	AAGCGGTAAACCCCTCTGA	55	(Strommenger et al., 2003)
	ErmAR	TTCGCAAATCCCTTCTCAAC		
tet(K)	Tet(K)F	GTAGCGACAATAGGTAATAGT	55	(Strommenger et al., 2003)
	Tet(K)R	GTAGTGACAATAAACCTCCTA		
vatA	VatAF	TGGTCCCGGAACAACATTTAT	55	(Strommenger et al., 2003)
	VatAR	TCCACCGACAATAGAATAGGG		
vatB	VatBF	GCTGCGAATTCAGTTGTTACA	55	(Strommenger et al., 2003)
	VatBR	CTGACCAATCCCACCATTTTA		
vatC	VatCF	AAGGCCCCAATCCAGAAGAA	55	(Strommenger et al., 2003)
	VatCR	TCAACGTTCTTTGTCACAACC		
strA/B	StrA/BF	AACGCCGAAGAGAACTGG	54	(Smalla et al., 2000b)
	StrA/BR	AGGTGTCCGCAATGAGAA		
strA	StrAF	CCTGTGATAACGGCAATTC	54	(Gebreyes and Thakur, 2005)
	StrAR	CCAATCGCAGATAGAAGGC		
aadA1	AadA1F	TATCAGAGGTAGTTGGCGTCAT	57	(Randall et al., 2004)
	AadA1R	GTTCCATAGCGTTAAGGTTTCATT		
oxa-1	Oxa1F	ACACAATACATATCAACTTCGC	54	(van Loon et al., 2004)
	Oxa1R	AGTGTGTTTAGAATGGTGATC		
Link between aadA1/oxa-1	AadA1F	TGACGGGCTGATACTGGG	58	(Randall et al., 2004; van Loon et al., 2004)
	OxaR	AAAACCCCCAAAGGAATGGAG		
tnpA	TnpAF	ATGACGGATTTCAAGTGGC	55	(Tauch et al., 2002)
	TnpAR	TCAGGCGGCTGCTGCG		
tnpB	TnpBF	ATGGGCATCTTCTTCGG	58	(Tauch et al., 2002)
	TnpBR	TCAGGCAGCCTCGAAAACTCGG		
traA	TraF	ATGCACGGCGAATCCG	52	(Tauch et al., 2002)
	TraR	CTAGAGATCCATACCG		
tetA/R	TetAF	TCACATCGAGTCGCCG	55	(Tauch et al., 2002)
	TetRR	TCATCGCTTTGATGCCAGC		
acrRA	AcrRAF	CAGTGGTTCCGTTTTTAGTG	53	(Olliver et al., 2005)
	AcrRAR	ACAGAATAGCGACACAGAAA		
acrSE	AcrSEF	TGGCGAAAGCGTTAAATCTG	56	(Olliver et al., 2005)

	AcrSER	CAGCGCAGCAGAGAATATGA		

All primers were produced by Tagn Newcastle Ltd., UK.

and then a final extension at 72 °C 15 min. The PCR master mix for the *qacE* and *qacEΔ1* genes had the addition of 1 mg/ml BSA and 5µl 100% DMSO. Genes *ermA* and *C, tet(K)* and *M, vatA, B* and C genes were screened using a multiplex PCR, the conditions varied from the standard protocol in that a 100 µl reaction was used with 10 pmol of each primer, 0.4 mM dNTPs, 5U *Taq* DNA polymerase and 4 mM $MgCl_2$. Primers for sequencing were designed using the online Primer 3 programme (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and produced by Tagn Newcastle Ltd, UK.

2.5.2 Agarose gel electrophoresis

Agarose (Helena) was mixed with 1 x TAE to a final concentration of 1 % (w/v) and then boiled (a 2.5 % agarose gel was used for the multiplex PCR) (Table 2.7). The agarose was cooled to "handhot" and ethidium bromide added to a final concentration of 0.25 µg/ml (Sigma). A 1Kb DNA ladder (Fermentas) was used to size products and sample (50 µl) was loaded into the wells with 5 µl of loading buffer 1 (Table 2.7). Gels were visualised using the Gene Flash Syngene Bio-Imaging System.

2.5.3 Sequencing

Sequencing reactions were performed with a terminator cycle sequencing kit (Applied Biosystems, Foster City, California) as described by the manufacturer, and electrophoresis and readout were carried out on an ABI Prism 3100 genetic analyzer

Table 2.7 Loading buffer and running buffer recipes.

Loading buffer 1	Loading buffer 2	50 x TAE	50 x TBE
10 mM Tris.HCl (pH7.6) 0.06 % Orange G 60 % Glycerol 60 mM EDTA	10 mM Tris.HCl (pH7.6) 0.1 % Bromophenol blue 50 % Glycerol 60 mM EDTA	242 g Tris Base 57.1 ml Acetic Acid 10 ml 0.5M EDTA (pH8.0) Up to 1l dw	54 g Tris Base 27.5 g Boric acid 20 ml 0.5M EDTA (pH8.0) Up to 1l dw

All chemicals obtained from Sigma.

(Applied Biosystems). Forward and reverse primers were used in the sequencing reactions to sequence both strands of each PCR product.

Resulting DNA sequences were edited using BioEdit (Isis Pharmaceuticals. Inc.) (Hall, 1999). The resulting DNA information was analysed using the programmes FASTA and BLAST for sequence comparisons (Altschul et al., 1990; Pearson, 1990).

2.5.4 Phylogenetic Analysis

Nucleotide sequences were analysed for similarities using BLAST (Altschul et al., 1990) and alignments were made using the CLUSTALW tool in Bioedit (Hall, 1999). Bioedit was used to translate open reading frames and edit alignments (Hall, 1999). Phylogenetic analysis was performed using Phylip phylogenetic programs (Felsenstein, 1985, 1988). Distance based analysis was performed using Dnadist for DNA sequences followed by Neighbor, in Phylip (Felsenstein, 1988). Trees were drawn using Treeview (Page, 1996).

2.5.5 Pulse field gel electrophoresis

PFGE was carried out to size plasmids using a Chef Mapper XA (Bio-Rad, US) set on the auto algorithm programme for 10-200 Kb separation on a 1 % PFGE (Bio-Rad) agarose gel made with 1 x TBE running buffer (Table 2.7). A 1-500 Kb ladder (Sigma) was used on all PFGE gels to size products. The gels were Southern blotted and probed with PCR products labelled and visualised using ECL Direct Nucleic Acid Labelling and Detection System Kit (Amersham Biosciences LTD, Buckinghamshire, U.K.).

2.5.6 Southern blotting

Southern blotting was carried out with the ECL Direct Nucleic Acid Labelling and Detection System Kit (Amersham Biosciences LTD, Buckinghamshire, U.K.) according to the manufacture's instructions, with the exception that all hybridisation and labelling was carried out at room temperature.

2.6 MICROBIOLOGY TECHNIQUES

2.6.1 Strains and Plasmids

P. putida UWC1 (Rif^r) and *E. coli* K-12 CV601 (Rif^r Thr^- Leu^- Thi^-) were used as recipients in exogenous plasmid isolations (Smalla et al., 2000b). Plasmids isolated in *P. putida* UWC1 were transferred to *E. coli* K-12 strain DH5α by transformation (Sambrook et al., 1989). *E. coli* K-12 DH5α served as the general host for preparation of plasmid DNA and determining antibiotic resistance patterns conferred by the plasmids.

2.6.2 Conjugation isolations

Conjugative matings were performed according to Smalla et al., (2000a), selection was carried out on 50 µg/ml rif (Sigma) and 8 µg/ml SCP on Iso-Sensitest agar plates, and screened by PCR.

2.6.3 Stability experiments

Stability experiments were carried out according to Gormley and Davies (1991) except cultures were grown in Iso-Sensitest broth and plated onto SCP containing medium. Minimum inhibitory concentrations (MIC) were carried out on stability cultures before and after the subbing.

2.6.4 MIC determinations

MICs were calculated on Iso-Sensitest plates according to the NCCL method (NCCLS, 1997).

2.6.5 Capsule stain

A clean slide was inoculated with a thin smear of culture from a plate. The smear was left to air dry and the slide flooded with crystal violet (Oxoid) for 4-5 min. Any excess crystal violet was then washed gently off with a $CuSO_4$ (Sigma) solution. The slide could then be observed with oil under a microscope. A positive capsule stain would give purple cells surrounded by a clear or faint blue halo on a purple background.

2.6.6 SCP/Tylosin resistance induction

Overnight cultures of isolates were grown at 28 °C and used to inoculate 50 mls Iso-Sensitest broth at an OD_{600} 0.05. Each treatment was set up in triplicate. The cultures were incubated at 28 °C and the starting cultures and subsequent time points were

measured for OD_{600} and an aliquot taken for MIC determinations. Depending on the antibiotic of induction, after 115 mins HCl (SCP control), 8 μg/ml SCP, 16 μg/ml SCP or 1 μg/ml tylosin was added to 3 different cultures (in triplicate) and one was left without any treatment. After growth overnight, 100 μl of each culture was plated onto Iso-Sensitest plates containing increasing concentrations of the respective induced antibiotics for MIC determination.

2.7 ISOLATE C442 (*Acinetobacter baumanni*) INVESTIGATIONS

2.7.1 Microcosm studies

The soil used in microcosms was taken from a local wheat field site near the University of Warwick, UK. Soil was prepared by passage through a 2 mm sieve and 10 g was placed in 50 ml Universal glass vials and double sterilized at 121°C for 15 min with 24 hours between each treatment (Wellington et al., 1990). Separate triplicate microcosms were prepared for sacrificial sampling at each time point. 1.5 g sterile dH_20 was added to all conditions at -20 kPa and left overnight to equilibrate at 28 °C (Wellington et al., 1990). Four separate conditions were used in triplicate and the conditions with SCP were left overnight to equilibrate on a roller in the dark at 28 °C. The conditions were;

1) soil + dH_20 only

2) soil + dH_20 + 0.2 μg/l SCP (the highest concentration recorded in the field trial (Kay et al., 2004)

3) soil + dH_20 + C442 (1×10^7)

4) soil + dH_20 + C442 (1×10^7) + 0.2 μg/l SCP.

The microcosms were incubated in the dark, to prevent photo-degradation of the antibiotic, at 28 °C. MIC plates for SCP or other antibiotics and viable count plates were conducted every week on all conditions.

2.7.2 MIC stability assay

From treatment 4 above, 1 g of soil was diluted 10^5 with sterile dH_20 and 100 µl were plated onto Iso-Sensitest agar containing 0, 10, 25 and 50 µg/ml SCP. A set of liquid cultures were also prepared with cells at a final concentration of 10^5 in 50 mls of Iso-Sensitest Broth also containing 0, 10, 25 and 50 µg/ml SCP. Viable counts were taken from the plates after 24 hours at 28 °C and the cells from agar plates containing 0 µg/ml SCP were resuspended in 1 ml dH_20 and 100 µl spread onto Iso-Sensitest agar containing 0, 10, 25 and 50 µg/ml SCP. Viable counts were also taken from the liquid cultures by plating 100 µl media onto Iso-Sensitest agar containing 0 µg/ml SCP and incubating at 28 °C overnight. After the aliquot for viable counts from the liquid cultures were taken the cells from 0 µg/ml SCP were pelleted, washed with Iso-Sensitest broth and resuspended with 50 mls broth. 100 µl from the resuspension was used to inoculate fresh media containing 0, 10, 25 and 50 µg/ml SCP. These procedures were conducted in triplicate and repeated for 7 days.

To investigate freeze/thawing, 1g of soil from treatment 4 of the microcosm study was added to 50 % sterile glycerol (Sigma) and incubated overnight at -20 °C. The sample was defrosted at room temperature and used to make a dilution of 10^5 in sterile dH_20. Viable counts plates were prepared by plating 100 µl of the sample onto Iso-Sensitest agar containing 0, 10, 25 and 50 µg/ml SCP and incubated at 28 °C overnight.

2.7.3 Efflux pump experiment

A fluorimetric assay was carried out according to (Jones and Midgley, 1985). Strains were grown overnight in LB broth at 28 °C or 37 °C depending on the genus. The overnight cultures were centrifuged at 1500 rpm for 10 minutes and the cells re-suspended to their original OD_{600}. Flasks of 50 ml LB broth were inoculated to a starting OD_{600} of 0.05 and incubated at 28 °C. The cells were cultured to mid-log phase (OD_{600} 0.4-0.5), washed in fresh LB broth and re-suspended to an experimental OD_{600} of 0.2. Cells were kept on ice until needed and incubated for 15 mins at 28 °C, before their use in the fluorometeric assay. Ethidium bromide (Sigma) was added to a final concentration of 2 μg/ml and carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) (Sigma) was added to a final concentration of 50 μM. Fluorescence was measured on a SimAminsco Fluorometer with a MC320 Monochromator, at an excitation wavelength of 530 nm and an emission wavelength of 600 nm. Ethidium bromide is highly fluorescent when bound to DNA intracellularly, therefore when the molecule is transported via efflux pumps out of the cell the fluorescence will decrease. CCCP is a protonophore which uncouples the membrane potential and therefore deprives the MDR pumps of energy, decreasing efflux and hence an increase maybe observed in fluorescence measurements.

2.8 STATISTICAL ANALYSIS

2.81 ANOVA single variance analysis

CFU counts were analysed using Anova single factor variance (Microsoft Excel). Anova single factor variance performs a simple analysis of variance, testing the

hypothesis that two or more samples are equal (drawn from populations with the same mean).

2.82 Association analysis

Pearson's chi-square exact test was used to evaluate the linkage between genes (Uitenbroek, 1997). An association between two genes can result in a positive result, indicating that the genes are found together or linked in some way. A negative result may indicate that the genes are not found together or linked.

CHAPTER 3:

CHARACTERISATION OF BACTERIAL ANTIBIOTIC RESISTANCE FROM PIG SLURRY AND SOIL

Chapter 3: CHARACTERISATION OF BACTERIAL ANTIBIOTIC RESISTANCE FROM PIG SLURRIES AND SOILS

3.1 INTRODUCTION

Pharmaceutical antibiotics can enter agricultural soils and adjacent environmental compartments through the use of contaminated slurry (Boxall, 2000). The three antibiotics used in this study; SCP, tylosin and OTC have a range of sorption (low to high) and degradation properties which effect their presence and availability in the environment. This in turn determines the selective pressure on the bacterial populations. The period over which antibiotics are effective depends on their persistence in the environment as an active compound, its bioavailability to the bacteria and its potential for bioaccumulation in soil over time to effective concentrations at which it could exert a selective pressure.

Strict regulations exist for concentrations of usage and consumption of antibiotics in the food chain in humans and animals but until recently no such regulations existed to govern limits in soils or water other than drinking. The non-therapeutic use of antibiotics as growth promoters was banned in the EU, in 1999, but increasing knowledge into the persistence and a lack of knowledge as to bioavailability and biological activity of these antibiotics present in the environment has led to a plethora of studies into these area (Boxall et al., 2002; Boxall et al., 2003; EMEA, 1999).

3.1.1 Environmental availability of antibiotics

Different antibiotics have different mobility and sorption properties in the soil. Factors affecting antibiotic sorption and movement in the soils include; the structure of the antibiotic and polarity, the pH of the soil/slurry, soil minerals and metals, composition

of soil organic matter and chemical characteristics of the antibiotic compounds (Tolls, 2001).

Slurry and manure are heterogeneous and complex matrices varying between farms and contain a number of metal, ammonium and acetate ions as well as bicarbonate, fatty acids and phenols derived from bacterial decomposition of proteins in pig intestines at a pH of between 7 to 9 (De la Torre et al., 2000; Japenga and Harmen, 1990; Tunney and Molloy, 1975), all effecting the distribution behaviors of antibiotics and its application to soils, decreases soil pH, again effecting antibiotic movement. Slurry and manure also contains high quantities of organic matter affecting the sorption of the antibiotics in a number of ways, detailed individually for each of the three study compounds.

Sorption is defined as the processes of adsorption and absorption considered jointly. Sorption, in soils and slurries/manure, is represented by calculating the sorption coefficient (K_d (kg/l)), a measure of the ratio between the chemical concentration in the solid-phase and free water at equilibrium which is used in environmental fate models and risk assessments (Boxall *et al.*, 2002; Stuer-Lauridsen *et al.*, 2000). The K_d is calculated in batch-equilibrium experiments and does not accurately reflect the normal application of antibiotics via slurry or manure, hence it can be normalised to the K_{OC} (organic carbon normalised sorption coefficient) for comparison between studies (Loke *et al.*, 2002). Low sorption coefficients indicate high mobility and availability in soil. The order of sorption ranking, lowest to highest, in soil and manure, for our study compounds was as follows; SCP<tylosin<OTC (Loke *et al.*, 2002). Sorption is thought to occur rapidly but this may vary between antibiotic compounds (Thiele-Bruhn, 2003).

Table 3.1 Sorption coefficients of sulphachloropyridazine, oxytetracycline and tylosin in soils and slurry.

Antibiotic	Concentration (mg/g)	Sample soil:texture/pH/OC %	Kd (kg/l)	KOC (kg/l/C)	Reference
SCP		clay-loam/6.2/3.1	4	129	Tolls, (2001)
	0.05-20	clay-loam/6.5	1.8		Boxall et al., (2002)
	0.05-20	sandy-loam/6.8	0.9		
OTC	0.033-2	pig manure 6h/24h	83.2/77.6	195	Loke et al.,
	2.5-50	loamy-sand/6.1/1.6	680	42500	(2002)(2002)
	2.5-50	sand/5.6/1.4	670	47880	
	2.5-50	sandy-loam/5.6/1.1	1026	93320	Rabølle and Spliid
	2.5-50	sand/6.3/1.5	417	27790	(2000)
Tylosin	0.1-2	pig manure 6h/24h	45.7/240	110	
	1.25-25	loamy-sand/6.1/1.6	128	7990	
	1.25-25	sand/5.6/1.4	10.8	771	Loke et al.,
	1.25-25	sandy-loam/5.6/1.1	62.3	5660	(2002)(2002)
	1.25-25	sand/6.3/1.5	8.3	553	Rabølle and Spliid
	0.1-3	pig manure 24h	38.6/107.5	241/831	(2000)
					Kölz et al.,(2005b)

Abbreviations; OC % = Percentage of Organic content, h = hours of study

A summary of studies calculating sorption coefficients based on soil type or slurry are displayed in Table 3.1.

Bioavailability is a factor determining antibiotic effects on the soil environment. Adsorbed compounds may not be bioavailable to bacteria and it is an area of current

research where little or no data is available for some compounds. It is affected by a number of factors including: sorption to soil and sediment in water, dissociation of the chemical, degradation and transfer from its source (Boxall, 2000).

Biodegradation and photo-degradation may also have an effect on the concentrations of antibiotics that remain in the soil or reach the soil from slurry/manure. These processes are governed by the antibiotics structure and will vary from class to class, for example, macrolides are targets for fast degradation in soils, whereas a slower degradation by de-methylation is observed for sulphonamides, thereby decreasing their activity (Thiele-Bruhn, 2003). Sulphonamides and tetracyclines are both thought to be susceptible to photo-degradation (Halling-Sørensen et al., 2003a). Photo-degradation is known to decrease with increasing depth, therefore it may have no significant effect on the concentrations in soils, especially when they are spread onto soils as contaminants in slurry or manure (Thiele-Bruhn, 2003). Each antibiotic and their photo or bio-degradation is discussed in more detail in the following sections.

There predictive models have been produced for the fate and effects of pharmaceuticals, metabolites and degradation products in the environment which are commonly based on structure-property, structure-biodegradability and structure-activity relationships (Montforts, 1997; Spaepen et al., 1997). Two models are widely used in environmental risk assessment; the Uniform Approach for Estimating Concentrations of Veterinary Medicines in Soil and model developed by the Dutch National Institute of Public Health and the Environment (RIVM), but are treated with caution as they predict 'worst case' concentrations and scenarios in the environment (Montforts, 1997; Spaepen et al., 1997).

3.1.2 Sulphachloropyridazine in the environment

Between 40 % and 60 % of the parent sulphonamide is excreted when given orally to animals, which may be further degraded in storage or break down products converted back into the parent compound (Berger et al., 1986; Halling-Sørensen et al., 2001). Langhammer, (1989) reported up to 39 mg/l of sulphonamides in slurry after an oral dose to swine, decreasing to a maximum of 60 % of the initial concentration, over a 5 week period.

The sulphonamides low sorption and high mobility in soil and slurry/manure matrices is attributed to its structures low polarity resulting from variable protonation of the amino group and SO_2NH moiety at pHs 2-3 and 5-11 (Fig. 1.1) (Boxall et al., 2002; Thiele, 2000; Tolls, 2001). This group of antibiotics high mobility can subsequently lead to its leaching into ground waters and surrounding environments when the antibiotic is applied to soils via slurry or deposited in waste waters (Boxall et al., 2002; Karthikeyan and Meyer, 2005; Langhammer, 1989). Sulphonamide sorbance is increased with the application of slurry, possibly due to decreases in pH and a coupling with organic matter (Boxall et al., 2002). The type of soil greatly effects its mobility in comparison to the other antibiotics, with coarse silt adsorbing sulphonamides more strongly than fine silt (Thiele-Bruhn and Aust, 2004). Studies by both Thiele-Bruhn and Aust (2004) and Bialk et al., (2005) concluded it was the phenolic carboxylic groups, N-heterocyclic compounds and decomposition products, the "peroxide-mediated covalent cross-linking with syringic and protocatechoic acids" that were the preferred binding sites of sulphonamides in soil and slurry.

A number of studies have been carried out which have looked at the mobility of sulphonamides in the soils (Table 3.1). These studies have relied upon the accurate

measurement of sulphonamide concentrations using a number of methods; HPLC with fluorescence (Maudens et al., 2004) or UV detection (Blackwell et al., 2004) and ELISA methods (Spinks et al., 2001). With the exception of the study carried out by Boxall et al., (2002) of which this project samples originate, all the investigations into sulphonamide sorption and mobility have proceeded under laboratory conditions in batch column experiments (Table 3.1). One study alone has calculated sorption/mobility in soils and slurry for SCP, Boxall et al., (2002), but numerous investigations into other members of the sulphonamide group of antibiotics exist with K_d values varying between 0.9 to 10 kg/l, converting to K_{OC} values of between 80 and 323 kg/l (Tolls, 2001). These figures display no correlation between sorption and soil clay content, but do record an association between increasing organic carbon content and increased sorption for sulphonamides (Boxall et al., 2002; Tolls, 2001).

No experiments into degradation have specifically investigated SCP, but a study by Ingerslev and Halling-Sørensen, (2000) concluded that this process was identical in several different sulphonamide compounds and therefore all sulphonamides could be tested for biodegradability as a group and not individually. Ingerslev and Halling-Sørensen, (2000) observed a 50 % degradation of various sulphonamides in activated sludge simulation tests over a 28 day period, in contrast to Frankenberger and Tabatabai, (1982) who reported no biodegradation in various soils over a 14 day study and Langhammer et al., (1990) who only recorded levels of 0.2 to 0.7 % biodegradation over a 64 day trial. Although Halling-Sørensen et al., (2003a) did not observe a significant reduction of sulphadiazine concentrations in a series of soil interstitial water and activated sludge experiments, but they did report a 50 % decrease in potency under light conditions, concluding that photo-degradation had occurred.

3.1.3 Oxytetracycline in the environment

Approximately 2,500 tonnes of tetracycline compounds are used annually in Europe through veterinary therapies and of a given dose only 20 % is metabolised by livestock, the remainder passing through the animal, extruded as an active parent compound with a high potential of reaching the environment (Boatman, 1998; Halling-Sørensen et al., 2002).

OTC has a high absorbance dictated by its structure which can readily complex with divalent cations. The structure of OTC permits a number of potential binding mechanisms to soil and slurry matrices; complexing to divalent cations, ion exchange, and hydrogen bridging from acidic groups of humic acids to polar groups of the tetracycline (Sithole and Guy, 1987a, b).

As with the sulphonamides, a number of studies investigated the mobility of tetracyclines in the soil, also relying on techniques such as HPLC (Kay et al., 2004) and mass spectrometry (Delepee et al., 2000), as well as a radioimmunoassay (Yang and Carlson, 2004), a potentiometric sensor (Sun et al., 2004) and an ELISA method for accurate measurements of OTC concentrations in soils or slurries (Aga et al., 2003). OTC has a calculated K_{OC} of above 4000 kg/l and K_d values which range from 290 to 1620 kg/l (Rabølle and Spliid, 2000). These figures reflect OTC's immobility and high persistence in soils, although its sorption is dependent on soil type and pH, adsorbing more strongly to clay soils and those with a low pH, but with no significant desorption occurring over time (Figueroa et al., 2004; Kulshrestha et al., 2004; Rabølle and Spliid, 2000). OTC's high sorption and immobility is possibly influenced by the formation of strong complexes with the manure and soil matrices by ionic binding to divalent cations such as Mg^{2+} and Ca^{2+}, humic acids and metal ions (Loke et al., 2002). MacKay and

Canterbury (2005) investigated OTC sorption to organic matter with respect to metal-bridging, concluding that with increased K_{OC} there was an increase in formation of OTC-FeII complexes within the humic acids of manures and soils. OTC therefore ranked as the antibiotic which was sorbed the highest to the soils and manure used in their study.

OTC has been observed to be photo-degraded within the first mm of soil, producing up to 6 different bi-products with toxicity levels lower than the parent compound and in some cases by bio-degradation (Balmer et al., 2000; Oka et al., 1989). The photo-degradation half-life of OTC has not been measured in soils, but Bjørklund et al., (1990) and Ingerslev et al., (2001) calculated the t½ in aquatic systems to be between 9 to 419 days, these rates were not effected by seasonal variation or location. With regards to bio-degradation, van Gool, (1993) observed no significant bio-degradation of OTC in soil and contaminated manure over 180 days, in contrast to studies in which a 50 % bio-degradation of the compound was recorded in aerobic sediments over 43.8 days (Ingerslev and Halling-Sørensen, 2001; Ingerslev et al., 2001). A study in which Halling-Sørensen et al., (2003b) investigated SCP degradation in soil interstitial water also investigated photo and biodegradation of OTC observing a 5.3 and 5.9 % dark/light reduction in potency of the antibiotic, MIC^{50} results indicating by-products of degradation were still biologically active. Resistance to these degradation compounds can be conferred by the *tet(B)*, *C* and *E* genes in *E. coli* (Halling-Sørensen et al., 2002).

The bioavailability of sorbed OTC in soils is still unclear but a number of whole-cell and cell-free biosensors have been developed which could answer this question. As yet, a biosensor has not been developed for use in the field and only one study has been carried out in soil microcosm studies; Hansen et al., (2001) produced GFP constructs

linked to a tetracycline-inducible promoter (P_{tet}) and a regulatory gene (*tetR*) for use in combination with FACs. This whole-cell biosensor was used in the qualitative detection of OTC production by *S. rimosus* in soil microcosms. Other studies have used whole-cell biosensors to detect OTC where it has been used in animals, for example in fish (Pellinen et al., 2002), pig faeces (CTC) (Hansen et al., 2002) and in milk from cows (Hansen and Sørensen, 2000). Pellinen et al., (2004) produced a similar, but cell-free system too Hansen et al., (2001), based on *tetR* and luciferase reporting increased sensitivity than the whole-cell system and omitting the use of GMOs. Chander et al., (2005) investigated the antimicrobial properties of sorbed tetracycline and tylosin in soils, on resistant and sensitive strains of *Salmonella* and *E. coli*. The study examined the sorption of the antibiotics with altering concentrations of spiked doses in clay and sandy soils, binding being higher in the clay soils and levels of tetracycline sorption calculated to be higher than tylosin. The study also observed that with increasing sorbancy, the CFU of all the bacterial strains decreased, a greater decline observed with tetracycline either due its higher concentrations in the soils compared to tylosin or a lower MIC, concluding that the antibiotics were sorbed to the soils whilst retaining their antimicrobial properties resulting in the growth inhibition of the bacteria.

3.1.4 Tylosin in the environment

Of a given oral dose of tylosin to livestock, 22 % is excreted, the majority identical to the parent compound mix, having a high potential of reaching the environment (Section 1.2.3) (van Leeuwen, 1997). The chemical study of tylosin (A) confers a pKA value of 7.7, therefore in manure or soil, the compound is both protonated and an uncharged molecule, binding to negatively charged sites such as soil and manure but not

complexing with metal ions as OTC (Loke et al., 2002; McFarland et al., 1997; Pharmacopoeia, 1999). HPLC with UV detection is the common technique for quantitative detection of tylosin in the environment (Loke *et al.*, 2000).

Table 3.1 summaries a number of studies into tylosin sorption with K_d values ranging from 8.3 to 240 kg/l and K_{OC} values ranging from 110 to 7990 kg/l (Kölz et al., 2005a; Loke et al., 2000; Rabølle and Spliid, 2000). These K_d values are significantly lower than OTC, demonstrating soils low sorption of tylosin and its high mobility through the soil matrix, although sorption is higher and mobility lower than for the sulphonamide class of antibiotics (Rabølle and Spliid, 2000).

Biodegradation of tylosin has been demonstrated; Loke et al., (2002) determining that a decrease in the free fraction not adsorbed by pig manure over time may have been due to biodegradation, or the use of non-sterile manure and observed by Loke et al., (2000) in an earlier study where a decrease in the concentration of tylosin A was examined under aerobic and methanogenic conditions, with an increase in detectable degradation products in slurry tanks, advising that under average farming practices of leaving slurry in tanks for 90 days before spreading, the concentration of active tylosin being applied to farmland would be negligible. Both Ingerslev, (2001) and Kölz et al., (2005a) calculated similar degradation rates to Loke et al., (2000; 2002) with a degradation rate quoted in slurry/sand or sandy loam combinations of 50 % over a period of 4.2 and 5.7 days in the former study and 22 % as an initial rapid rate loss and a following slow removal phase of 90 % over periods of 30 to 130 hrs for anaerobic slurry and 12-26 hrs for aerated slurry in the latter. In contrast, Gavalchin and Katz, (1994) calculated no significant biodegradation in a sandy loam and manure mix over a period of 30 days. Halling-Sørensen et al., (2003b) demonstrated that tylosin may be photo-degraded in an

activated sludge experimental system where it became less biologically active in light compared to a dark system.

3.1.5 Microbial diversity in soils and slurries

It is estimated that only 0.1 % to 10 % at the most of microorganisms have been cultured and characterised, leaving the soil environment to a large extent uncharted (Amann et al., 1995). The stumbling block for characterising the soil bacterial community has been the diversity, complexity and variability dependent upon a large number of factors including soil type, chemical composition and amount of organic compound, ecosystem and biological organisation; the bacterial species present, their abundance, evenness and seasonal variations (Sessitsch et al., 2001; Smit et al., 2001; Torsvik and Ovreas, 2002). Smit et al., (2001) reported that soils with a higher concentration of available nutrients had higher numbers of Alpha and Gammaproteobacteria, potentially a selection of high growth rate bacteria and displayed seasonal variation, in a study of agricultural soils from Lovinkhoeve, The Netherlands.

Soils from a wide range of ecosystems have been assessed for their microbial communities, differing in the presence of a number of specific bacterial phyla which define the soil type, in general the percentage distributions of a defined number of phyla change in relation to the soil type and environmental disturbances, the changes in phyla possibly linked to a change in biochemical activities in the soils. A study into the distributions of bacterial phyla in agricultural soils, observed dominance (27 % of isolates) of Proteobacteria and 11.1 % identified as Actinobacteria (Nagy et al., 2005). One further investigation into the microbial diversity of agricultural soils used a culture-independent method to interrogate a clover-grass pasture in Southern Wisconsin, in the US. The report observed that 98.4 % of sequences belonged to bacteria, and of this,

16.1 % belonged to the Proteobacteria group, 21.8 % to low GC, Gram positive groups and 21.8 % were characterised as members of Cytophaga-Flexibacteria-Bacteriodes (Borneman et al., 1996).

An investigation by Snell-Castro et al., (2005) reported the microbial diversity of pig manure storage pits by small subunit rDNA partial sequences, observing that 36 % of sequences belonged to bacteria, of which 15 % were *Clostridium* and 20 /10 % members of the groups Bacillus-Lactobacillus-Streptococcus, *Mycoplasma* and relatives respectively. Archeal and eukaryotic sequences were also identified. The investigation concluded that the diversity in the pits bore a close resemblance to the diversity of bacteria found in the pig gastrointestinal tract.

A number of reports have also highlighted the effects of manure application to agricultural land on the soil microbial community. Ibekwe et al., (2003) reported a community shift towards dominating bacteria originating in the gastrointestinal tract of animals and Cotta et al., (2003) also identified predominating Gram positive, low GC, obligate anaerobes in microbial communities in swine faeces, manure storage pits and soils with freshly applied slurries. Onan and LaPara, (2003) investigated the soil bacterial community in 6 agricultural soils with different subtherapeutic levels of antibiotic use and observed a dominance of tylosin resistant Alpha and Betaproteobacteria in soils with a subtherapeutic level of antibiotic usage compared to *Streptomyces*-like tylosin resistant bacteria (high GC content) in soils where no usage has occurred.

Antibiotic resistance genes have been observed in clinical isolates from a wide range of bacterial genera but it is thought the reservoir for these genes exists in environmental bacteria (Arthur et al., 1987), originating (Suller and Russell, 1999), at least in part, from antibiotic-producers such as the Actinobacteria (Matsuoka and Sasaki, 2004; Normark and Normark, 2002; Sundin and Bender, 1996).

3.2 AIMS

The a number of the reports summarized above provide evidence for the persistence of the three study antibiotics in the environment after entry through slurry applications and a number of these already discussed, have reported changes in the bacterial soil community diversity (Buckley and Schmidt, 2003; Onan and LaPara, 2003), effects on microbial activity (De la Torre et al., 2000) and effects on resistance profiles as a result (Aarestrup et al., 2001; Sengeløv et al., 2003). The release of continual sub-lethal doses of antibiotics via slurry into the environment may cause the development or acquisition of single, cross and multiple resistance in bacteria over time within the indigenous bacterial population or surviving enteric bacteria applied through slurry serving as potential reservoirs of resistance genes and mechanisms (Thiele-Bruhn, 2003; Wegener, 2003). Few studies have investigated the effects of continual doses or doses of antibiotic directly applied through slurries, but of those few there is evidence to suggest that effects are observed on the soil microbial population (Buckley and Schmidt, 2003); decreases in activity, growth (Chander et al., 2005), community diversity and numeration of resistant bacteria or numbers and types of resistance genes (Schmitt et al., 2004; Westergäard et al., 2001). These studies are lacking total community analysis and tracking of specific resistance genes within slurries and within soils before and after slurry application, especially where slurries are known to harbour resistant bacteria with

the potential for the HGT of resistant genes and manure enhancement of transfer (Götz and Smalla, 1997).

As well as the effects on microbial resistance, due to the application of slurry, diversity studies using 16S rRNA sequences from culturable isolates provided an ideal opportunity to investigate environmental reservoirs of resistant bacteria and genes, and, determined shifts in the microbial population.

This chapter aimed to enumerate the resistant and sensitive culturable bacteria in a number of different slurries; a control (from tylosin fed-pigs), organic and from the Cranfield study (tylosin fed-pigs with SCP and OTC amendment), and soils before and after the application of the Cranfield slurry. The objectives were to observe whether there was an impact and therefore a shift in the numbers of resistant bacteria and their community diversity due to the application of slurry containing antibiotics in the Cranfield soil. Specifically the study aimed to investigate the prevalence of resistant bacteria with respect to antibiotic application using direct bacterial count methods and phylogenetic studies of cultured isolates.

3.3 RESULTS

3.3.1 Bacterial resistance to sulphachloropyridazine

Bacterial CFU counts and RQ values were calculated for three different slurry types, with a range concentration of SCP from 0-100 μg/ml (Figs. 3.1a and d). Due to the differences in sample treatment, the Cranfield and control slurries were frozen before analysis, no comparison could be made between these and the organic slurry as this was used fresh.

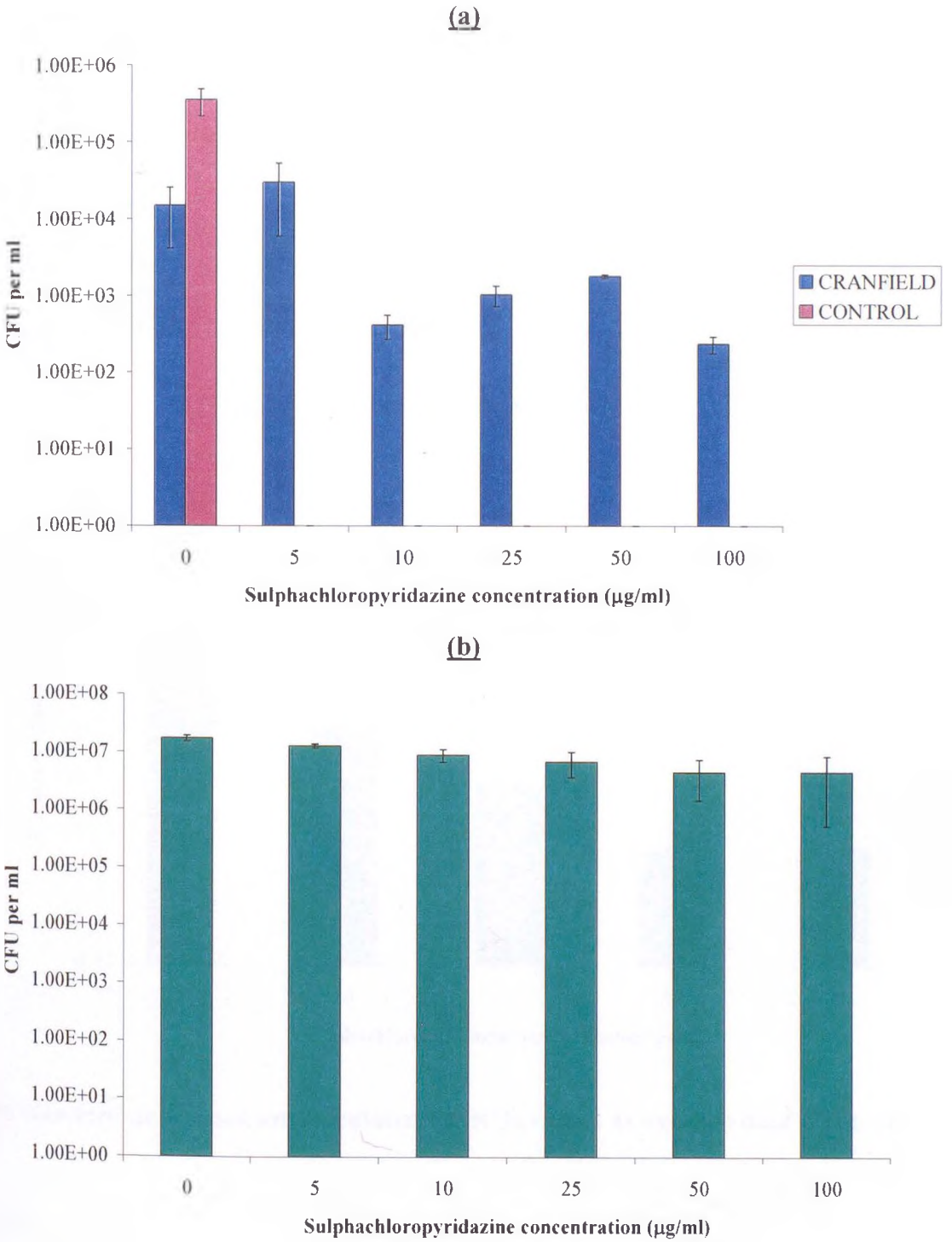
High bacterial CFU counts of between 10^7 and 10^8 CFU per ml of slurry were observed in the organic slurry, with little or no decrease in counts with increased concentrations of SCP (Fig. 3.1b). The RQ values for the organic slurry at 5 µg/ml SCP were 73 %, which decreased with increasing concentrations of SCP to 2 % at 100 µg/ml SCP (Fig. 3.1d).

The CFU counts were observed to be between 10^2 and 10^4 CFU per ml of Cranfield slurry and RQ values were recorded at 100 % with 5 µg/ml SCP decreasing with increasing concentrations of the antibiotic (Fig. 3.1a and c). The control slurry displayed no counts of SCP resistant bacteria1.

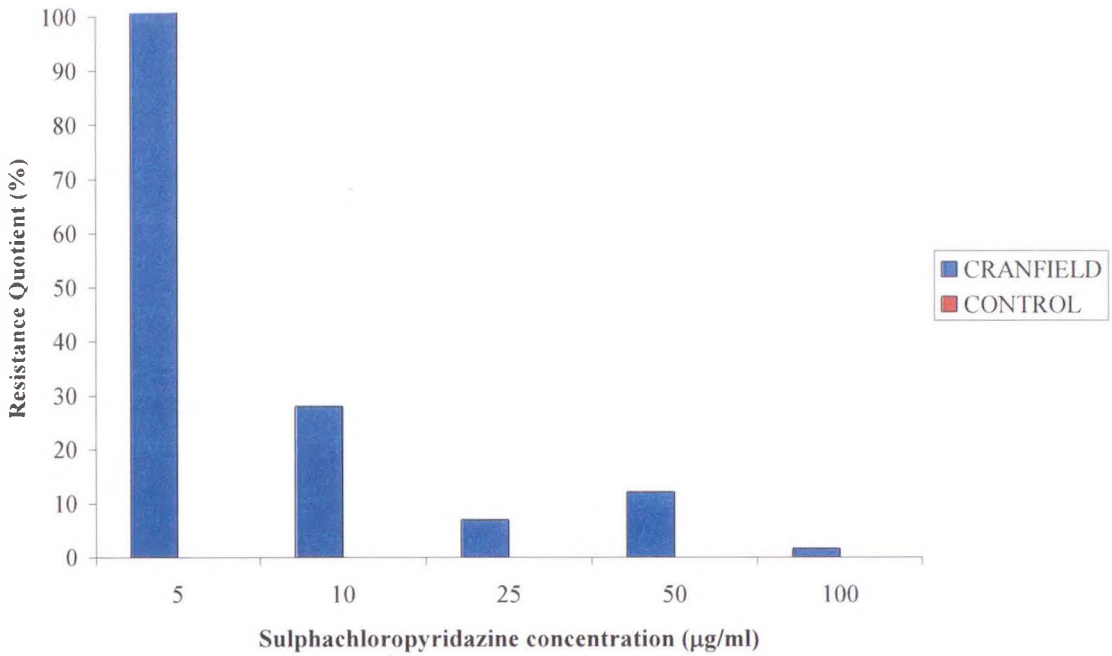
Fig. 3.2b exhibited a bimodal curve for the Cranfield slurry bacterial RQ values, decreasing from over 100 % at 5 µg/ml SCP to 7 % at 25 µg/ml and increasing to a second peak at 50 µg/ml. This trend was also observed to a lesser extent in the organic slurry.

Bacterial CFU counts and RQ values were calculated with increased concentrations of SCP and compared between soil cores taken at 12 different time points throughout the two years of the study and compared to the Cranfield slurry, which was applied at day 0 (Figs. 3.2a to d). Counts for CFU, in the Cranfield soil cores, decreased with increasing concentrations of SCP and with time, over both years (Figs. 3.2 a and c). High CFU counts were recorded throughout year 1 and year 2, up to 50 µg/ml SCP at year 1, day 289 (Fig. 3.3a) and year 2, day 1 (Fig. 3.3c), whereas bacterial colonies were present at 100 µg/ml SCP only in the Cranfield slurry (Fig. 3.1a). When CFU were normalised to RQ values, resistance was observed to be higher in the Cranfield slurry than the soil cores and recorded on 100 µg/ml SCP plates compared to 50 µg/ml for year 1, day 289 and year 2, day 1 soil cores (Figs. 3.2 b and d).

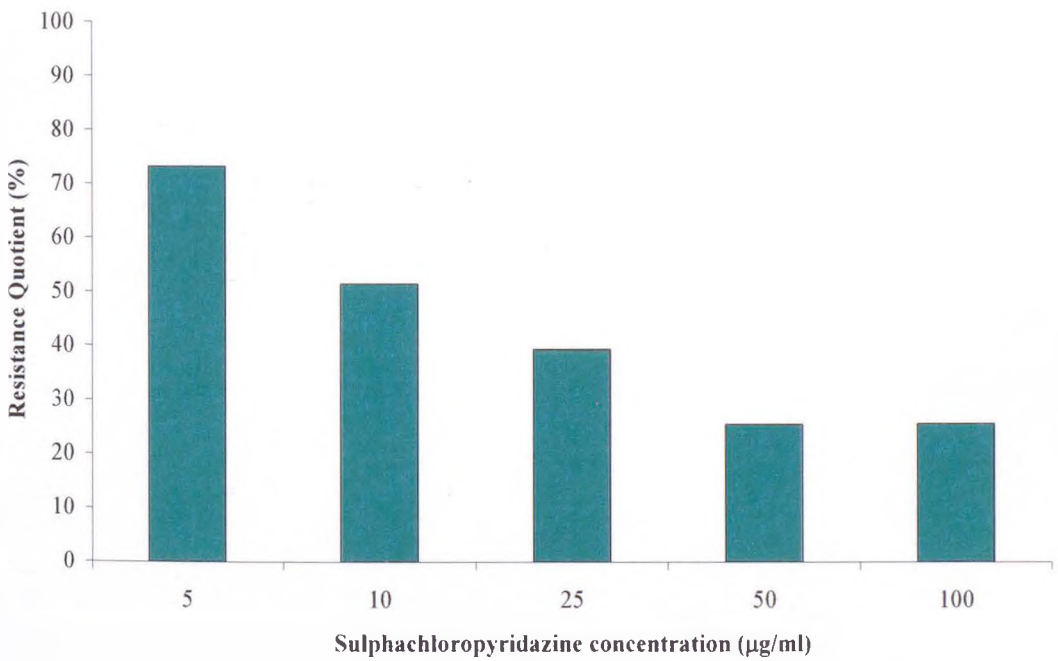
Fig. 3.1 Bacterial CFU (± standard deviations) for (a) Cranfield and control pig slurries (b) organic slurry and RQs (c) for Cranfield and control slurries and (d) from organic slurry, with increased concentrations of sulphachloropyridazine.



(c)



(d)



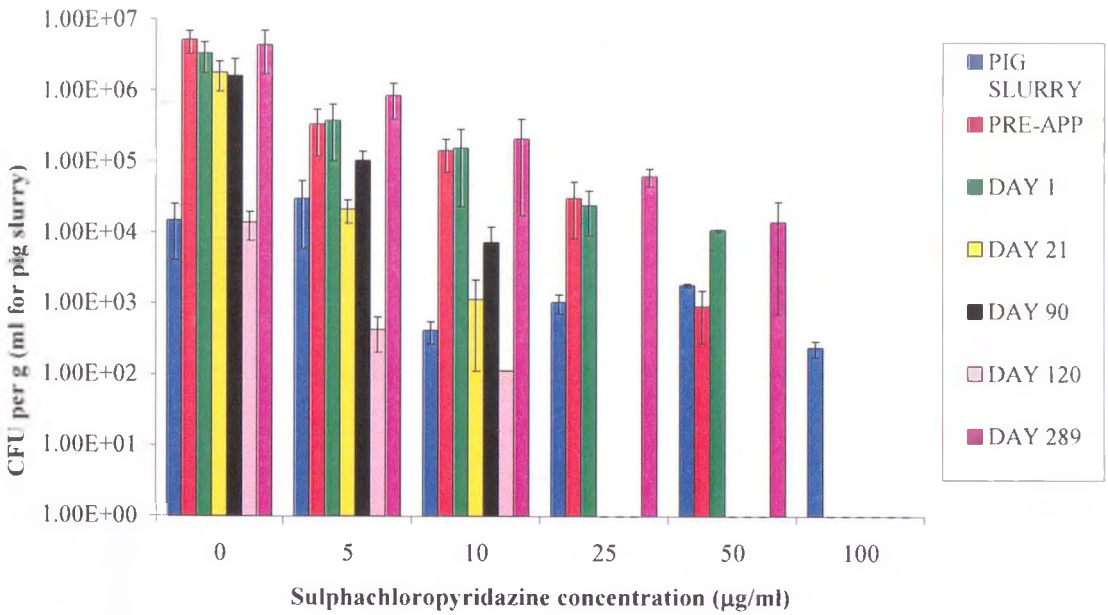
No standard deviations are calculated for RQs values as average data was used.

The RQ value for year 2, day 120 for 5 μg/ml seemed elevated in comparison to the other data points for year 2, day 120 SCP concentrations and other time points.

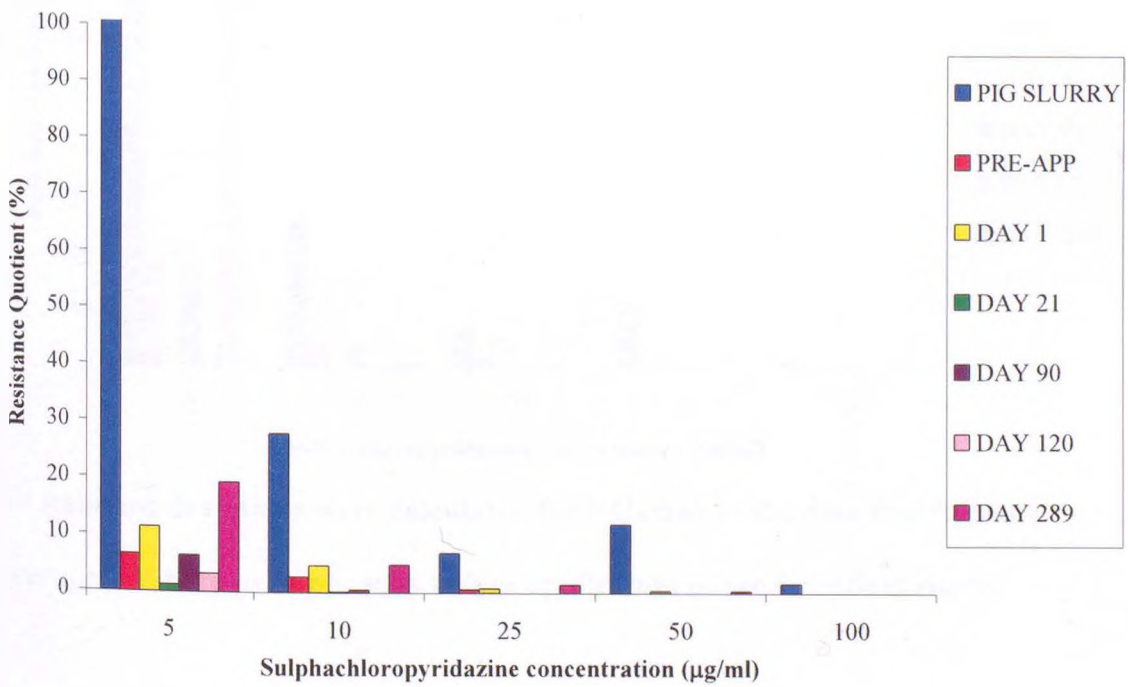
ANOVA tests were performed and determined that there was a significant difference, at each time point, between the CFU counts with increasing concentrations of antibiotic in both years of the study (Tables 3.2 and 3.3) and established that there was also a significant difference, at each concentration of antibiotic, between the CFU counts over time from pre-application to year1, day 289 to year 2, day 240 (Tables 3.4 and 3.5). This significant difference between time points and concentrations was observed for each of the study compounds.

Fig. 3.2 Bacterial CFU (± standard deviations) (a) and RQs (b) of Cranfield slurry and soils from years 1 and 2 (c and d) with increased concentrations of sulphachloropyridazine.

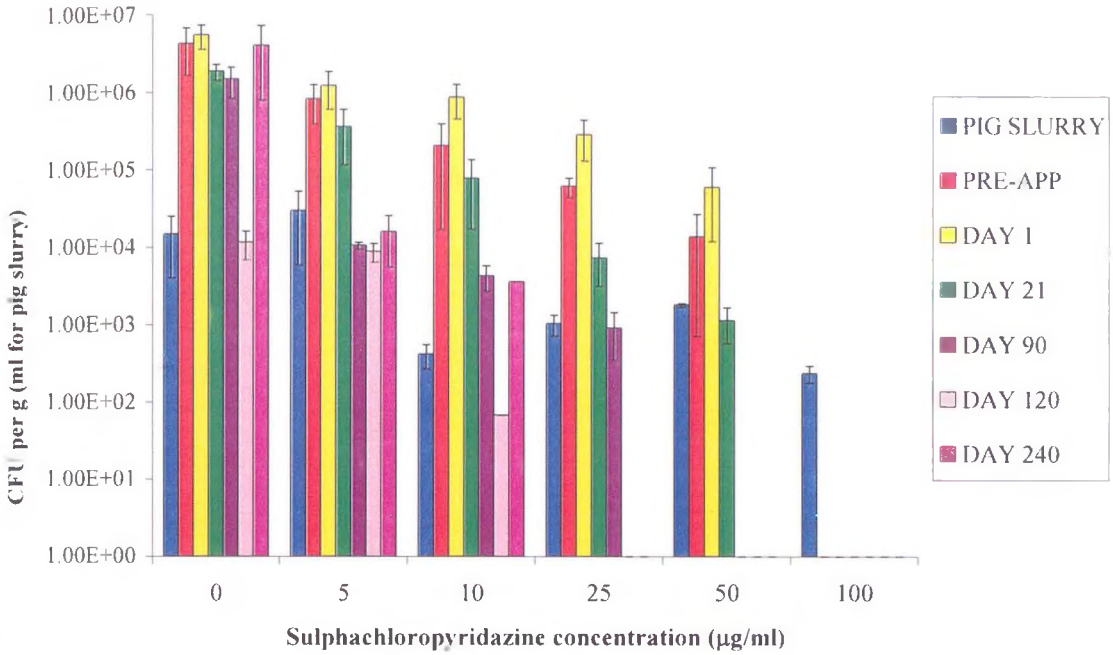
(a)



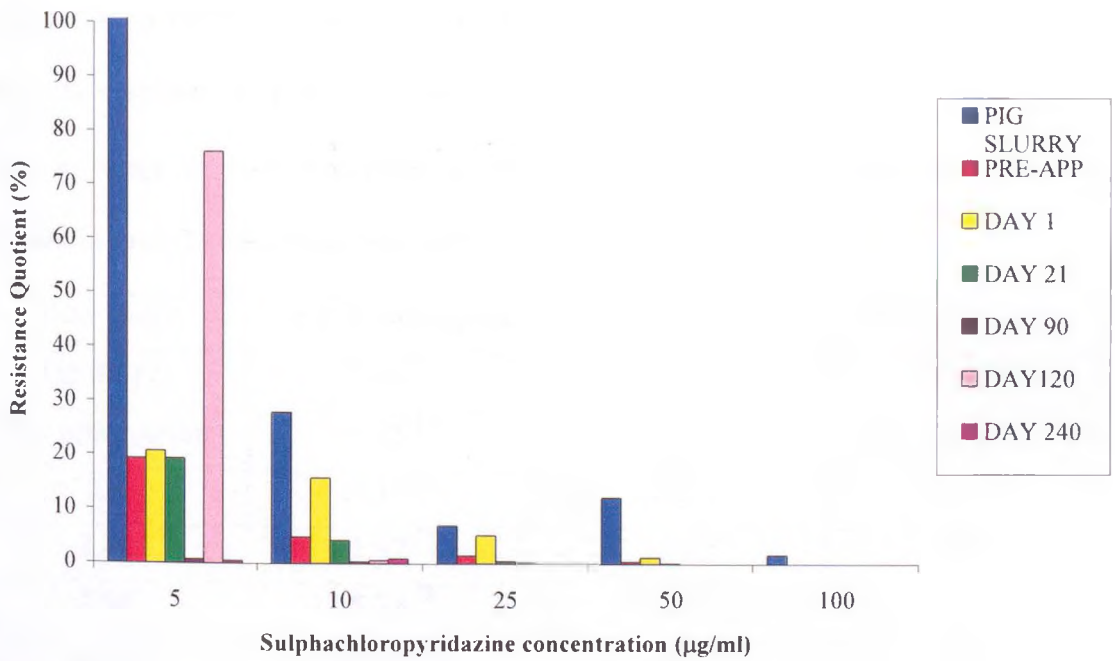
(b)



(c)



(d)



No standard deviations were calculated for RQs due to the data drawn using averages. Where pre-app: soils before application of the Cranfield slurry.

Table 3.2 p values for ANOVA single factor variance analysis assessing whether there is a statistical difference between the CFU counts at different time points over a range of concentrations of sulphachloropyridazine, oxytetracycline and tylosin in year 1 soils, where p= 0.05.

Time point	SCP 0-100 µg/ml	OTC 0-25 µg/ml	TY 0-50 µg/ml
Pig Slurry	2.43^{-7}	0.000309	0.001258
Pre-application	9.22^{-17}	6.70^{-17}	7.50^{-17}
1,1	7.98^{-16}	1.16^{-13}	8.18^{-9}
1,21	8.26^{-17}	7.64^{-17}	6.75^{-19}
1,90	8.49^{-9}	3.71^{-9}	2.46^{-7}
1,120	2.51^{-17}	6.65^{-11}	3.77^{-20}
1,289	1.85^{-11}	4.31^{-12}	3.33^{-2}

Table 3.3 p values for ANOVA single factor variance analysis assessing whether there is a statistical difference between the CFU counts at different time points over a range of concentrations of sulphachloropyridazine, oxytetracycline and tylosin in year 2 soils, where p= 0.05.

Time point	SCP 0-100 µg/ml	OTC 0-25 µg/ml	TY 0-50 µg/ml
Pig Slurry	2.43^{-7}	0.000309	0.001258
Pre-application	1.85^{-11}	4.31^{-12}	3.33^{-2}
2,1	7.19^{-19}	1.63^{-10}	1.34^{-12}
2,21	1.97^{-25}	2.78^{-21}	9.94^{-22}
2,90	8.75^{-18}	1.0^{-17}	4.12^{-18}
2,120	2.63^{-16}	1.79^{-10}	1.2^{-25}
12,240	1.94^{-8}	1.98^{-8}	1.39^{-9}

Table 3.4 p values for ANOVA single factor variance analysis assessing whether there is a statistical difference between the CFU counts at different concentrations of sulphachloropyridazine, oxytetracycline and tylosin over all time-points in year 1 soils, where p= 0.05.

Antibiotic concentration (µg/ml)	SCP	OTC	TY
0	8.58^{-7}	1.44^{-10}	1.44^{-10}
0.2	ND	4.03^{-8}	ND
1	ND	1.68^{-5}	0.005029
5	2.73^{-5}	4.80^{-7}	0.002227
10	2.96^{-5}	1.42^{-2}	0.005391
25	1.07^{-2}	4.10^{-3}	0.003037
50	2.63^{-2}	ND	8.62^{-9}
100	No bacteria detected	ND	2.32^{-8}

Table 3.5 p values for ANOVA single factor variance analysis assessing whether there is a statistical difference between the CFU counts at different concentrations of sulphachloropyridazine, oxytetracycline and tylosin over all time points in year 2 soils, where p= 0.05.

Antibiotic concentration (µg/ml)	SCP	OTC	TY
0	5.5^{-7}	4.31^{-12}	2.19^{-7}
0.2	ND	5.46^{-22}	ND
1	ND	1.65^{-8}	0.000904
5	1.24^{-10}	1.47^{-5}	0.000187
10	1.2^{-13}	0.013234	0.001284
25	3.64^{-11}	0.013741	0.003584
50	3.81^{-7}	ND	3.4911
100	No bacteria detected	ND	9.6^{-6}

3.3.2 Bacterial resistance to oxytetracycline

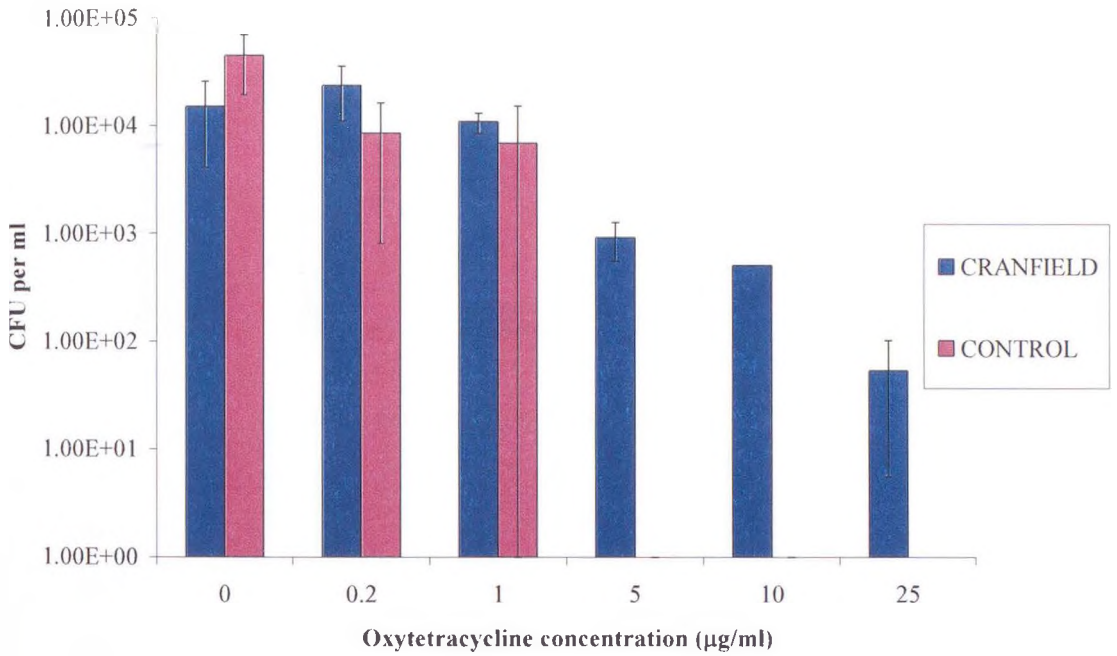
Bacterial CFU counts and RQ values were calculated for three different types of slurries, with increased concentrations of OTC from 0 to 25 µg/ml (Figs. 3.3a and 3.3d). As with the CFU counts on SCP, high numbers of bacteria (between 10^6 and 10^8 CFU per ml of slurry) were enumerated on selective plates containing OTC from the organic slurry (Fig. 3.3b). CFU counts were collected up to 25 µg/ml OTC, in the case of the Cranfield slurry, with counts of between 10^4 CFU per ml on non-selective media and 10^2 on plates containing 25 µg/ml. A decrease in CFU was observed in the Cranfield slurry with increasing concentrations of OTC. The control slurry displayed CFU on selective plates containing up to 5 µg/ml OTC, compared to no SCP resistance bacteria at this concentration.

When the CFU data was converted to RQ values, the Cranfield slurry displayed resistant bacteria up to 25 µg/ml OTC, as also recorded in the CFU counts (Fig. 3.4a and C). The organic and Cranfield slurries exhibited lower RQ values in comparison to their respective RQ values on SCP selective media and neither the CFU counts nor the RQ values of OTC followed the bimodal curves demonstrated with increasing concentrations of SCP (Figs. 3.1c and 3.3b).

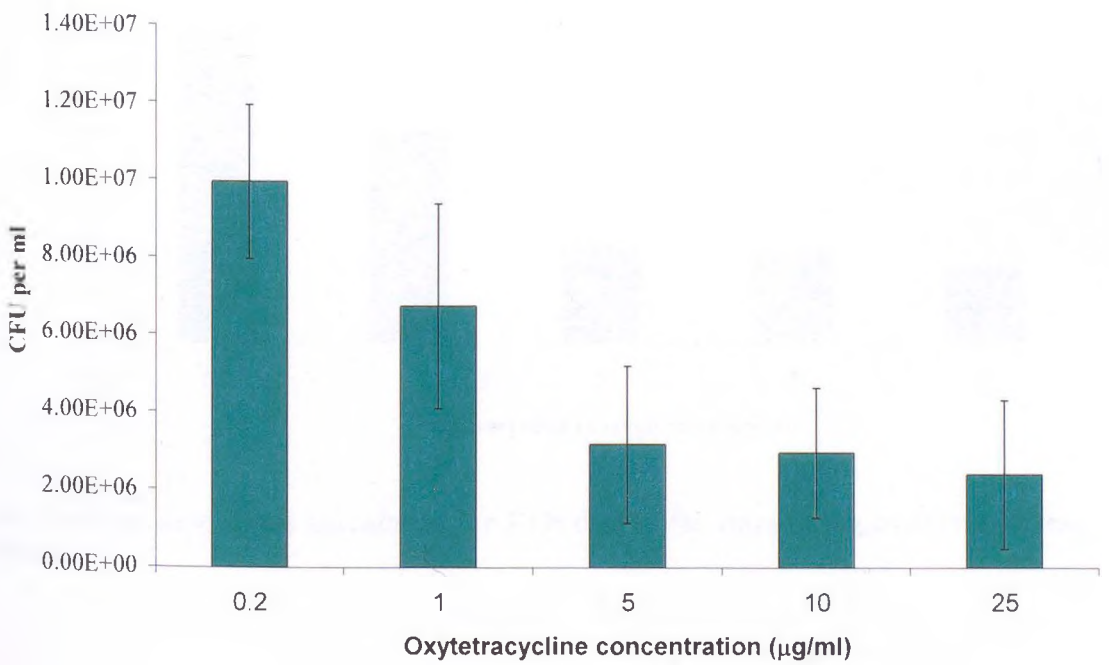
Bacterial CFU counts and RQ values were calculated and compared between the Cranfield soil cores taken at 12 different time points throughout the two years of the study and compared to the Cranfield slurry which was applied at day 0 (years 1 and 2) and a control soil with increased concentrations of OTC (Figs. 3.4a to d). In summary for all samples there was a general trend of a decrease in CFU with time and with increased concentration of OTC (Fig. 3.5a and c).

Fig. 3.3 Bacterial CFU (± standard deviations) for (a) Cranfield and control pig slurries (b) organic slurry and RQs (c) for Cranfield and Control slurries and (d) from organic slurry, with increased concentrations of oxytetracycline.

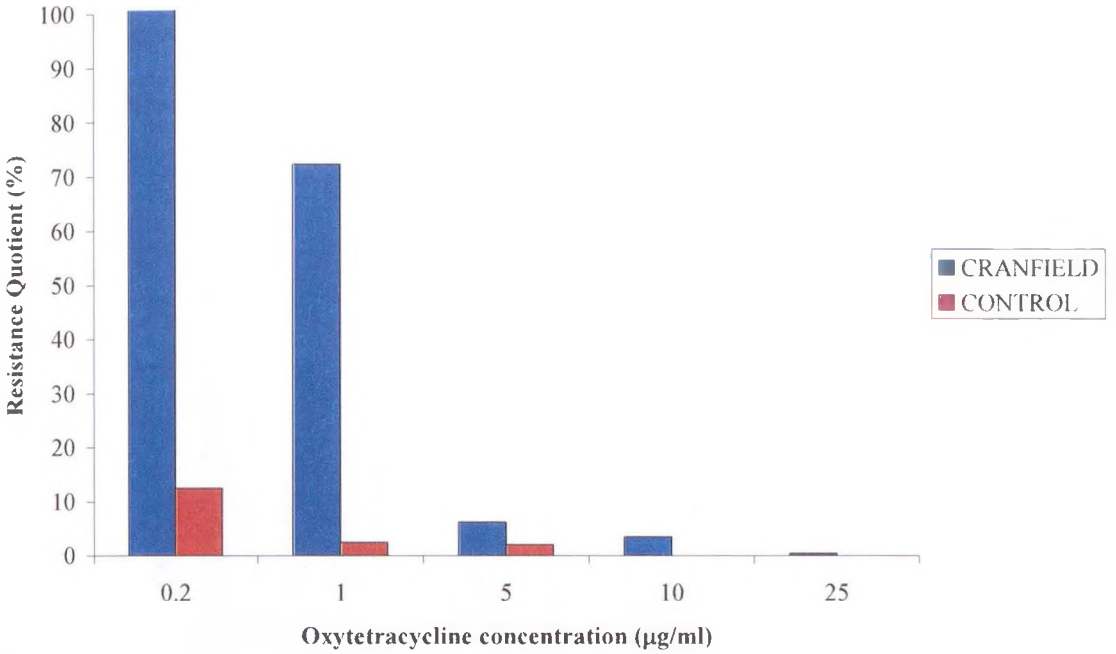
(a)



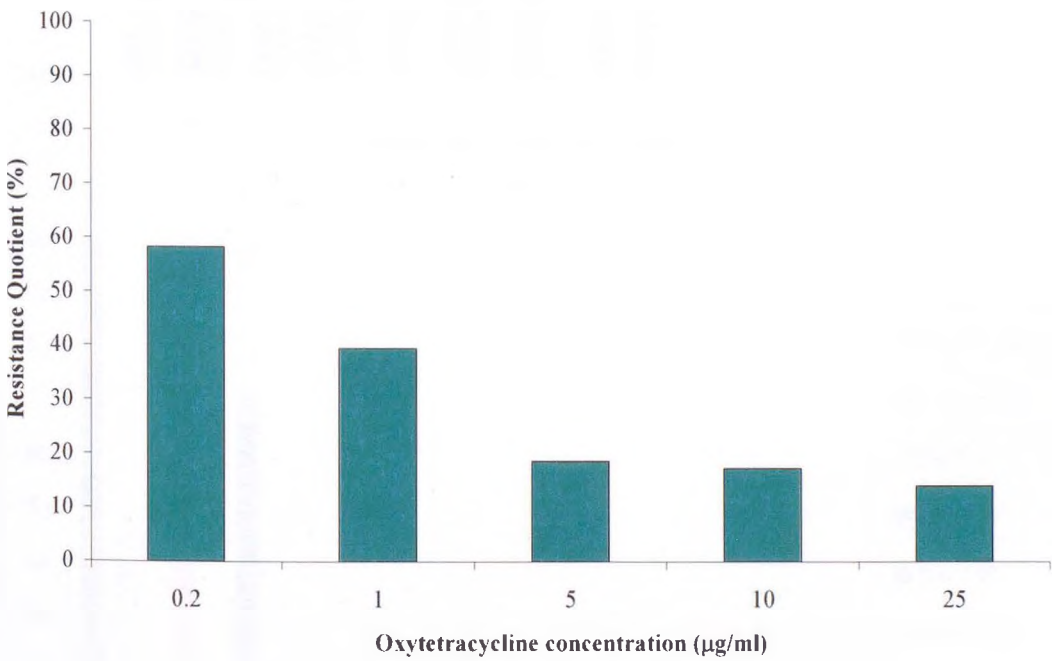
(b)



(c)



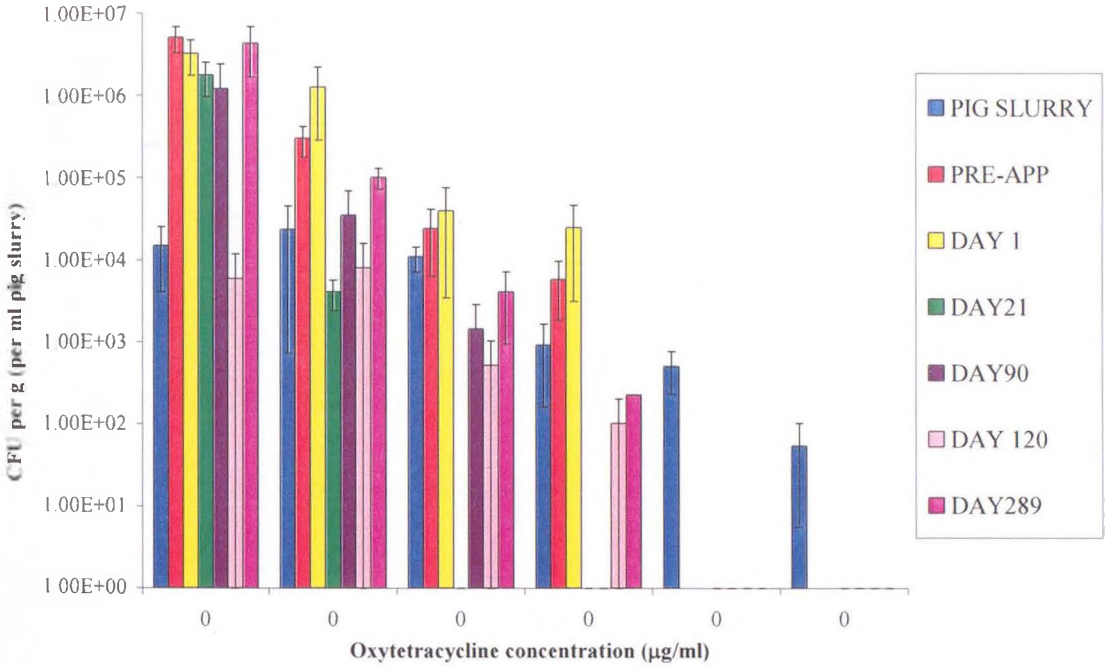
(d)



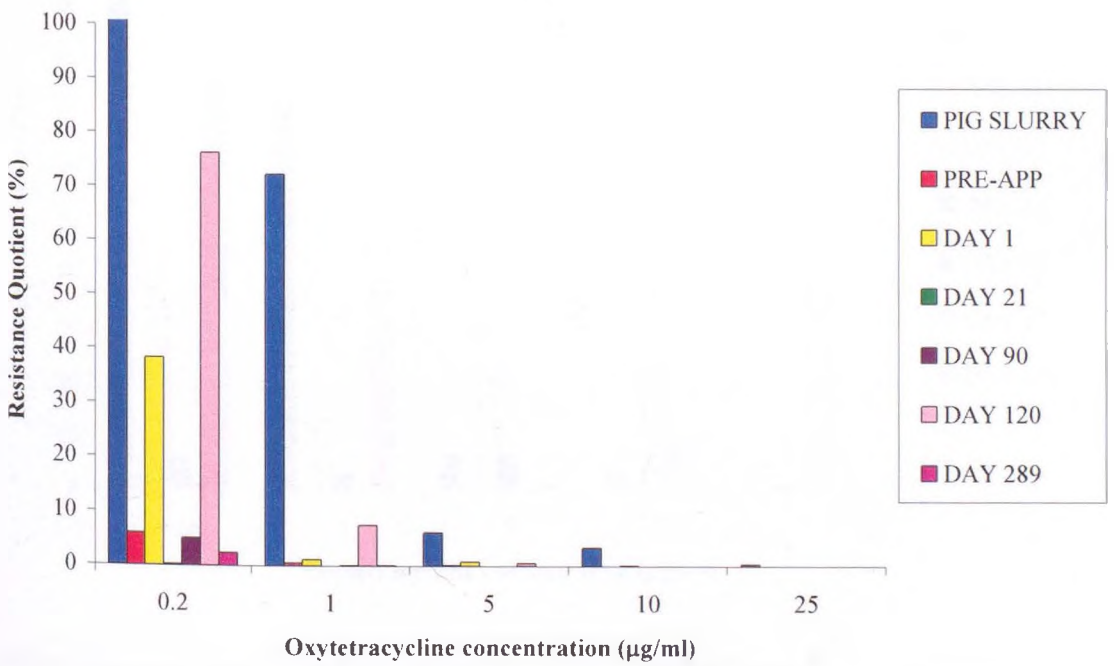
No standard deviations calculated for RQs due to the data being converted using averages.

Fig. 3.4 Bacterial CFU (± standard deviations) (a) and RQs (b) of Cranfield slurry and soils from year 1 and 2 (c and d) with increased concentrations of oxytetracycline.

(a)

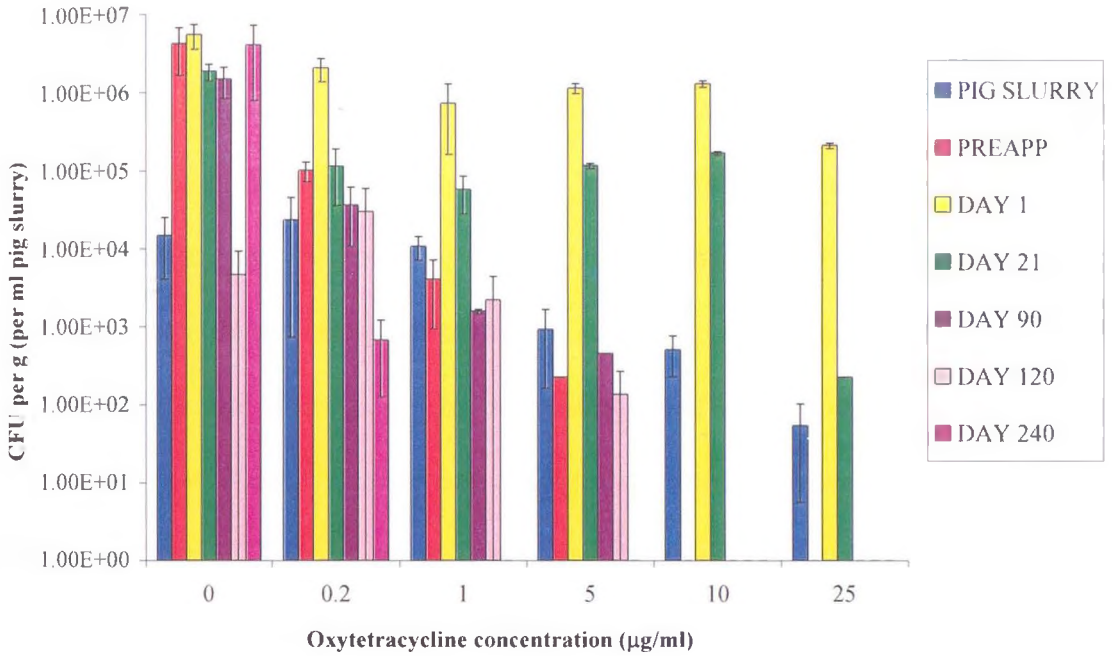


(b)

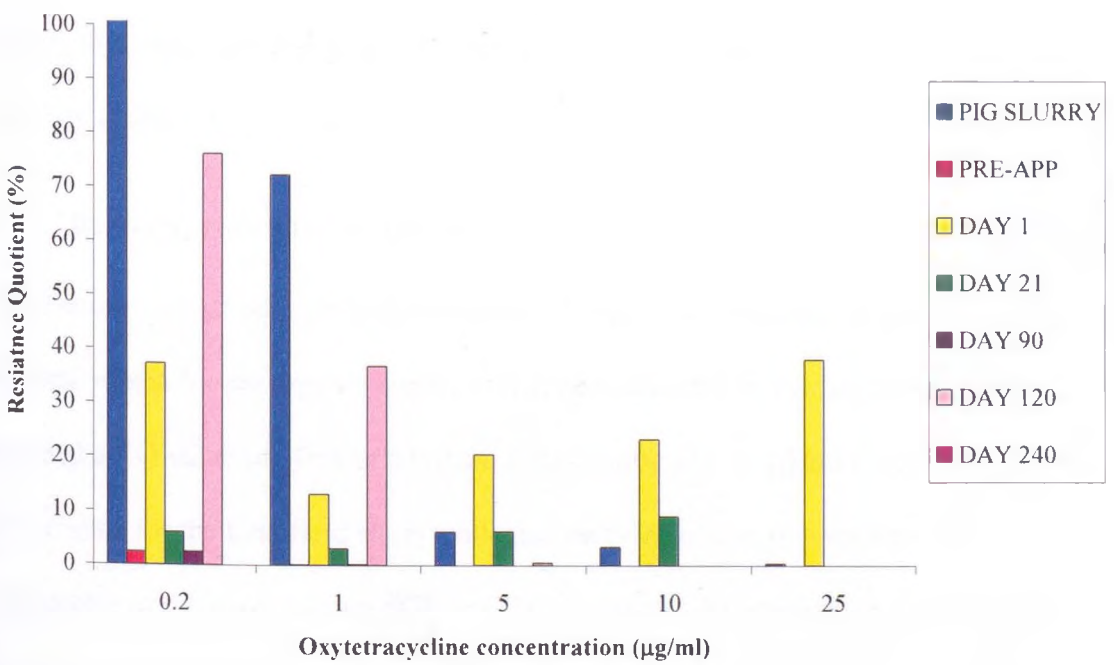


No standard deviations for RQs due to the data being calculated using averages.

(c)



(d)



Where pre-app: soil before application of the Cranfield slurry.

The control soils displayed CFU at all concentrations of OTC and high numbers of resistant bacteria which both decreased with increased OTC concentrations (Fig. 3.4b). Investigations into the soils from year 1 revealed CFU were recorded at day 289 up to a concentration of 25 μg/ml OTC but for RQ values the latest soil sample taken with the highest recorded resistance was day 120 at 5 μg/ml OTC. In the year 2 soils, CFU counts were only observed up to day 21 at 25 μg/ml and day 240 at 0.2 μg/ml, the RQ values were also lower in this year compared to year 1, observing resistance only up to day 1 with 25 μg/ml (Figs. 3.4). An interesting trend was displayed in the soils RQ values from year 2 day 1, exhibiting a decrease with increasing concentration until reaching 1 μg/ml OTC after which it reversed the trend, increasing resistance values with increasing concentrations of antibiotic.

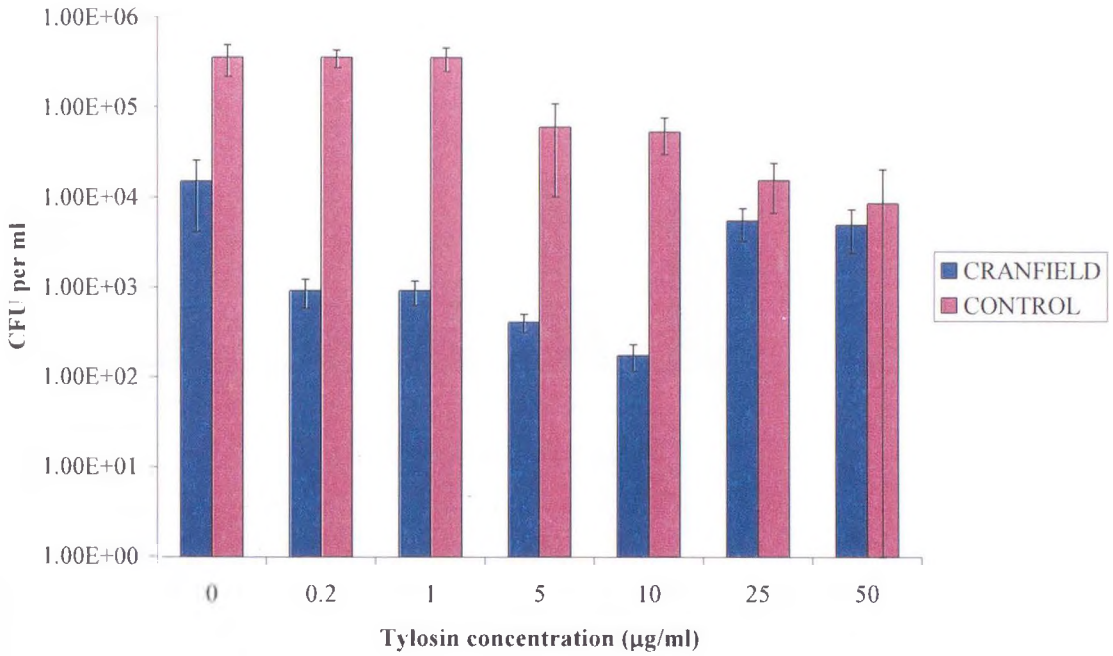
Statistically there was a significant difference recorded for resistance to OTC between the different soils at different time points and over increasing concentrations of OTC, but this difference did decrease with increasing concentrations of OTC for both years of the study (Tables 3.2 to 3.5).

3.3.3 Bacterial resistance to tylosin

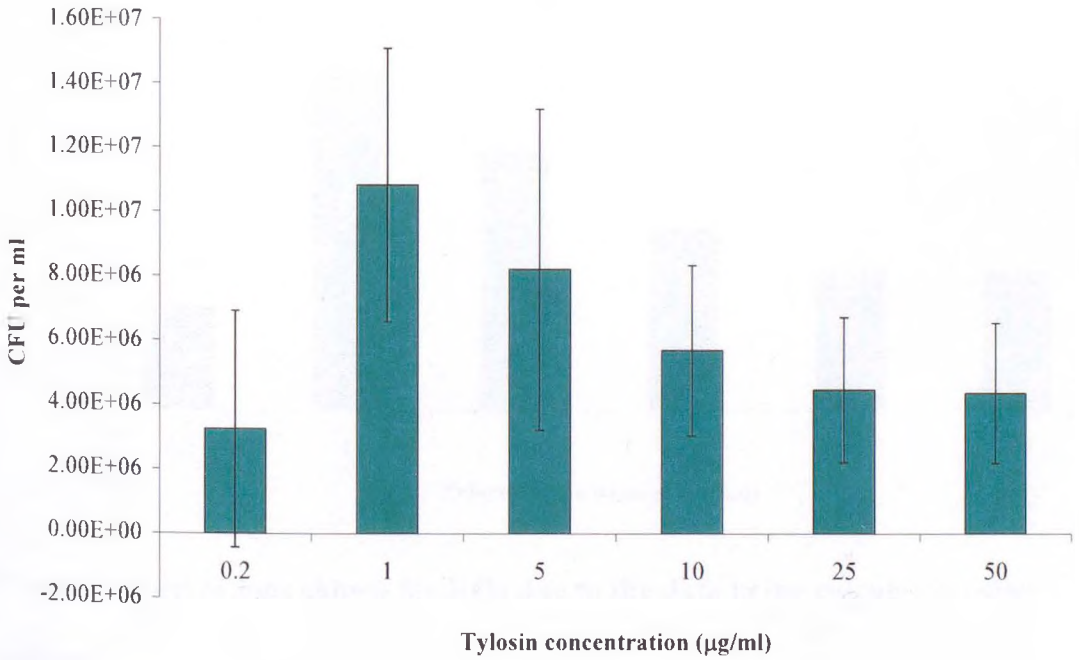
CFU counts remained high in the presence of high concentrations of tylosin in the selective media for the organic slurry, which was reflected in the RQ counts which recorded a RQ value of 30 % at a tylosin concentration of 25 μg/ml (Figs. 3.6a and d). CFU counts for the Cranfield slurry collected on tylosin selective media were comparable to those containing SCP or OTC, but with CFU present up to 50 μg/ml tylosin. Whereas the RQ values for the same slurry were higher than recorded for SCP and OTC, displaying an RQ value of 35 % on media containing 25 μg/ml tylosin (Fig.

Fig. 3.5 Graph to show bacterial CFU (± standard deviations) for (a) Cranfield and control pig slurries (b) organic slurry and RQs (c) for Cranfield and Control slurries and (d) from organic slurry, with increased concentrations of tylosin.

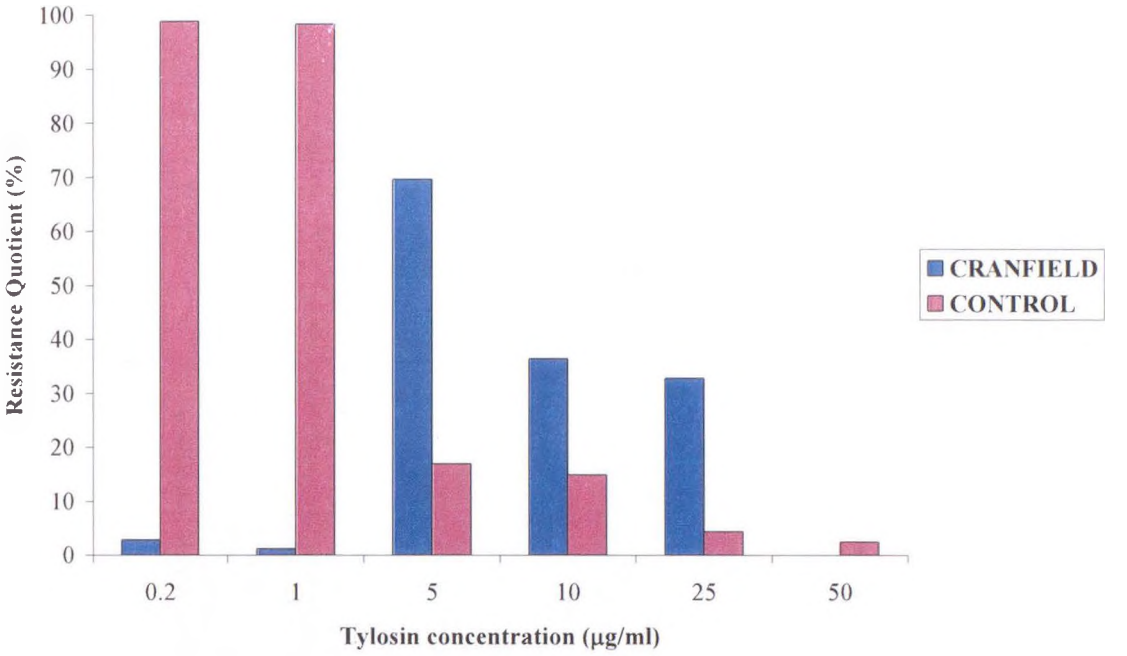
(a)



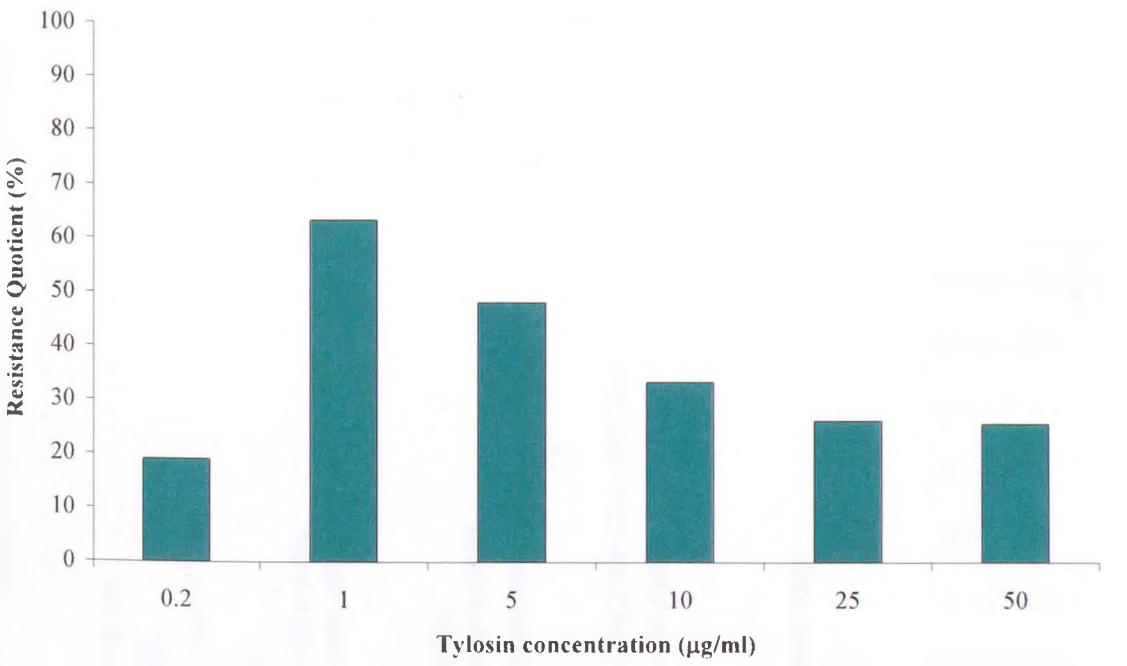
(b)



(c)



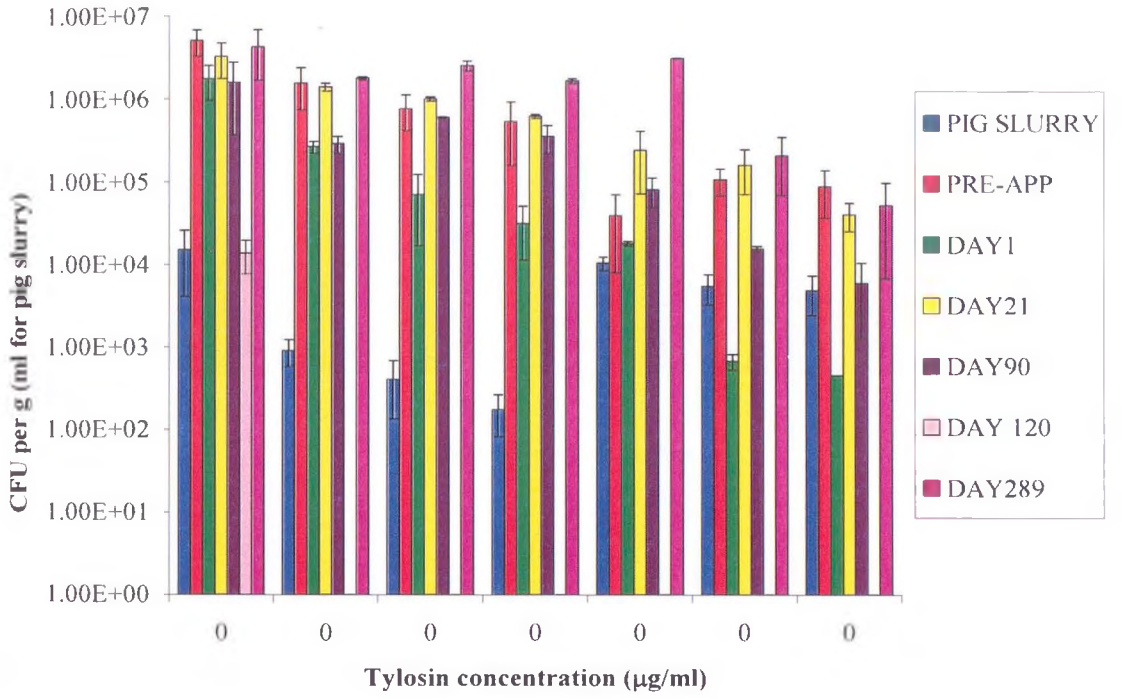
(d)



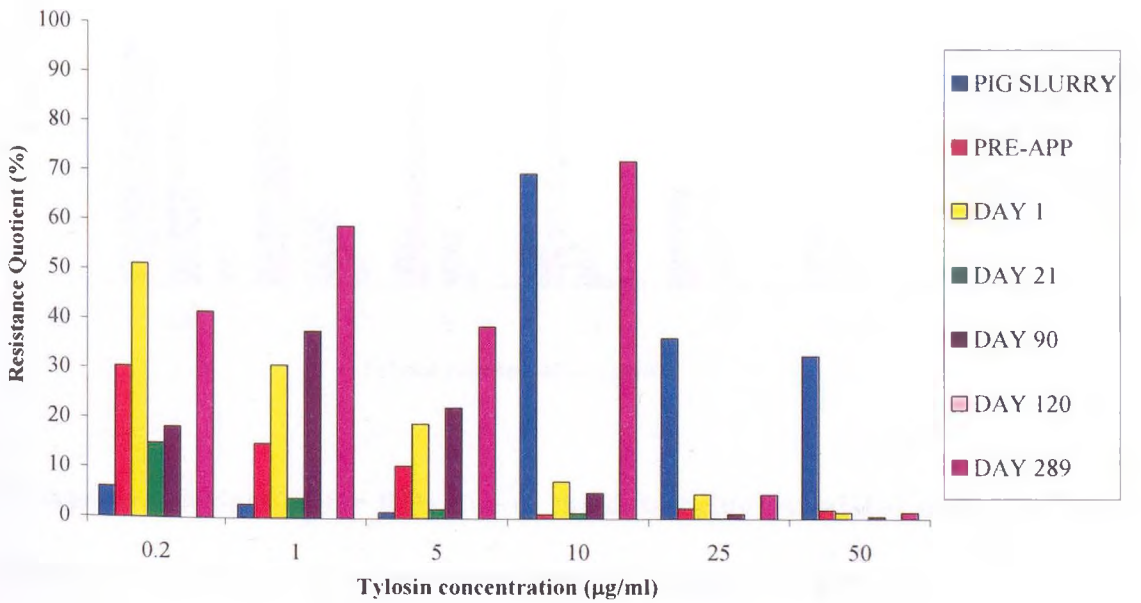
No standard deviations shown for RQs due to the data being calculated using averages.

Fig. 3.6 Bacterial CFU (± standard deviations) (a) and RQs (b) of Cranfield slurry and soils from year 1 and 2 (c and d) with increased concentrations of tylosin.

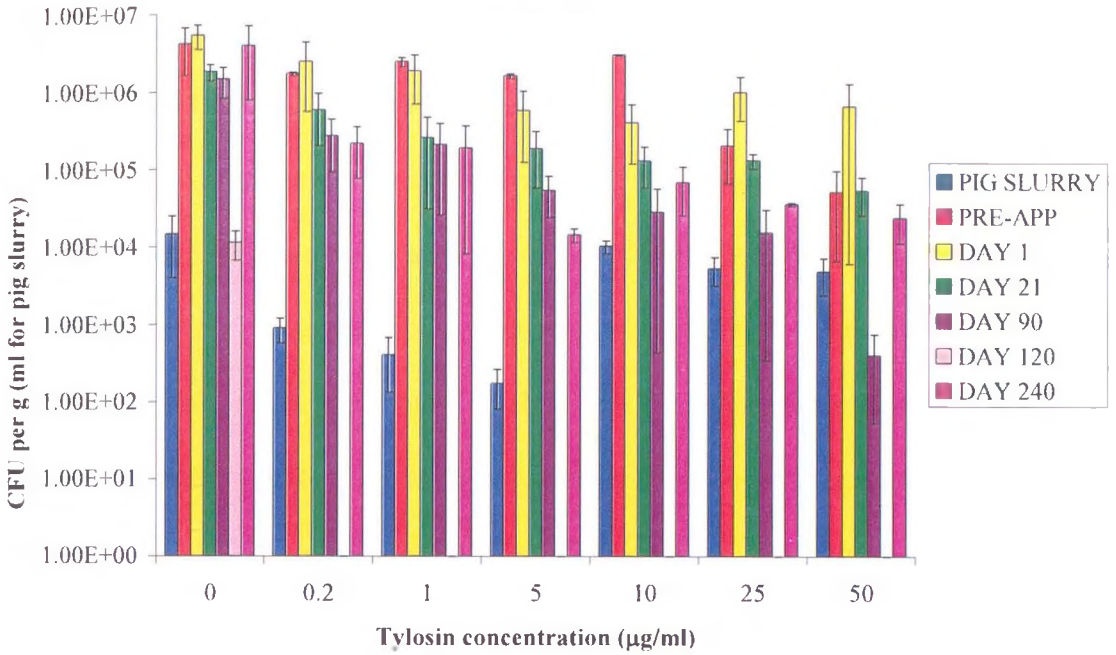
(a)



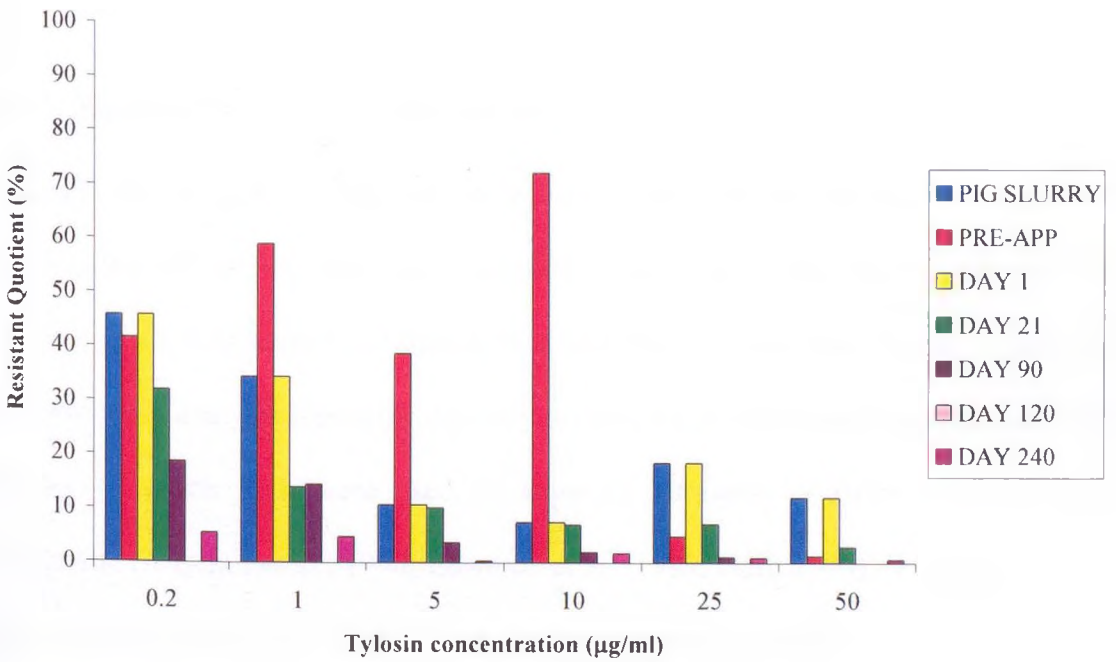
(b)



(c)



(d)



No standard deviations for RQs due to the data being calculated using averages.

Where pre-app: soils before the application of Cranfield slurry.

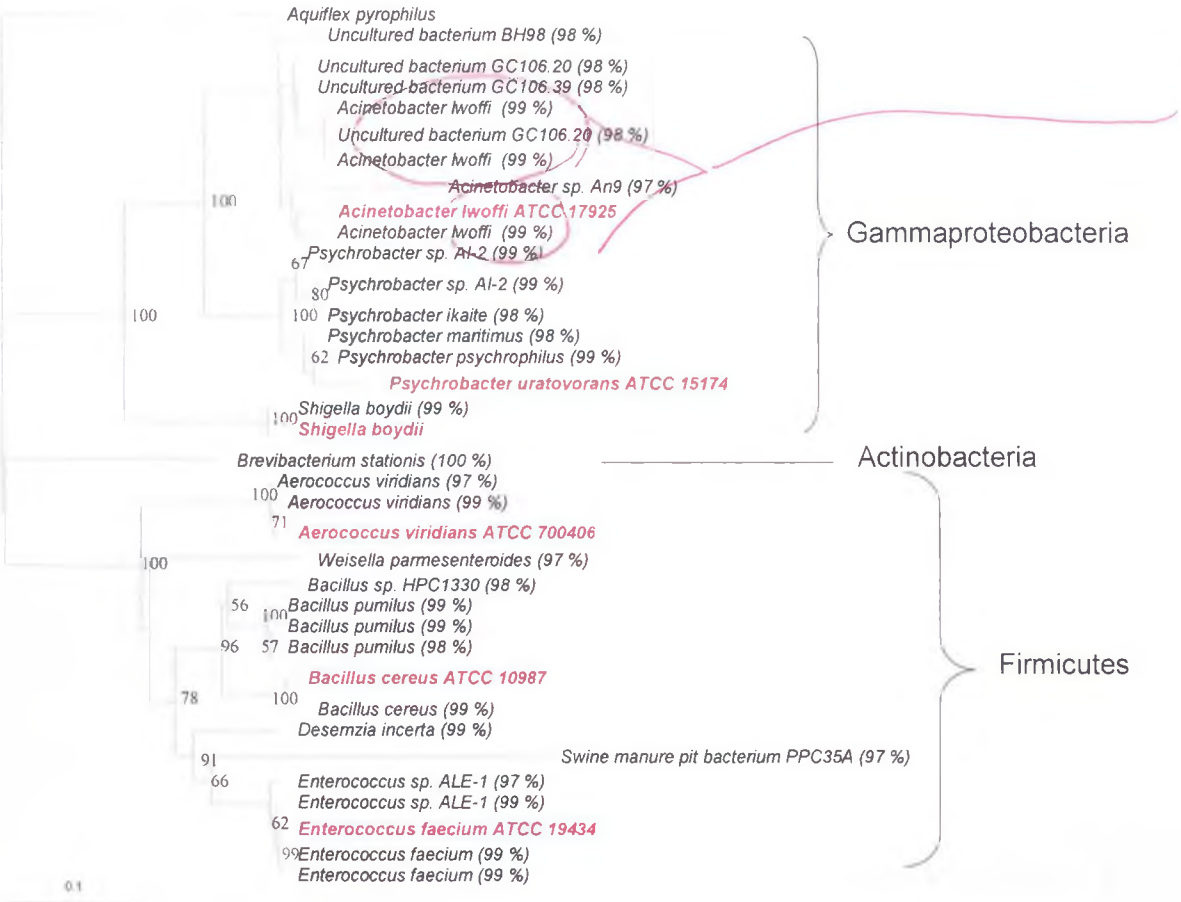
3.5b). The Cranfield slurry RQ values did display trends of bimodal curves. Resistance was reported in the control up to 50 μg/ml tylosin for CFU counts and RQ values.

In the Cranfield soil study, to a lesser degree than OTC and SCP, there was a recorded decrease in CFU with increasing concentration of tylosin and over time in both years of the study (Figs. 3.6a and c). The addition of slurry bacteria did increase the CFU counts above those of the pre-application counts on day 1, in both years, but for 0 and 0.2 μg/ml tylosin only (Figs. 3.6a and c). Resistant bacteria were observed in the soils, CFU and RQ values, up to the last days of the study in both years; year 1, day 289 and year 2, day 240 at 50 μg/ml tylosin (Figs. 3.6a to d). RQ values were higher and persisted for a longer period than observed for OTC and SCP, with bimodal curves displayed in the RQ values, particularly for year 1, day 289 and year 2, pre-application soils (Figs. 3.6b and d).

3.1.6 Microbial diversity in soils and slurries

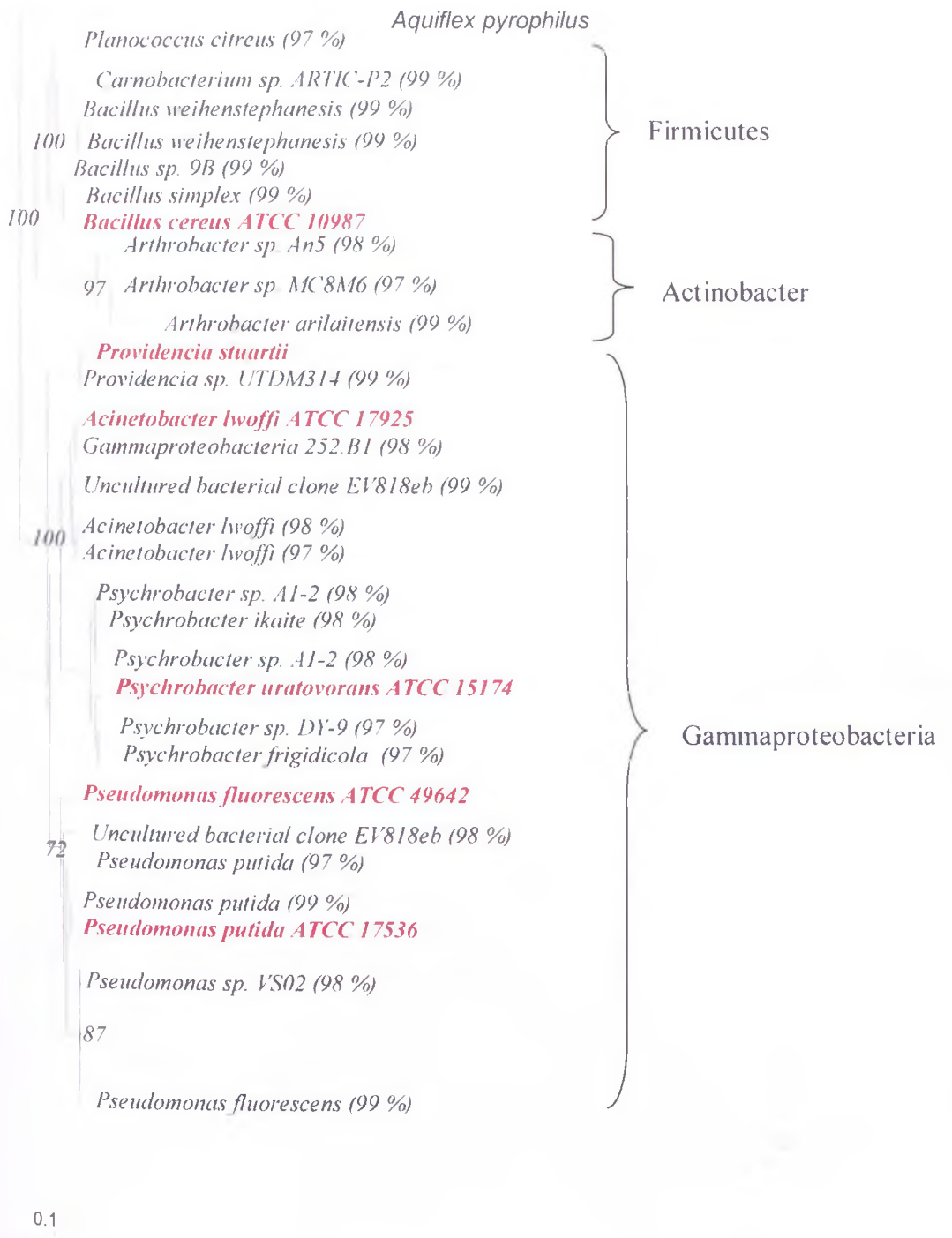
Through the progress of this project a number of cultured bacterial isolates were identified by 16S rRNA, those to be studied in more detail had the full PA-PH PCR fragment of 1500 bp sequenced from both directions, whereas those isolates sequenced for community analysis were only partially sequenced in both directions, approximately 600 bp, of which both were used in sequence similarity analysis in the BLAST programme (Altschul et al., 1990; Edwards et al., 1989; Hall, 1999). Phylograms have been compiled from the partial 16S rRNA sequences for the slurries and pre-application, year 1 and year 2 soils to compare the bacterial community in each, investigating whether the diversity shifted with the application of these slurries to the soils over the two year period of the Cranfield study (Figs. 3.7-3.10).

Fig. 3.7 Rooted phylogram, using phylip, based on partial 16S rRNA gene sequences from bacterial isolates from three different slurry samples to observe the community diversities. Sequence similarity is indicated in brackets and type strains are displayed in red. Bootstrap values are displayed when over 50 % as calculated by phylip.



Overall, the bacteria cultured from the slurries were low in perceived diversity, spanning 3 bacterial phyla; the Firmicutes, Actinobacteria, and the dominating phylum of Gammaproteobacteria (Fig. 3.7). No diversity indices were calculated. A number of enteric bacteria were identified in the slurry samples, *Aerococcus* and *Enterococcus* species from the Firmicutes and *Shigella* from the Gammaproteobacteria. An opportunistic bacterial group of *Acinetobacter* spp. were also identified in the slurries.

Fig. 3.8 Rooted phylogram, using phylip, based on partial 16S rRNA gene sequences from bacterial isolates from Cranfield pre-application soils from both years of the study, to observe the community diversities. Sequence homology is indicated in brackets and type strains are displayed in red. Bootstrap values are displayed when over 50 % as calculated by phylip.



The community diversity of the bacterial isolates from the pre-application soils was lower in comparison to the slurries (Fig. 3.7 and 3.8). Isolates belonging to three bacterial phyla were identified, the dominating group being formed by members of the Gammaproteobacteria; *Pseudomonas*, *Psychrobacter*, *Providencia* and *Acinetobacter* spp., the former three, common soil dwelling bacteria. In the pre-application soils from both years, one member of the Actinobacteria was isolated which was not observed previously in the slurries; *Arthrobacter* spp. A smaller number of Firmicutes were identified in the soils compared to the slurries, *Bacillus*, *Planococcus* and *Carnobacterium* spp. (Figs. 3.7 and 3.8).

No differences were observed between the bacterial communities identified in pre-application soils or years 1 and 2.

Table 3.6 displays the bacterial species identified using partial 16S rRNA sequencing, presenting the dominating phylum to be Gammaproteobacteria in this study. A large number of bacterial isolates were identified as having homology with either *Acinetobacter lwoffi* or uncultured Gammaproteobacteria. No relevance can be drawn between species isolated and sample due to the low numbers of isolates collected and identified.

Fig. 3.9 Rooted phylogram, using phylip, based on partial 16S rRNA gene sequences from bacterial isolates from soil samples taken from year 1 of the Cranfield study, to observe the community diversities. Sequence homology is indicated in brackets and type strains are displayed in red. Bootstrap values are displayed when over 50 % as calculated by phylip.

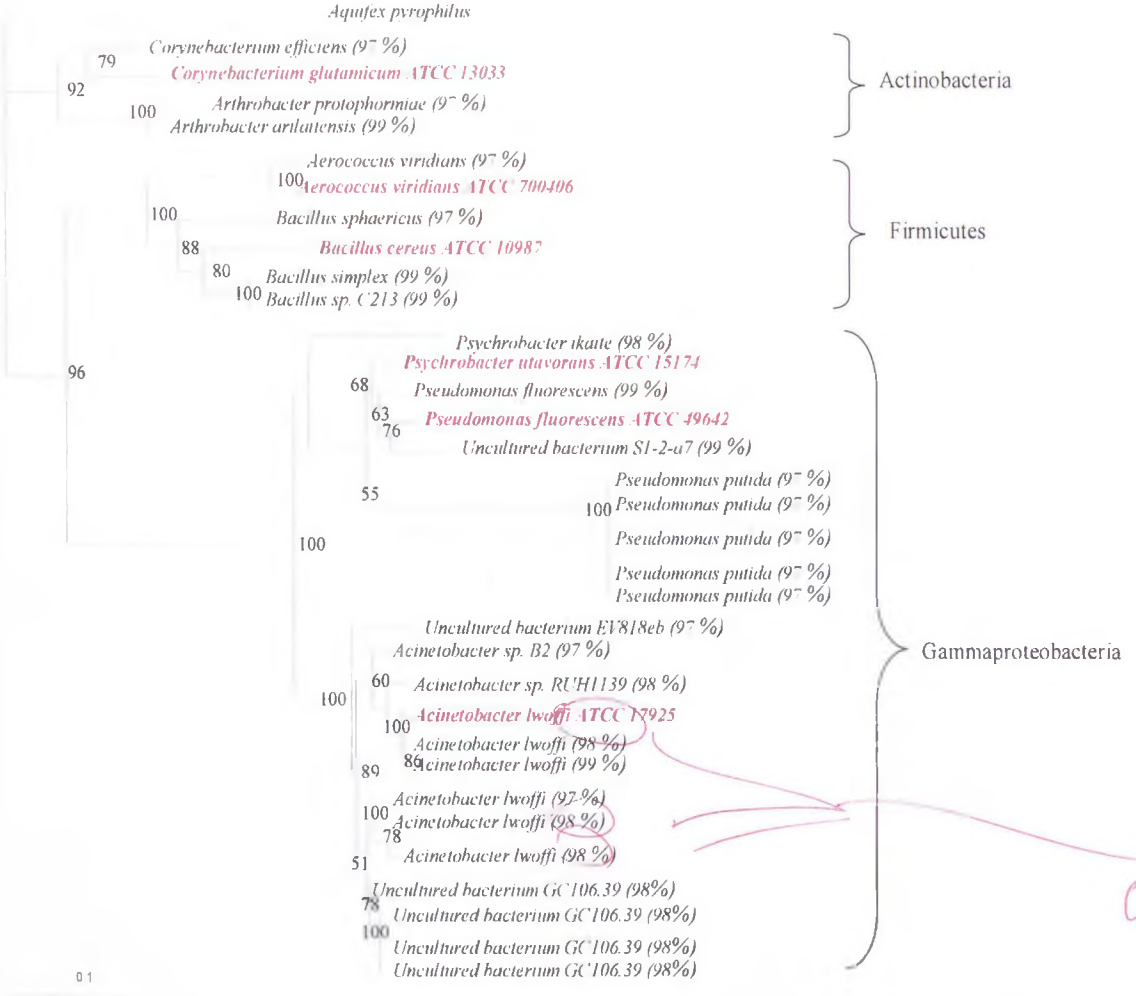


Fig. 3.10 Rooted phylogram, using phylip, based on partial 16S rRNA gene sequences from bacterial isolates from soil samples taken from year 2 of the Cranfield study, to observe the community diversities. Sequence homology is indicated in brackets and type strains are displayed in red. Bootstrap values are displayed when over 50 % as calculated by phylip.

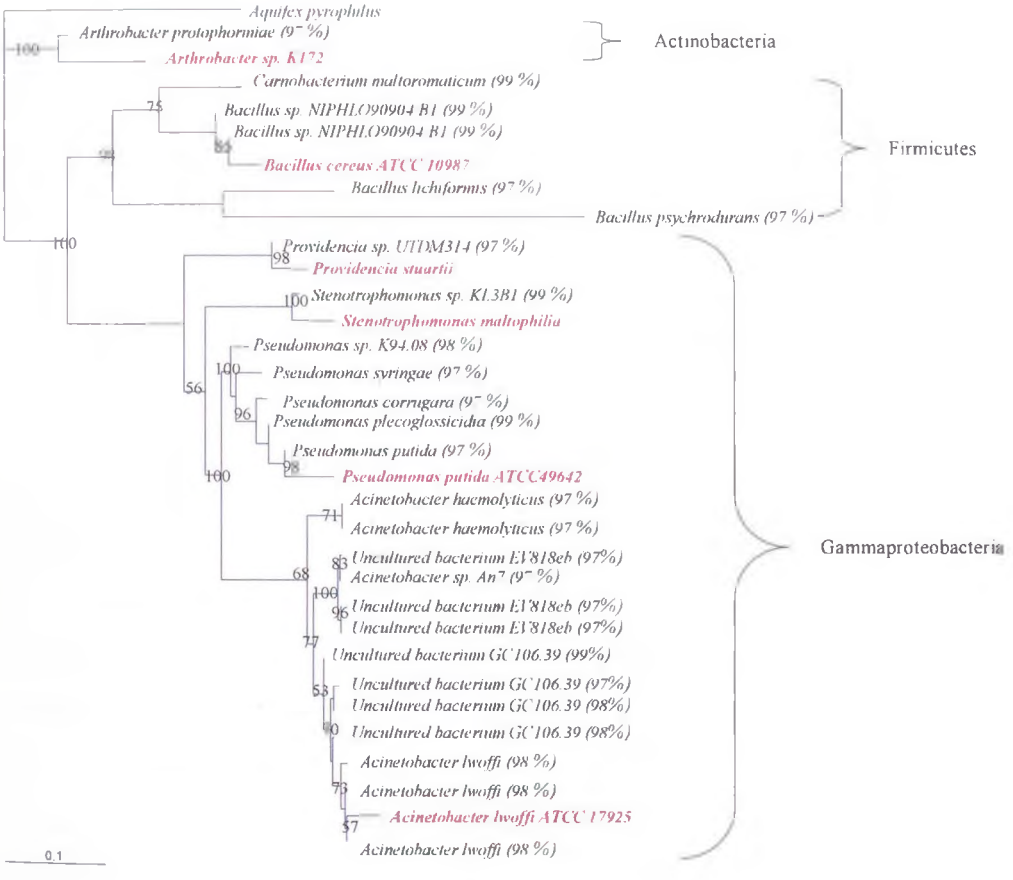


Table 3.6 Bacterial species cultured and identified by partial 16S rRNA sequencing with over 97 % similarity and their prevalence in the soils and slurries examined in this study.

Species	**Total identified**	**Number identified in soil/slurry samples**
Arthrobacter sp.	2	1,P (2)
Arthrobacter arilaitensis	1	1,289 (1)
Arthrobacter protophormiae	2	1,1 (1): 2,21 (1)
Brevibacterium stationis	1	C (1)
Corynebacterium efficiens	1	1,21 (1)
Aerococcus viridians	3	CR (2): 1,90 (1)
Bacillus sp.	5	C (1): 1,P (1) :1,289 (1): 2,240 (2)
Bacillus cereus	1	C (1)
Bacillus lichiformis	1	2,240 (1)
Bacillus psychrodurans	1	2,1 (1)
Bacillus pumilus	3	CR (3)
Bacillus sphaericus	1	1,21 (1)
Bacillus simplex	3	2, P (1): 1,90 (1)
Bacillus weihenstephanesis	1	1,P (1)
Carnobacterium sp. ARTIC-P2	1	1,P (1)
Carnobacterium maltoromaticum	1	2,1 (1)
Desemzia incerta	1	CR (1)
Enterococcus ALE-1	2	CR (2)
Enterococcus faecium	2	O (2)
Planococcus citreus	1	1,P (1)
Swine pit bacterium	1	CR (1)
Weisella parmesenteroides	1	O (1)
Acinetobacter sp.	3	CR (1): 1,1 (2)
Acinetobacter haemolyticus	2	2,1 (1): 2,21 (1)
Acinetobacter lwoffi	13	CR (3): 1,P (1): 2,P (1): 1,1 (5): 2,1 (1):

		2,21 (1): 2,90 (1)
Providencia sp. UTDM314	2	1,P (1): 2,1 (1)
Pseudomonas corrugura	1	2,90 (1)
Pseudomonas fluorescens	2	1,P (1): 1,90 (1)
Pseudomonas sp.	2	1,P (1): 2,90 (1)
Pseudomonas plecoglossicidia	1	2,90 (1)
Pseudomonas putida	8	1,P (2): 1,21 (2): 1,90 (3): 2,90 (1)
Pseudomonas syringae	1	2,240 (1)
Psychrobacter sp. A1-2	5	CR (2): 1,P (3)
Psychrobacter frigidicola	1	1,P (1)
Psychrobacter ikaite	3	CR (1): 1,P (1): 1,1 (1)
Psychrobacter maritimus	1	CR (1)
Psychrobacter psychrophilus	1	CR (1)
Shigella boydii	1	O (1)
Stenotrophomonas sp. KL3B1	1	2,90 (1)
Uncultured Gammaproteobacteria	22	CR (3): O (4): 1,P (1): 2,P (2): 1,1 (5): 2,90 (3): 2,1 (4)

Where; Actinobacteria members, Firmicutes and Gammaproteobacteria. O: organic slurry, C: control slurry, CR: Cranfield slurry, 1,P: year 1 pre-application soils, 1,1: year 1, day 1 soils, 2,P: year 2 pre-application soils, 2,1: year 2 day 1 soils.

3.4 DISCUSSION

There has been increasing concern about the release of antibiotics into the environment through the application of polluted animal slurries. Reports have investigated antibiotic mobility and under taken risk assessments in soils and slurries but few have focused on the phenotypic effects on the slurry and indigenous soil bacteria due the antibiotic compounds present in the slurry (Boxall et al., 2002; Boxall et al., 2003; Kay et al.,

2004). The aims of part of this study were therefore to investigate whether there was a phenotypic effect on antibiotic resistance in the slurry and soil bacterial populations due to the study antibiotic compounds and to investigate the effects of long-term applications of antibiotic containing slurries on bacterial antibiotic resistance to tylosin.

3.4.1 Bacterial resistance in slurry samples

Due to the Cranfield and control slurries being frozen immediately after collection, where as those collected from the organic farm were all used fresh, no comparisons could be drawn between these samples due to the loss of large numbers of bacteria. A 100-fold decrease has been observed in CFU counts before and after a freeze thaw event (N. Abouslam, pers. comm.). The organic and Cranfield farms also had different land usage, pasture compared to winter cereal crops, and the swine were reared in a number of different ways. The animals on the organic farm were free-range Saddleback pigs, direct descendants from organically farmed pigs, and had neither been prophylactic or therapeutically treated with antibiotics or other agents such as $CuSo_4$ during their lives of approximately 13 months, according to Soil Association guidelines and the farmer (Soil-Association, 2001). This is in direct contrast to the Landrace pigs farmed at Cranfield, not of organic descent, which had been constantly fed antibiotics prophylactically and therapeutically, of differing ages, kept in very close proximity.

High RQ values for resistance to SCP, OTC and tylosin were observed in the organic slurry possibly as a result of contamination of the soil from other animal faeces, such as fowl and cattle, as the animals were rotated through the fields or linkage with metal resistance such as $CuSo_4$ due to undisclosed usage. $CuSo_4$ and zinc oxide have been used as alternatives to antibiotics for growth promotion and can also be effective against parasitic infections. Resistance has been determined for copper on conjugative plasmids

in *Pseudomonas* sp. from the environment (Vargas et al., 1995) and Hasman and Aarestrup (2005) demonstrated a relationship between copper and macrolide resistance on a Tn*1546* element in *E. faecium* strains isolated from pigs.

Bacterial resistance was lower in the control than the Cranfield slurry with a complete lack of SCP resistant bacteria in the control slurry possibly due to the lack of a selective pressure to force the evolution, gain and transfer of resistance genes in the slurry bacterial population.

Overall, bacterial resistance was higher in the pig slurry samples, than the soil cores, for all three study compounds, possibly as a result of the constant selective pressure of tylosin in the animal gut and pre-selection of resistant bacteria in the slurry due to the spiking before application to the soils. The constant addition of sub-lethal doses of tylosin to the feed, could have produced a tylosin resistant population (Chander et al., 2005; DuPont and Steele, 1987; Muller et al., 2002). This resistant population may also have co-selected a number of other antibiotic resistance genes linked on mobile genetic elements to tylosin resistance or the co-selection of antibiotic resistance may have taken place due to the use of MD efflux pumps for resistance (Poole, 2005).

3.4.2 Bimodal curves: a reflection of functional resistance mechanisms

The bimodal curves, exhibited in a number of the graphs representing RQ values, may represent an effect of the antibiotics on the bacterial cells' physiology and metabolism. This trend has been reported once in the literature, in a similar study conducted in the same lab with increasing concentrations of QACs (Gaze et al., 2005).

At lower antibiotic concentrations, in the first phase of the curve, the effect on the constitutive expression, production and rate of general efflux pumps may be being observed. These may be up-regulated with increased exposure, or increasing

concentrations of antibiotics. At higher concentrations, in the second phase of the curve, an induction effect may be being displayed for acquired genes, but many acquired genes, such as *sulI* which encodes for sulphonamide resistance, are not thought to be inducible (Sundström et al., 1988). This means there maybe an unidentified process effecting regulation of antibiotic resistance in the cells. Co-regulation with one or all of the study compounds may also cause the bimodal effect.

The bimodal curve was not exhibited with increasing concentrations of OTC. A number of known *tet* genes have been reported to be repressed in the absence of tetracycline; the tetracycline resistance repressor protein (TetR) has alternating structural conformations which allow its binding to α-helix-turn-α-helix motifs on the Tet operator blocking transcription of the structural genes for both the repressor and the efflux protein (TetA), thus allowing precise regulation of these genes (Chopra and Roberts, 2001). The absence of the bimodal trend may mean OTC resistance was due to Tet efflux pumps or MD efflux pump mechanisms, for example members of the MFS and ABC efflux transport systems (Paulsen and Lewis, 2001; Roberts, 1996).

3.4.3 Patchy resistance

Resistance in the CFU counts and RQ values often decreased at one time interval to increase at the next without plausible explanation. This patchy resistance and bacterial counts was possibly due to the Cranfield clay soils characteristics; large cracks were observed in the soil during the summer months and macro-pores developed in the winter months which may have effected the distribution of the SCP especially during slurry application causing uneven distribution of both the bacteria and the antibiotic/slurry (Kay et al., 2004). Composite sampling may have elevated this patchy resistance in the results by grouping a larger number of soil cores together.

3.4.4 Bacterial resistance to sulphachloropyridazine

The results illustrated that bacterial resistance to SCP was low in pre-application soils, increasing with the application of slurry but then decreased over time, in both years of the study to below pre-application levels. The presence of SCP resistant bacteria in the soils prior to application of the SCP spiked slurry may be due to its continual application, with exposure to tylosin, and the co-selection of antibiotic resistance occurring in the slurry bacteria. A numbers of explanations for the increase of bacterial SCP resistance after slurry application are the addition of resistant enteric bacteria to the soil, their survival over time and the presence of a selective pressure on the soil indigenous bacterial population allowing resistance genes to be transferred from the enteric bacterial population to dominate and persist in new recipients. After this initial increase, RQ values decreased, possibly as a result of the lack of survival of enteric bacteria from the slurry and a decrease in numbers of bacteria with newly acquired resistance due to a lack of a selective pressure shown by the HPLC data, decreased levels of sensitive bacteria due to cell death or stasis. The lack of a selective pressure was predicted by the mobility of SCP through the soil profile as indicated by its calculated K_d value of 1.8 l kg/l and HPLC concentrations from different soil core depths over both years of the study (Fig. 2.1) (Boxall *et al.*, 2002). Schmitt et al., (2004) reported similar findings with increasing concentrations of SCP, soil microbial communities developed a 10 % pollution-induced community tolerance with initial exposure to the antibiotic. This investigation did not include the effects over time, therefore it is unknown as to whether this tolerance would decrease with time, but the investigators did conclude that the selective pressure was probably not in presence long enough for step-wise development of resistance.

3.4.5 Bacterial resistance to oxytetracycline

HPLC data recorded that OTC remained at high concentrations in the soils throughout both years of the study and the K_d value for OTC in this study was calculated to be 500 kg/l, suggesting that OTC had a low mobility and was sorbed to the soils (Fig. 2.2) (Kay et al., 2004). However, the RQ values for years 1 and 2 suggest the OTC was not bio-available and hence was not providing a selective pressure for resistance, in contrast to Chander et al., (2005) who's study illustrated bio-availability of tetracycline after its sorbance to soils with respect to *Salmonella* and *E. coli* strains.

The general trend of an increase in the presence of resistant bacteria with the application of slurry and then a decrease over time was possibly due to the same factors detailed for the same trend observed for SCP, which was also observed in the study by Sengeløv et al., (2003), in which they reported a similar relationship between tetracycline resistance and soils fertilized by manure with an initial increase after application which decreased over time to normal levels. The increase could be explained by transfers of resistant genes and transfer of resistant enteric bacteria.

3.4.6 Bacterial resistance to tylosin

Tylosin resistance was higher in the slurry samples and throughout all soil core time intervals as a potential result of continual sub-lethal doses of the antibiotic applied to the soils via slurry for over 20 years, in comparison to SCP and OTC CFU and RQ values. This constant application could have maintained a constant selective pressure, although the HPLC data indicated no tylosin was present in the soil or slurry samples (Kay *et al.*, 2004). This effect of continual sub-lethal doses has been described in a number of studies; Langlois et al., (1978) reported the excretion of multiple resistant microorganisms after pigs received therapeutic antibiotic doses and van Den Bogaard et

al., (2000) recorded a high prevalence of multiple resistant bacteria where antibiotics were used as growth promoters. The higher resistance for tylosin in the study represented in this thesis may be a result of a shift in the microbial community in this perturbed soil, also observed in an investigation by Westergäard et al., (2001) where the treatment of soils with tylosin caused a shift in the soil microbial community even after traces of tylosin or its degradation products could no longer be detected, but a brief investigation into bacterial community diversity for this thesis did not support a shift without a comparison to a suitable control soil.

3.4.7 Microbial community diversity

The low microbial diversity in the slurries may have been a result of the pH and anaerobic conditions. The freezing of the control and Cranfield slurries may have reduced the numbers of culturable bacteria and pre-selected for certain bacterial phyla, possibly supported by the low diversity. The use of culturable techniques to address the diversity of the slurries may have limited the microbial species identified, in comparison to the report by Snell-Castro et al., (2005) who observed the characterization of species from a much wider diversity of bacterial phyla. Although the Firmicutes were present in the study by Snell-Castro, they represented a much smaller percentage than the isolates presented in this study.

The isolates identified as *Aerococcus*, *Enterococcus* and *Shigella* were probably of pig gastrointestinal tract origin, and represented survival during slurry storage, where the storage is a means of allowing the death of pathogenic and potentially harmful bacteria. The presence of Actinobacteria and a number of Gammaproteobacteria may have been through pig ingestion of soil or contamination of the storage pits with soils. The presence of these bacterial phyla represents the potential for HGT of resistance genes

into soil indigenous bacteria before the slurry is applied to the soils, before the biodegradation of antibiotics and thus with a selective antibiotic pressure.

In comparing the bacterial diversity between pre-application soils and those of years 1 and 2, no differences between species despite the application of slurries was observed, therefore there was no effect on the microbial communities due to SCP and OTC. This lack of effect was also observed in the phenotypic studies of the isolates. There was a slight difference in the diversity of bacterial isolates between the pre-application soils and post-application soils in both years, but only in year 2 did this relate to effects of the spiked SCP, resulting in a shift to higher numbers by selection on this antibiotic. This perceived effect may also have been a consequence of low isolate numbers collected and assessed.

The domination of Gammaproteobacteria such as *Pseudomonas* spp. and *Acinetobacter* spp. may have been due to their increased intrinsic resistance (over most other Gram negative rods) to antibiotics such as β-lactamases, tetracyclines, chloroamphenicol and fluroquinolones, the mechanisms thought to be responsible are low permeability of outer membranes (Li et al., 1994; Poole, 2002), efflux pumps (Li et al., 1995) or biofilm formation (Poole, 2002).

3.5 CONCLUSIONS

This chapter has observed, as have a number of published reports, that pig slurries contain a high number of bacteria resistant to a number of antibiotics and a higher number compared to soils even where the slurry has been applied. The results following the application of slurry to soils displayed the long-term survival and die off of enteric bacteria and the possible persistence of resistance genes acquired through HGT from the enteric bacteria. The death of sensitive bacteria in the soils due to the

selective pressure of antibiotics and increased domination of resistant indigenous bacteria was also indicated by the results. The data suggested a shift in the overall microbial community due to the effects of tylosin entering and persisting in the environment, with the development of an environmental reservoir of resistant bacteria and antibiotic resistance genes, possibly through co-selection.

This study also demonstrated the low diversity of the culturable bacterial populations identified in the slurries and soils investigated. The pre-application soils displayed some effect of a community shift towards tylosin resistant Gammaproteobacteria and the year 2 soils exhibited some evidence for a shift of the same bacterial phyla towards a higher resistance to SCP, although isolate number were too low to confirm these initial observations.

3.6 FURTHER WORK

A number of points were brought up in the course of the study, one being that further CFU counts should be performed to compare the difference in bacterial numbers before and after a freeze thaw event in organic soil and slurry samples. This was not performed due to logistical problems but also meant the organic samples could not be directly compared to the control or Cranfield samples.

An alternative control using Landrace organic pigs, not in contact with other animals, may have given a truer background of resistance for the three antibiotics. This was not possible as an organic farm was needed with similar soil characteristics and organic farms commonly have rare or old breeds to increase their meat and novelty values, with theses breeds tending to come from a lineage which has not been treated with antibiotics.

Fitness or biological costs are important parameters in determining the stability and potential reversibility of resistance. The presence of a resistance plasmid may reduce the fitness of a resistant bacterial strain, thus being out-competed in a particular environment. The prevalence of resistant bacteria will then decline in a population without the antibiotic pressure. Fitness costs of antibiotic resistance have not been investigated in this study but a review by Andersson (2003) details that particular drug resistances and types of carriage of resistance may confer a fitness cost to the bacteria but this is often balanced by cost-free resistances and genetic linkage between non-selected and selected resistances. The fitness costs of sulphonamide resistance has been studied, Haasum et al., (2001) measured enzyme kinetics of DHPS where its encoded gene contained a number of different resistance conferring mutations in *S. pneumoniae* and suggested the fitness cost to the organism may be very low. A report by Enne et al., (2004) investigated plasmids encoding the *sulII* gene in *E. coli* hosts and concluded that their presence improved host fitness and possibly contributed to the maintenance of sulphonamide resistance in the UK in the absence of clinical selective pressure.

To investigate whether the bimodal curve was due to specific gene regulation or general efflux mechanisms, gene expression could be measured over time (Dumas et al., 2006). RT-PCR could be performed on isolates containing genes such as the inducible *tet(A)* and *tet(R)* (Hinrichs et al., 1994) or genes encoding efflux pumps in the MFS family for macrolides, such as *mefA* and *mefE* (Randall et al., 2004).

As detailed in the site introduction (Section 2.1) and the study by Boxall et al., (2002), a replicate set of data and soil cores on a sandy loam soil exists. If time permitted it would have been interesting to conduct replica studies and compare the data between the two soils due to the antibiotics differing sorbance in the two soils.

Time and money allowing all the culturable bacterial isolates collected in this study would have been identified through 16S rRNA sequencing to allow a more accurate picture of diversity. A comparable study of non-culturable bacteria using total community DNA sequence cloning and DGGE would also have allowed a more thorough study of diversity in the slurries and more phyla may have been identified.

CHAPTER 4:

DETECTION OF ANTIBIOTIC RESISTANCE GENE DETERMINATES IN BACTERIAL ISOLATES FROM SLURRIES AND SOILS

Chapter 4: Detection of antibiotic resistance gene determinates in bacterial isolates from slurries and soils

4.1 INTRODUCTION

The widespread and indiscriminate use of antibiotics has led to the development of antibiotic resistance in pathogenic, commensal and indigenous soil microorganisms and its maintenance due to the selective advantage conferred on the cell. Antibiotic resistance genes are found in clinical isolates from a wide range of bacterial genera but it is thought the reservoir for these genes exists in environmental bacteria (Biyela et al., 2004), originating, at least in part, from antibiotic-producers such as the Actinobacteria (Arthur et al., 1987; Heuer et al., 2002; Normark and Normark, 2002). Few studies have evaluated the diversity of antibiotic resistant bacteria or the antibiotic genes present in the environment (Alonso et al., 2001; Miranda et al., 2003; van Overbeek et al., 2002). Studies commonly rely upon traditional microbiological techniques, and as only 1-10 % of the soil microbia has been reported to be culturable, this will greatly underestimate the reservoir of resistant bacteria in the environment (Torsvik and Ovreas, 2002).

Bacterial HGT has been demonstrated to occur through the biosphere, especially in nutrient-rich sites such as aquatic systems (Biyela et al., 2004), sediments, soils, rhizospheres (Götz and Smalla, 1997) and in the sludge of the biological sewage treatment plants (Lorenz and Wackernagel, 1994; Pukall et al., 1996; Smalla and Sobecky, 2002). These environments contain a large diversity of bacterial species and gene transfer of resistance genes has been likely to proceed to its current recipients in a step-wise manner involving a number of different genera, the transfer commonly involving mobile genetic elements such as integrons.

4.1.1 Environmental occurrence of integrons

Epidemiological studies into integrons, whether clinical or environmental, commonly focus on a particular species, but there are a small number of investigations covering a diverse species range. One of the first studies was conducted by Sallen et al., (1995) who systematically screened 49 clinical isolates from one hospital in France, of which 59 % were demonstrated to carry integrons, the isolates belonging to six different genera of Enterobacteriacae, some of these isolates carried multiple integrons. A Chilean study found that the Class 2 integrons were the most prevalent in *A. baumanni* isolates, a number carrying both classes 1 and 2 (Gonzalez et al., 1998). Investigations into bacteria isolated from agricultural animals have found similar integron frequencies to clinical studies; 59 % in *E. coli* from calf diarrhoea cases and 44 % and 23 % in *E. coli* isolates from poultry and swine commensal isolates (Du et al., 2005; Kang et al., 2005). Of the environmental studies, Class 1 integrons have been detected in 3.6 % of isolates from an estuarine environment (Rosser and Young, 1999), 7.98 % of isolates in a QAC polluted environment (Gaze et al., 2005) and in 24 % of coliform isolates from an aquatic environment (Park et al., 2003).

The prevalence data for Class 1 integrons compared to Class 2 is variable between studies. A study into MD resistant *S. enterica* from poultry reported that 100 % of isolates screened carried a Class 2 integron and a similar percentage was reported in a study into epidemic *S. sonnei* strains in Australia (McIver et al., 2002; Miko et al., 2003). Alternatively other investigations have reported cases where the Class 1 integron is more prevalent (Roe et al., 2003a; Roe et al., 2003b; Seward, 1999). The two reports by Roe et al., (2003a and 2003b) reported Class 1 integron incidence higher

than Class 2 in studies into total-community DNA from poultry carcasses and *E. coli* isolates from irrigation water and sediment collected from the Rio Grande.

Integrons are now widely found in the hospital setting worldwide and their sources are unknown. Evidence suggests that the environment is a potential reservoir (Leverstein-Van Hall et al., 2002). Integrons have been detected in a wide variety of environments such as; apple orchards (Schnabel and Jones, 1999), fish farms (Schmidt et al., 2001a), estuarine environments (Rosser and Young, 1999) and polluted environmental sites (Gaze et al., 2005; Szczepanowski et al., 2004), and Class 1 integrons have been described in all types of agricultural animals, as well as pets and zoo animals, with antibiotic use on farms long considered a significant factor contributing to antibiotic resistance (Fluit and Schmitz, 1999). The link between antibiotic resistance in humans and Class 1 integrons in the environmental reservoir is well documented in the cases of *E. coli* and *Salmonella* spp. (Fluit and Schmitz, 2004).

4.1.2 Sulphonamide resistance

As stated in the introduction, resistance to sulphonamides is mediated by 3 mechanisms: efflux pumps, mutations or recombination changes in the chromosomal DHPS gene (*folP*) and acquired mechanisms which encode for alternative DHPS enzymes with an altered binding site and a lower affinity for the antibiotic group (Swedberg et al., 1993). The focus of this study was to investigate antibiotic genes which could be acquired through HGT, therefore only sulphonamide resistance genes which could be acquired were screened for.

There have been 3 identified acquired genes which encode for alternative DHPS enzymes; *sulI* (Sundström et al., 1988), *sulII* (Rädström and Swedberg, 1988) and *sul3* (Perreten and Boerlin, 2003). The *sulI* and *sulII* genes from *E. coli* share 57 % DNA

homology, and their origins remain unknown as their sequences are distinct from all the known chromosomal DHPS genes (Rädström and Swedberg, 1988). The *sulI* gene was identified on the transposon, Tn*21*, in a highly conserved region designated a Class 1 integron (Sundström et al., 1988). The *sulII* gene is frequently found on large plasmids in Gram negative bacteria and was first described on the IncQ plasmid RSF1010 (Rädström and Swedberg, 1988). The third acquired gene, *sul3*, was identified in sulphonamide resistant *E. coli* isolated from pigs in Switzerland (Perreten and Boerlin, 2003).

The presence of *sulI*, *sulII* and *sul3* in a genome does not confer resistance to an isolate in all cases and resistance may vary between bacterial species (Guerra et al., 2003). The MIC for *E. coli* susceptible to sulphonamides is <0.01 mM (approximately 28 μg/ml), this increases to 2 mM (570 μg/ml) with the encoding of a *sulI* gene and up to >4 mM in an *E. coli* expressing the *sulII* or *sul3* genes (Sköld, 2001).

The prevalence and distribution of each of the sulphonamide resistance genes alters between studies, environment and bacterial species and a number of these investigations are summarised in Table 4.1. The majority of studies have concentrated on Enterobacteriaceae isolates, specifically *E. coli* and *Salmonella* spp. Guerra et al., (2003) conducted a phenotypic and genotypic characterization of antimicrobial resistance in German *E. coli* isolates from cattle, swine and poultry, 30 % of isolates were resistant to the sulphonamide, sulphamethoxazole. This resistance was conferred by the *sulII* gene in 66 % of sulphonamide resistant isolates, 42 % and 14 % were carrying the *sulI* and *sul3* genes respectively. The *sulII* gene was found with either *sulI* or *sul3* but not both.

Table 4.1 Summary of studies investigating sulphonamide resistance gene frequencies.

Study	Bacteria	Isolation Environment	Gene frequencies (%)
Enne et al., (2001)	*E. coli*	Human	*sulI* 16.4-17.5 *sulII* 26.7-36.5
Grape et al., (2003)	*E. coli*	Human	*sulI* 61 *sulII* 75 *sulI + II* 39 *sul3* 3
Guerra et al., (2003)	*E. coli*	Cattle Swine Poultry	*sulI* 66 *sulII* 42 *sul3* 14
Lanz et al., (2003) Perreten and Boerlin (2003)	*E. coli*	Swine	*sulI* 30 *sulII* 28 *sul3* 30 *sulI + II* 12
Maynard et al., (2003)	*E. coli*	Swine	*sulI* 62 *sulII* 39
Guerra et al., (2004)	*S. enterica*	Animal Food	*sul3* 4.3
Maynard et al., (2004)	*E. coli*	Human Swine Poultry Cattle Pets	*sulI* 50 (Animal) *sulII* 67 (Animal) *sulI* 69 (Human) *sulII* 60 (Human)
Antunes et al., (2005)	*S. enterica*	Human Food	*sulI* 59 *sulII* 22 *sul3* 2 *sulI + II* 12 *sulI + 3* 22 *sulI + II + 3* 3
Infante et al., (2005)	*E. coli*	Human	*sulI* 20 *sulII* 55 *sulII + 3* 5 *sulI + II* 15

In the study by Perreten and Boerlin (2003), in which they first described the *sul3* gene, the distribution of *sulI* was 30 % , *sulII* 28 % , *sulI* and *sulII* (12 %) and *sul3* 30 % from *E. coli* isolates (Lanz et al., 2003; Perreten and Boerlin, 2003). Maynard et al., (2003) studied antimicrobial resistance genes in *E. coli* isolates collected over a 23 year period from pigs, finding an average 62 % of strains carried *sulI* and 39 % carried *sulII*, concluding that the percentage of strains positive for *sulI* did not vary significantly during the period investigated and that the *sulII* gene had proliferated, no localisation of the genes were performed in these studies. No correlation between carriage and increased usage on the farms was deduced. A study into *S. enterica* strains isolated from livestock and food reported an incidence of only 4.3 % carrying the *sul3* gene, with only 1 out of the 22 positive isolates carrying a *sulII* and *sul3* gene (Guerra et al., 2004). Isolates were only screened for *sulI* and *sulII* genes if they were positive for *sul3*, therefore comparisons in prevalence cannot be made in this study. In summary, *sulI* and *sulII* were the dominant sulphonamide resistance genes in most environments and bacterial species studied. This may reflect either the later evolution of the *sul3* gene, a slower distribution from its original host organism or the more stable integration of *sulI* and *sulII* genes in transposons, integrons and plasmids that are widely disseminated among mostly Gram negative bacteria.

The distribution of sulphonamide resistance in clinical isolates was similar to the dissemination reported in environmental isolates. The three alternative DHPS enzyme genes have been identified in a large number of sulphonamide resistant clinical isolates from around the world, eg. *V. cholerae* strains from the Lao People's Democratic Republic (Iwanaga et al., 2004). Clinical isolates are usually only screened for the phenotype of sulphonamide resistance, therefore, without more detailed studies of these

genes it is difficult to determine if *sul* genes are on the increase or decrease in clinical isolates and that any fluctuation in distribution maybe due to their prevalence in environmental bacteria and transfer into the clinical population.

The genotype containing *sulI*, *sulII* and *sul3* was first reported by Antunes et al., (2005) in sulphonamide resistant *S. enterica* strains from Portugal. The strains also encoded the multiple resistance genotype ACSSuT carried on the *S. enterica* genomic island (SGI1), which confers resistance to ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracyclines (Antunes et al., 2005; Boyd et al., 2000).

4.2 AIMS

The environmental aspect of distribution of key resistance genes has not been studied and studies in the clinical setting focus on clinically significant bacteria and not the wider distribution of genes in the clinical bacterial population. This study aimed to investigate the environmental distribution of key genes in the culturable environmental bacterial population and attempt to assess the environmental impact on the distribution of these genes as a result of manuring. This study aims to provide a balanced picture of the reservoir of resistance genes in the environment and their potential and ability to be mobilised. A comparison will be made between manured and non-manured soils as well as between organic and non-organic farms as a means of providing control environments. True control environments are difficult to obtain as the majority of farmland has been in contact, whether directly or indirectly, with antibiotics.

This study aimed to:

1. Screen, by PCR, for a number of specific genes which confer resistance to the sulphonamide group of antibiotics; *sulI, sulII* and *sul3,* from culturable bacterial isolates

obtained from environmental soil samples before and after the application of slurry and from bacterial isolates from the applied slurry;

2. To screen the same isolates as in 1, by PCR, for two integrase genes, *intI1* and *intI2*, which may indicate the presence of integrons and a possible vector for horizontal gene transfer and the mobilisation of the sulphonamide resistance genes;

3. To investigate the environmental frequencies and distribution of these screened genes with respect to antibiotic application via the slurry, sample type and longevity through the study.

Designations throughout results section:

1,Preapp.: year 1, pre-application soil, 1,1: year 1, day 1 soils, 1,21: year 1, day 21 soil, 2,1 : year 2, day 1 soils, DF: drain flow samples.

4.3 RESULTS

4.3.1 Prevalence of the *intI1* gene

The occurrence and frequencies of the *intI1* gene, by PCR screening, from the total bacterial isolates collected from all pig slurry and soil samples, are given in Fig. 4.1 and Table 4.2. Only 33 (5.64 %) of the 583 isolates screened carried the *intI1* gene that was characterised in isolates without other screened genes and in combination with the *intI2* gene and a number of sulphonamide resistance genes. The *intI1* gene was the sole screened gene identified in 36.37 % of positive isolates. A total of 3 (9 %) of positive isolates carried both integrase genes screened, a further 2 isolates carried both genes one with a *sulII* gene and the other with the *sulI* gene.

Fig. 4.1 Prevalence of *intI1* containing genotypes in the total number of bacterial isolates collected from all slurry and soil samples, as screened by PCR.

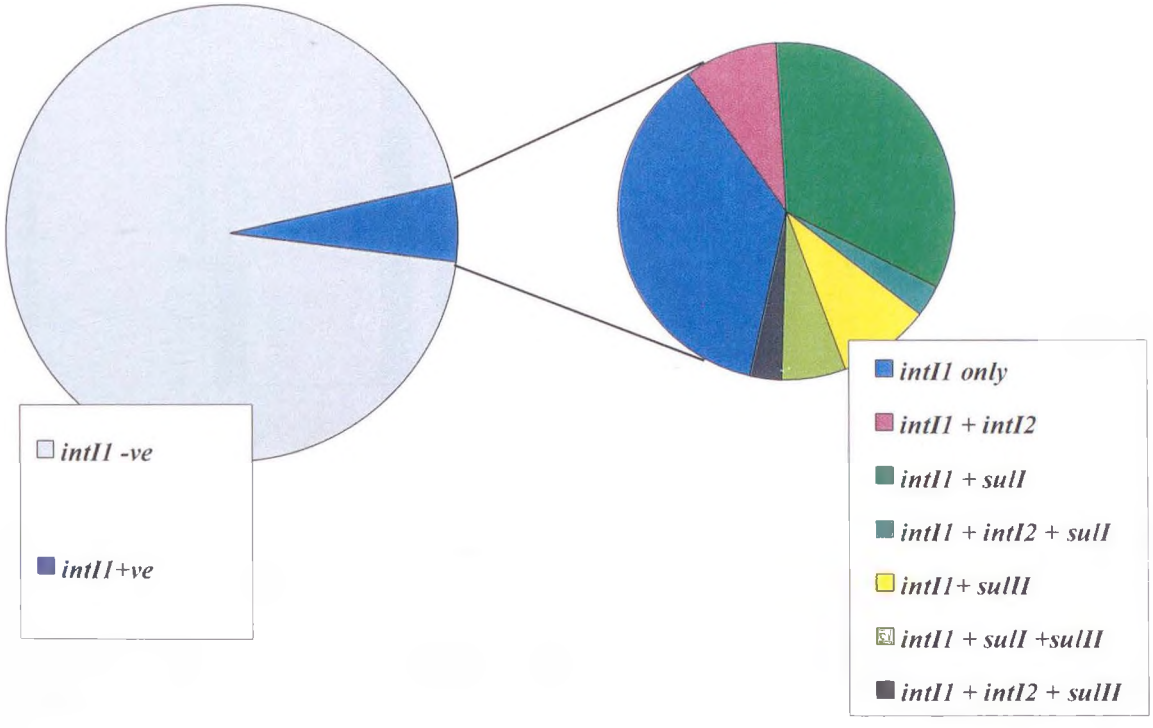


Table 4.2 Prevalence and numbers of *intI1* containing genotypes in all the bacterial isolates collected and within the *intI1* positive isolates.

Genotype	Number of isolates PCR positive for *intI1*	% from total number of collected isolates	% from total number of positive isolates
intI1 only	12	2.03	36.37
intI1 + *intI2*	3	0.51	9.09
intI1 + *sulI*	11	1.88	33.33
intI1 + *intI2* + *sulI*	1	0.17	3.03
intI1 + *sulII*	3	0.51	9.09
intI1 + *intI2* + *sulII*	1	0.17	3.03
intI1 + *sulI* + *sulII*	2	0.34	6.06
intI1 + *sul3*	0	0	0
TOTAL	**33**	**5.64**	**100**

Fig 4.2 Distribution of the *intI1* gene positive isolates according to the day and year of the study in which they were collected.

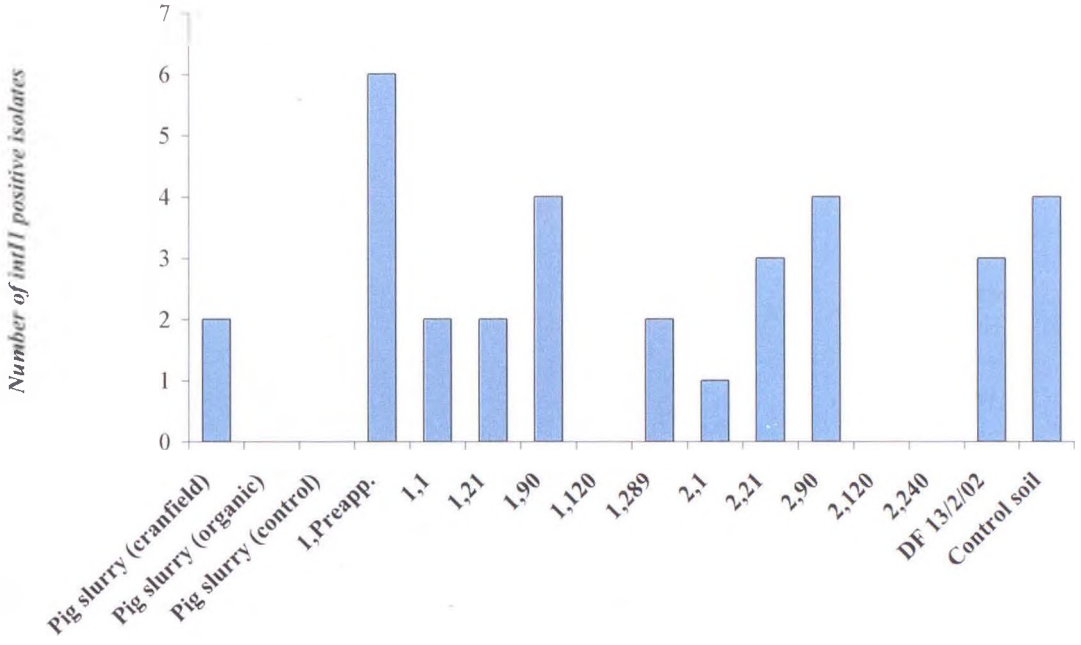


Table 4.3 Frequencies of isolates carrying *intI1* in each slurry and soil core sample.

Sample	Number of *intI1* positive	% of *intI1* positives from each sample	Number of screened isolates from each sample
Pig slurry (Cranfield)	2	6.06	78
Pig slurry (organic)	0	0	32
Pig slurry (control)	0	0	18
1,P	6	18.19	79
1,1	2	6.06	71
1,21	2	6.06	20
1,90	4	12.12	39
1,120	0	0	1
1,289	2	6.06	41
2,1	1	3.03	45
2,21	3	9.09	39
2,90	4	12.12	41
2,120	0	0	4
2,240	0	0	29
DF 13/2/02	3	9.09	13
Control soil	4	12.12	15
TOTAL	***33***	***100***	***583***

Fig. 4.3 Distributions of the *intI1* gene in isolates collected from selective plates containing different antibiotic concentrations.

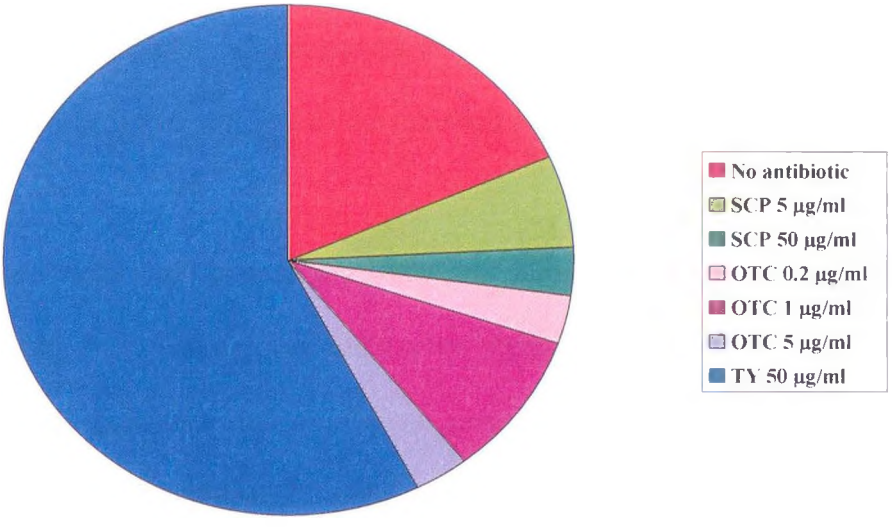


Table 4.4 Prevalence, on a number of selective plates containing antibiotics at different concentrations, for isolates PCR positive for the *intI1* gene.

Antibiotic concentration (µg/ml)	Number of *intI1* positives	% of *intI1* positives from each selective plate	Number of total isolates from each antibiotic selective plate
None	6	18.2	75
TY 0.2	0	0	3
TY 1	0	0	3
TY 5	0	0	40
TY 10	0	0	16
TY 25	0	0	18
TY 50	20	60.6	123
SCP 5	0	0	3
SCP 10	0	0	48
SCP 25	1	3.03	64
SCP 50	1	3.03	41
SCP100	0	0	10
OTC 0.2	1	3.03	29
OTC 1	3	9.09	21
OTC 5	1	3.03	36
OTC 10	0	0	23
OTC 25	0	0	30
TOTAL	***33***	***100***	***583***

The most frequent combination of *intI1* and sulphonamide resistance genes was the genotype *intI1* + *sulI*, at a prevalence of 1.88 % in positive isolates or 33.33 % of the total number of bacterial isolates (11 isolates). The *sulII* gene was identified with *intI1* in 3 isolates (9 % of positive isolates) and a further 2 encoded the integrase gene with *sulI* and *sulII* genes. No isolates were characterised carrying the *intI1* and *sul3* genes in this study.

The occurrence of the *intI1* gene in pig slurry and soils was recorded in Table 4.3 and graphically in Fig. 4.2. The organic and control slurry (tylosin-fed pig slurry with no antibiotic spiking) contained no *intI1* gene positive isolates from those cultured, whereas the control soil contained 12 % (4) *intI1* positive isolates. The slurry that was applied in this study contained 2 *intI1* gene encoding isolates. The pre-application soil cores contained the highest number of *intI1* positive isolates (18.75 % of *intI1* positive isolates). The highest number of *intI1* positive isolates identified in the soils after slurry application were in the Year 1, day 90 cultured bacterial isolates, 12.12 % (4 isolates), with days 1, 21 and 289 encoding 2 each (3.03 %). In year 2, the highest numbers of *intI1* positive isolates were again observed 90 days after the slurry application, with days 1 and 21 playing host to 1 and 3 respectively. Year 2, days 120 and 240 were PCR –ve for the *intI1* gene. The drain-flow samples collected from a high rainfall event (Fig. 2.2) had a screened prevalence of 21.4 % of its cultured isolates containing the *intI1* gene, which was 9 % of the total *intI1* positive isolates. The data, represented in Fig. 4.2, therefore indicates there was no increase in *intI1* prevalence following application of pig slurry to agricultural land.

High frequencies, 57.56 %, of *intI1* positive bacteria were isolated on plates containing 50 µg/ml tylosin (Fig. 4.3 and Table 4.4). This is in contrast to the frequencies of

bacteria containing *intI1* isolated from SCP and OTC plates, 9 % and 15 % respectively, which in total only accounts for 24 % of the positives. A number of positive bacteria were also isolated from plates containing no antibiotics.

4.3.2 Prevalence of the *intI2* gene

The *intI2* gene frequencies in the bacterial isolates from this study are given in Figs. 4.4 to 4.6 and Tables 4.5 to 4.7. PCR screening identified a higher number of *intI2* gene positive isolates than *intI1*; 57 (9.7 %) and 33 (5.64 %) respectively. The genotypes of highest occurrence were *intI2* + *sulI* (16 isolates) and *intI2* + *sulII* (11 isolates) in comparison to *intI1* frequencies where this gene on its own was the most frequent. A smaller number of bacteria with *intI2* than *intI1* were recorded without any other screened genes (8 and 12 respectively). There were a number of isolates that carried the *sul3* and *intI2* genes, which were observed in a number of different genotypes (Table 4.5).

The highest number of *intI2* positive isolates was identified in Cranfield slurry, in contrast to *intI1*, where the highest number was characterised in the pre-application soils from year 1 (Fig. 4.5). As with the *intI1* gene, in year 1 there did not appear to be a correlation between the addition of slurry and the occurrence of the *intI2* gene in soils following application. In year 2, an increase was observed at day 1 with the application of slurry, increasing to a peak at day 21. The largest drain-flow count occurred on 13/02/02 which correlates with the high prevalence at Year 2, day 21. No isolates carrying the *intI2* gene were cultured from the organic and control pig slurries or soils.

Fig. 4.4 Prevalence of *intI2* containing genotypes in the total number of bacterial isolates collected from all slurry and soil samples, as screened by PCR.

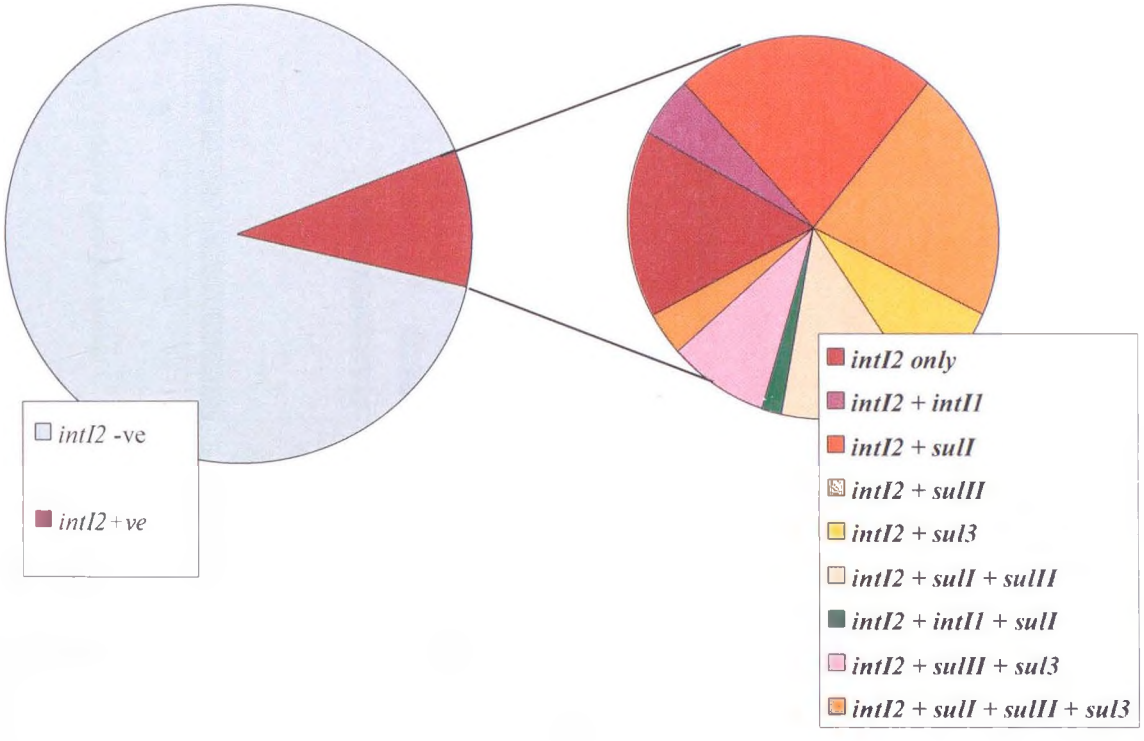


Table 4.5 Prevalence and numbers of *intI2* containing genotypes in the all bacterial isolates collected and within the *intI2* positive isolates.

Genotype	Number of isolates PCR positive for *intI2*	% from total number of collected isolates	% from total number of *intI2* positive isolates
intI2 only	8	1.37	14.04
intI2 + *intI1*	3	0.51	5.26
intI2 + *sulI*	16	2.74	28.07
intI2 + *intI1* + *sulI*	1	0.17	1.70
intI2 + *sulII*	11	1.88	19.30
intI2 + *sul3*	4	0.68	7.02
intI2 + *sulI* + *sulII*	6	1.03	10.52
intI2 + *sulI* + *sulII* + *sul3*	3	0.51	5.26
intI2 + *sulII* + *sul3*	4	0.68	7.02
intI2+ *intI1* + *sulII*	1	0.17	1.70
TOTAL	**57**	**9.7**	**100**

Fig. 4.5 Distribution of the *intI2* gene positive isolates according to the day and year of the study in which they were collected.

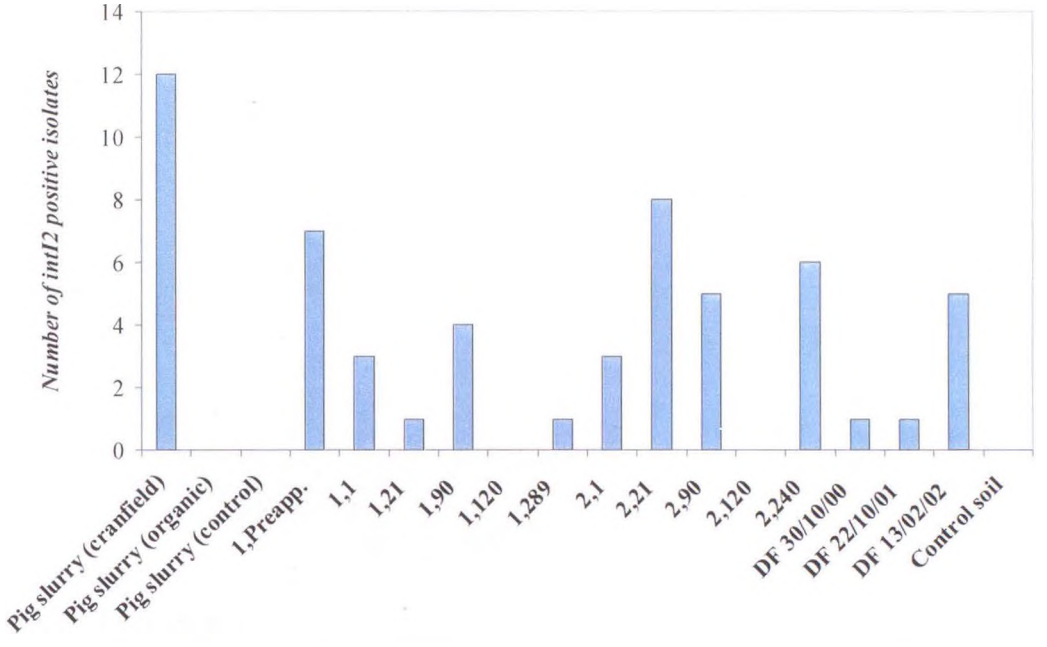


Table 4.6 Prevalence of isolates carrying *intI2* in each slurry and soil core sample.

Sample	Number isolates *intI2* positive	% of *intI2* positives from each sample	Number of screened isolates from each sample
Pig slurry (Cranfield)	12	21.06	78
Pig slurry (organic)	0	0	32
Pig slurry (control)	0	0	18
1,P	7	12.28	79
1,1	3	5.26	71
1,21	1	1.75	20
1,90	5	7.03	39
1,120	0	0	1
1,289	1	1.75	41
2,1	3	5.26	45
2,21	8	14.04	39
2,90	4	7.02	41
2,120	0	0	4
2,240	6	10.53	29
DF 30/10/00	1	1.75	8
DF 22/10/01	1	1.75	10
DF 13/2/02	5	8.77	13
Control soil	0	0	15
TOTAL	***57***	***100***	***583***

Fig. 4.6 Distributions of the *intI2* gene in isolates collected from selective plates containing different antibiotic concentrations.

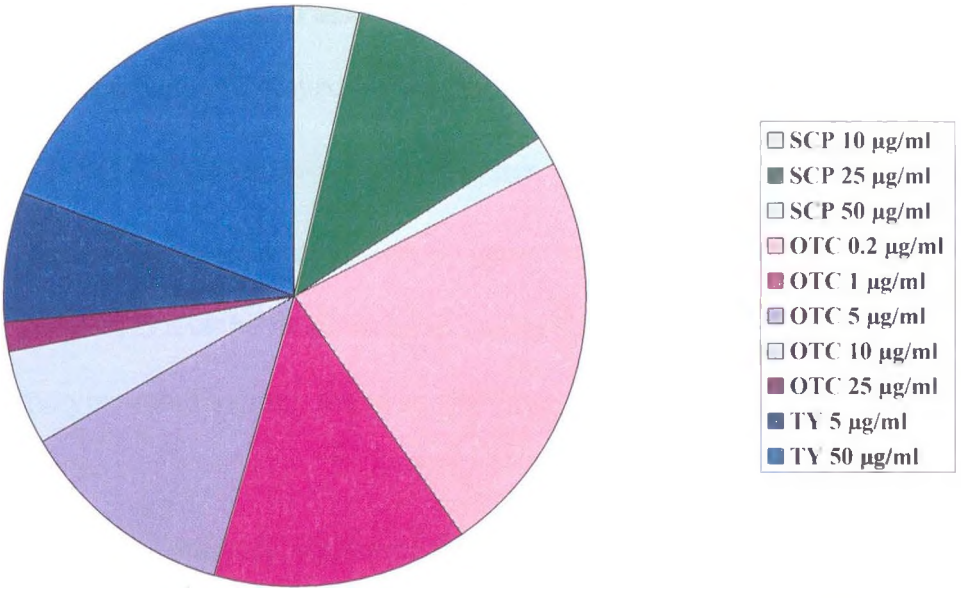


Table 4.7 Prevalence on a number of selective plates containing antibiotics at different concentrations for isolates PCR positive for the *intI2* gene.

Antibiotic concentration (µg/ml)	Number of *intI2* positive	% of *intI2* positives from each selective plate	Number of total isolates from each antibiotic selective plates
None	0	0	75
TY 0.2	0	0	3
TY 1	0	0	3
TY 5	4	7.02	40
TY 10	0	0	18
TY 25	0	0	16
TY 50	11	19.3	123
SCP 5	0	0	3
SCP 10	2	3.51	48
SCP 25	7	12.28	64
SCP 50	1	1.75	41
SCP100	0	0	10
OTC 0.2	13	22.81	29
OTC 1	8	14.04	21
OTC 5	7	12.28	36
OTC 10	3	5.26	23
OTC 25	1	1.75	30
TOTAL	***57***	***100***	***583***

A prevalence of 56 % *intI2* positive isolates were collected from plates containing OTC, 13 (22 %) were isolated from OTC 0.2 µg/ml (Fig. 4.6 and Table 4.7). A large number of positive isolates were also assembled from plates containing TY 50 µg/ml but at a lower prevalence than displayed for the *intI1* gene.

4.3.3 Prevalence of sulphonamide resistance genes

Screening of the three sulphonamide resistance genes was performed individually and trends in Figs. 4.7 to 4.15 and the frequencies in Tables 4.8 to 4.16 are displayed, with a summary of genotype combinations found in this study given in Fig. 4.16 and Table 4.17.

The most frequent sulphonamide resistance gene isolates from culturable bacteria in this study was *sulII*, 32.59 % of isolates (Table 4.11), with the *sulI* gene encoded in 31.05 % of isolates (Table 4.8) and 18.35 % carrying the *sul3* gene (Table 4.14). For all three genes, their highest prevalence was when identified singularly without other screened genes. Other frequent combination of genes were *sulII* + *sul3* (6.35 %), *sulI* + *sulII* (5.32 %) and *sulI* in combination with *intI2* (5.57 %) or *intI1* (1.89 %).

The *sulI* gene was observed more frequently in pre-application soil core isolates than either *sulII* or *sul3* which were recorded more in Cranfield slurry and year 1, day 1 soils respectively.

The *intI1* gene occurred more frequently in isolates from the pre- application soil cores (Fig. 4.2) and this trend was also observed in Fig. 4.8 with the *sulI* gene. No correlation was observed between application of slurry to the soil and prevalence of the sulphonamide resistance genes in culturable bacteria in year 1, but in year 2 an increase in *sulI, sulII* and *sul3* gene positive isolates was recorded the day after application and day 90 (day 21 in the cases of *sulII* and *sul3*), thereafter decreasing. A number of

sulphonamide resistance gene positive isolates were identified in the drain-flow samples and control slurries but only *sulII* was observed in the control soil.

The highest prevalence of *sulI* positive isolates was collected from plates containing tylosin 50 μg/ml (Fig. 4.9 and Table 4.10) and this trend was also observed with *intI1* indicating the *sulI* gene maybe linked to a Class 1 integron. Linkage data, however, suggests there is no physical link between the *sulI* and *intI1* genes (Table 4.18). The highest numbers of *sulII* containing bacteria were isolated from tylosin containing plates at a range of concentrations but isolates were also collected from SCP and OTC containing plates at concentrations of 100 μg/ml and 25 μg/ml. There was no correlation to SCP, with the lowest number of *sulI* positive isolates being collected from SCP containing plates, although 3 isolates were collected from plates containing SCP at a concentration of 100 μg/ml (Table 4.10).

In contrast to the *sulI* gene, both *sulII* and *sul3* were isolated at the highest prevalence from plates containing SCP (Figs. 4.12 and 4.15), 25 μg/ml SCP containing plates carrying the highest number of *sulII* and *sul3* positive isolates. 20% of *sulII* isolates were cultured from tylosin 50 μg/ml plates with a lower prevalence from OTC containing plates. Only 8.33 % of *sul3* positive isolates were collected from tylosin 50 μg/ml plates (Table 4.16).

Fig. 4.7 Prevalence of *sulI* containing genotypes in the total number of bacterial amples, as screened by PCR.

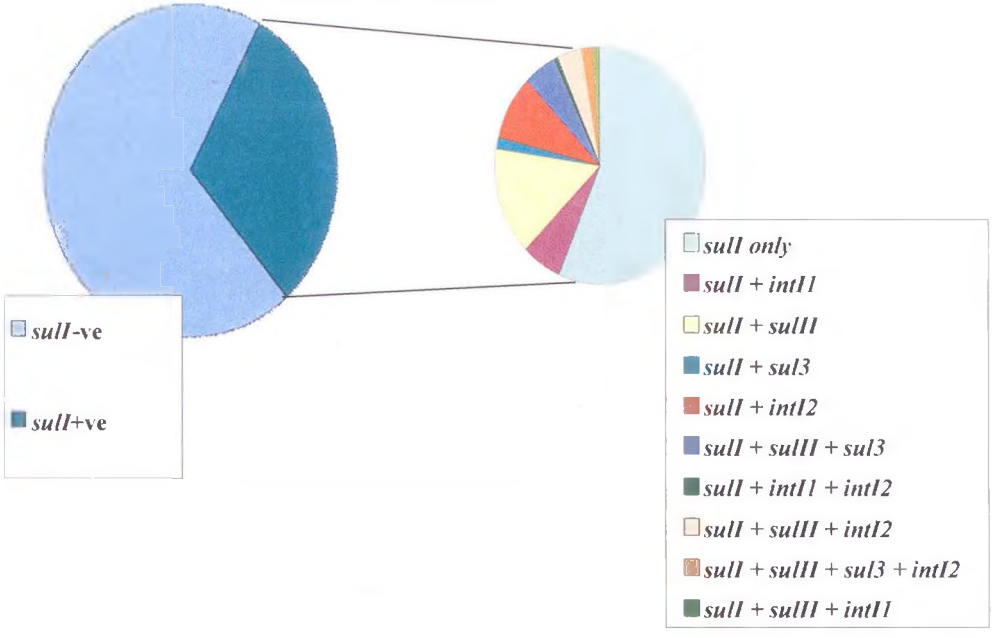


Table 4.8 Prevalence and numbers of *sulI* containing genotypes in all the bacterial isolates collected and within the *sulI* positive isolates.

Genotype	Number of isolates PCR positive for *sulI*	% from total number of collected isolates	% from total number of positive isolates
sul1 only	99	16.98	54.7
sul1 + *intI1*	11	1.89	6.08
sulI + *intI2*	15	2.57	8.29
sulI + *sulII*	31	5.32	17.13
sulI + *sul3*	4	0.69	2.21
sulI + *intI2* + *intI1*	1	0.17	0.55
sulI + *sulII* + *intI2*	6	1.03	3.31
sulI + *sulII* + *intI1*	2	0.34	1.10
sulI + *sulII* + *sul3*	9	1.54	4.97
sulI + *sulII* + *sul3* + *intI2*	3	0.34	1.66
TOTAL	**181**	**31.05**	**100**

Fig. 4.8 Distribution of the *sulI* gene positive isolates according to the day and year of the study in which they were collected.

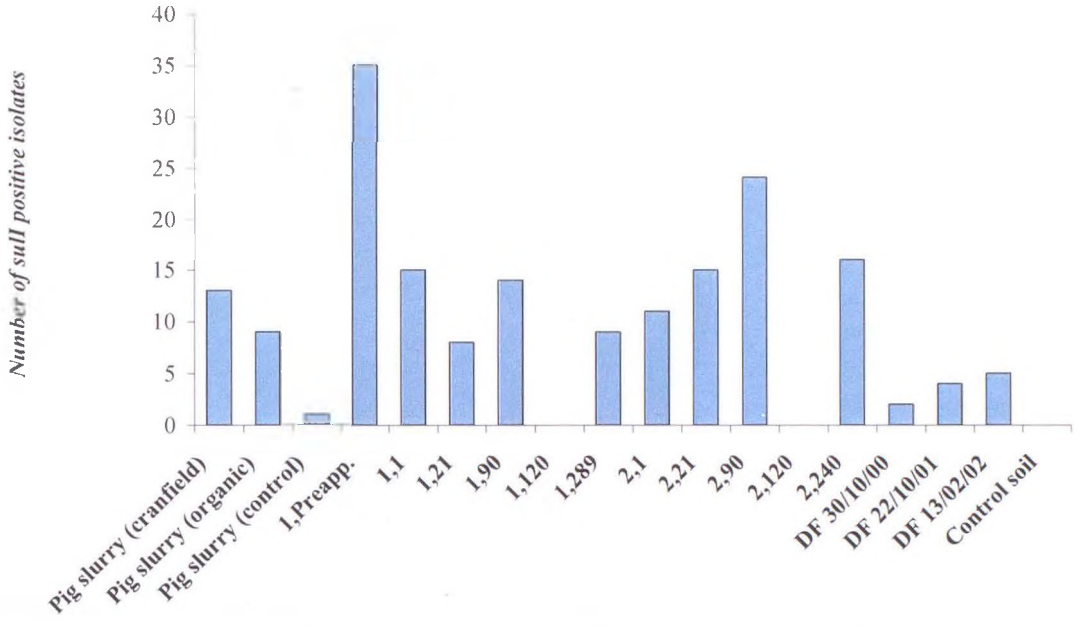


Table 4.9 Prevalence of isolates carrying *sulI* in each slurry and soil core sample.

Sample	Number of *sulI* positive	% prevalence of *sulI* positives	Total number of screened isolates from each sample
Pig slurry (Cranfield)	13	7.18	78
Pig slurry (Organic)	9	4.97	32
Pig slurry (Control)	1	0.55	18
Control soil	0	15	15
1,P	35	19.34	79
1,1	15	8.29	71
1,21	8	4.42	20
1,90	14	7.73	39
1,120	0	0	1
1,289	9	4.97	41
2,1	11	6.08	45
2,21	15	8.29	39
2,90	24	13.26	41
2,120	0	0	4
2,240	16	8.84	29
DF 30/10/00	2	1.10	8
DF 22/10/01	4	2.21	10
DF 13/2/02	5	2.76	13
TOTAL	***181***	***100***	***583***

Fig. 4.9 Distributions of the *sulI* gene in isolates collected from selective plates containing different antibiotic concentrations.

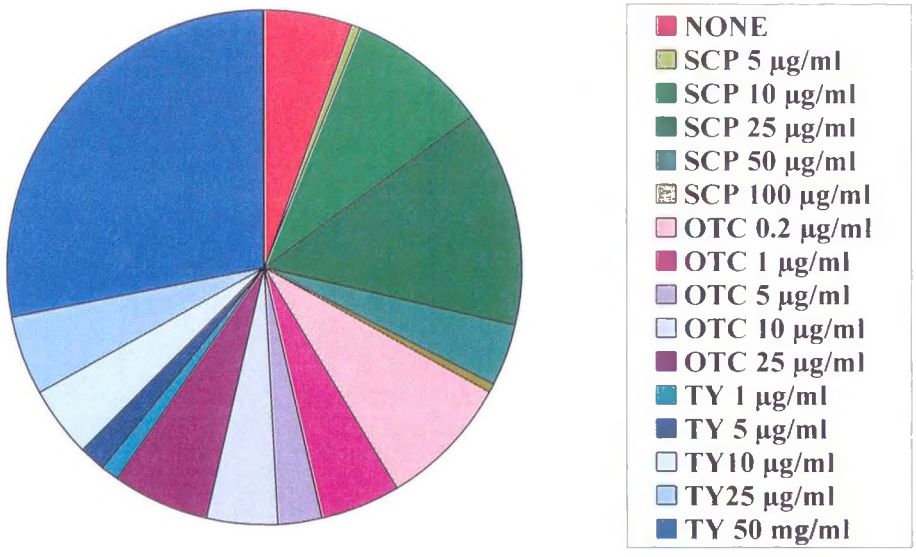


Table 4.10 Prevalence, on a number of selective plates containing antibiotics at different concentrations, for isolates PCR positive for the *sulI* gene.

Antibiotic concentration (µg/ml)	Number of *sulI* positive	% of *sulI* positives from each selective plate	Number of total isolates from each antibiotic selective plate
None	10	5.52	75
TY 0.2	0	0	3
TY 1	2	1.11	3
TY 5	3	1.66	40
TY 10	8	4.42	18
TY 25	9	4.97	16
TY 50	51	28.18	123
SCP 5	1	0.555	3
SCP 10	16	8.84	48
SCP 25	25	13.81	64
SCP 50	7	3.87	41
SCP100	1	0.555	10
OTC 0.2	15	8.29	29
OTC 1	9	4.97	21
OTC 5	5	2.76	36
OTC 10	8	4.42	23
OTC 25	11	6.08	30
TOTAL	***33***	***100***	***583***

Fig. 4.10 Prevalence of *sulII* containing genotypes in the total number of bacterial isolates collected from all slurry and soil samples, as screened by PCR.

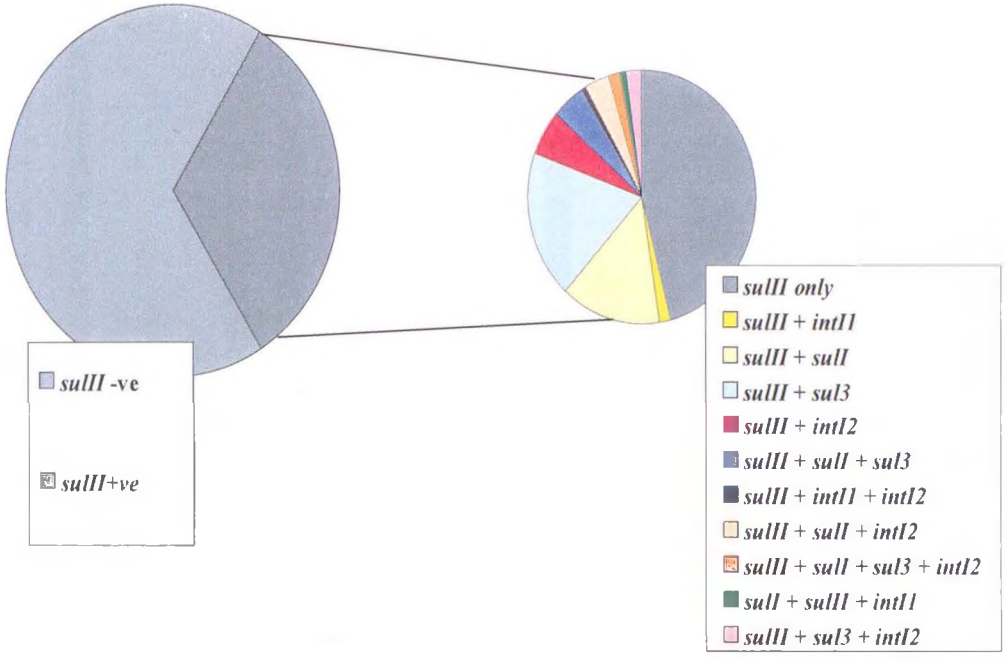


Table 4.11 Prevalence and numbers of *sulII* containing genotypes in the all bacterial isolates collected and within the *sulII* positive isolates.

Genotype	Number of isolates PCR positive for *sulII*	% from total number of collected isolates	% from total number of positive isolates
sulII only	83	14.24	45.68
sulII + intI1	3	0.51	1.58
sulII + sulI	31	5.32	16.32
sulII + intI2	11	1.89	5.79
sulII+ sul3	37	6.35	19.47
sulII + sulI + sul3	9	1.54	4.74
sulII + sulI + sul3 + intI2	3	0.51	1.58
sulII + sul3 + intI2	4	0.69	2.11
sulII + sulI + intI2	6	1.03	3.16
sulII + sulI + intI1	2	0.34	1.05
sulII + intI1 + intI2	1	0.17	0.53
TOTAL	**190**	**32.59**	**100**

Fig. 4.11 Distribution of the *sulII* gene positive isolates according to the day and year of the studying which they were collected.

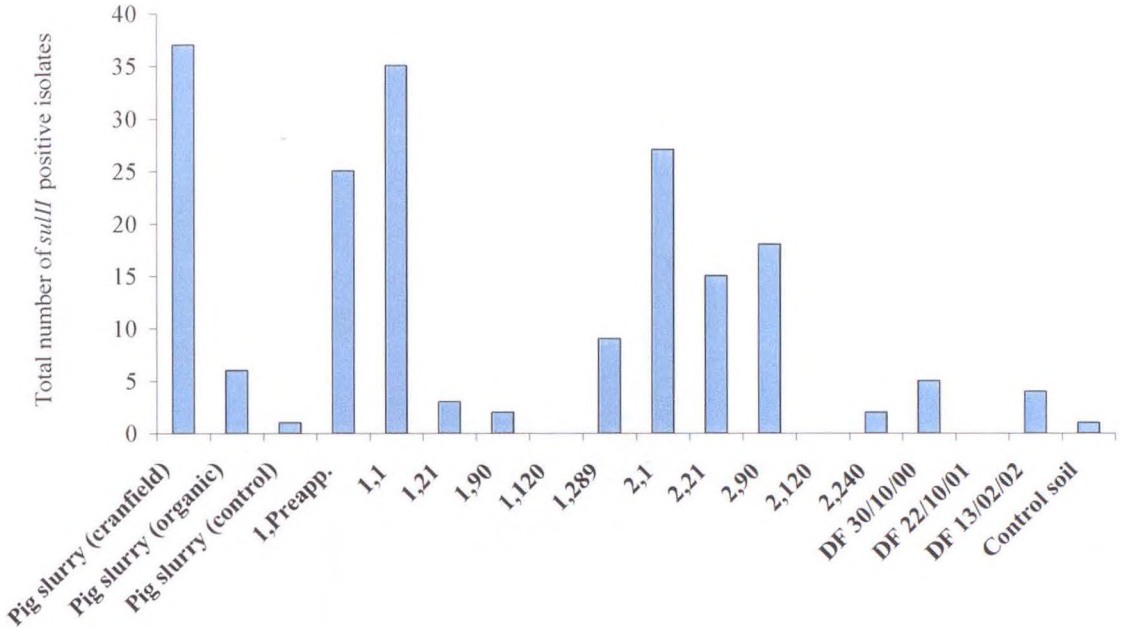


Table 4.12 Prevalence of isolates carrying *sulII* in each slurry and soil core sample.

Sample	Number of *sulII* positive	% prevalence of *sulII* positives	Total number of screened isolates from each sample
Pig slurry (Cranfield)	36	18.95	78
Pig slurry (organic)	6	3.16	32
Pig slurry (control)	1	0.53	18
control soil	1	0.53	15
1,P	25	13.16	79
1,1	34	17.89	71
1,21	4	2.11	20
1,90	2	1.05	39
1,120	0	0	1
1,289	10	5.26	41
2,1	28	14.74	45
2,21	14	7.37	39
2,90	18	9.47	41
2,120	0	0	4
2,240	2	1.05	29
DF 30/10/00	5	2.63	8
DF 22/10/01	0	0	10
DF 13/2/02	4	2.11	13
TOTAL	***190***	***100***	***583***

Fig. 4.12 Distributions of the *sulII* gene in isolates collected from selective plates containing different antibiotic concentrations.

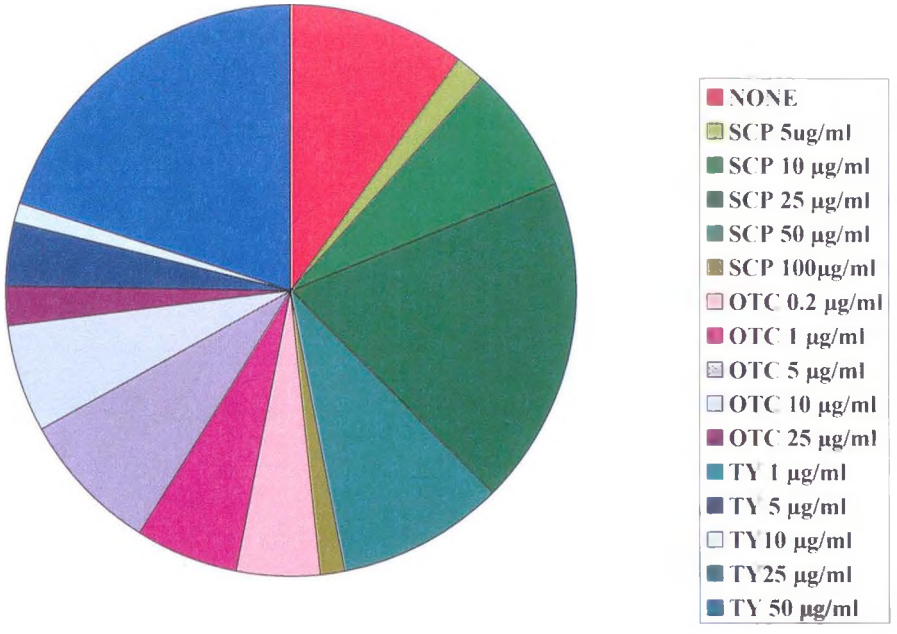


Table 4.13 Prevalence, on a number of selective plates containing antibiotics at different concentrations, for isolates PCR positive for the *sulII* gene.

Antibiotic concentration (µg/ml)	Number of *sulII* positive	% of *sulII* positives	Number of total isolates from each antibiotic selective plate
None	19	10.0	75
TY 0.2	0	0	3
TY 1	0	0	3
TY 5	7	3.68	40
TY 10	2	1.05	18
TY 25	0	0	16
TY 50	38	20.0	123
SCP 5	3	1.58	3
SCP 10	14	7.37	48
SCP 25	35	18.42	64
SCP 50	18	9.47	41
SCP100	3	1.58	10
OTC 0.2	9	4.74	29
OTC 1	11	5.78	21
OTC 5	15	7.89	36
OTC 10	12	6.32	23
OTC 25	4	2.11	30
TOTAL	***190***	***100***	***583***

Fig. 4.13 Prevalence of *sul3* containing genotypes in the total number of bacterial isolates collected from all slurry and soil samples, as screened by PCR.

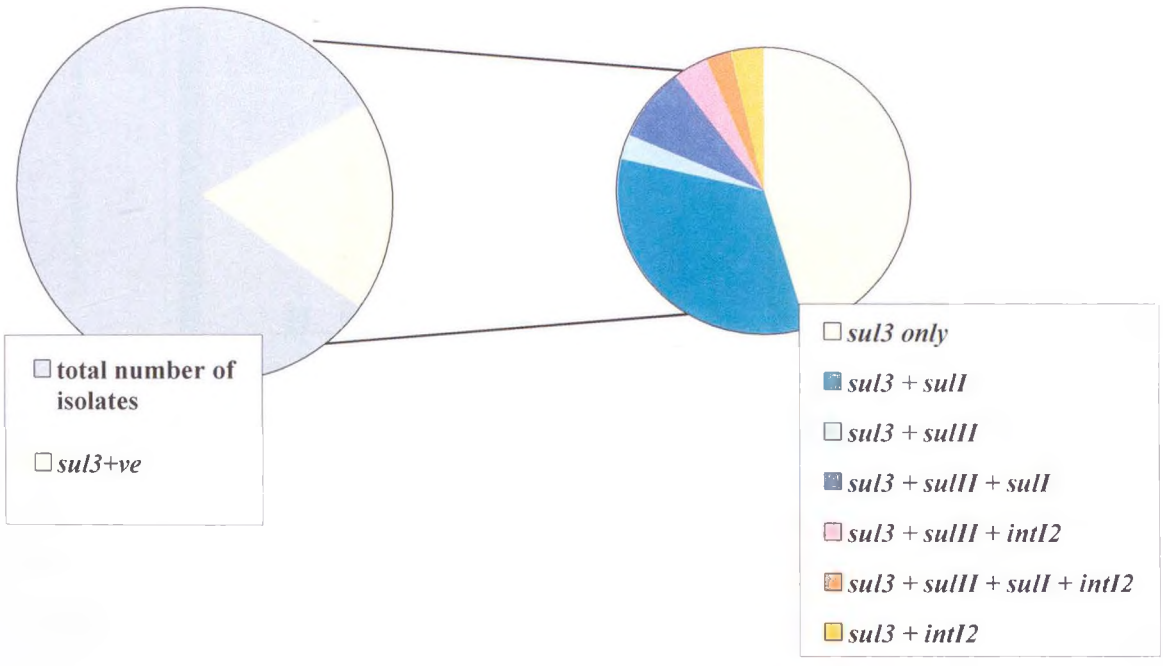


Table 4.14 Prevalence and numbers of *sul3* containing genotypes in the all bacterial isolates collected and within the *sul3* positive isolates.

Genotype	Number of isolates PCR positive for *sul3*	% from total number of collected isolates	% from total number of positive isolates
*sul3*only	46	7.89	42.99
sul3 + sulI	4	0.69	3.74
sul3 + intI2	4	0.69	3.74
sul3 + sulII	76	6.35	34.58
sul3 + sulII + sulI	9	1.54	8.41
sul3 + sulII + sulI + intI2	3	0.51	2.80
sul3 + sulII + intI2	4	0.69	3.74
TOTAL	**107**	**18.35**	**100**

Fig. 4.14 Distribution of the *sul3* gene positive isolates according to the day and year of the studying which they were collected.

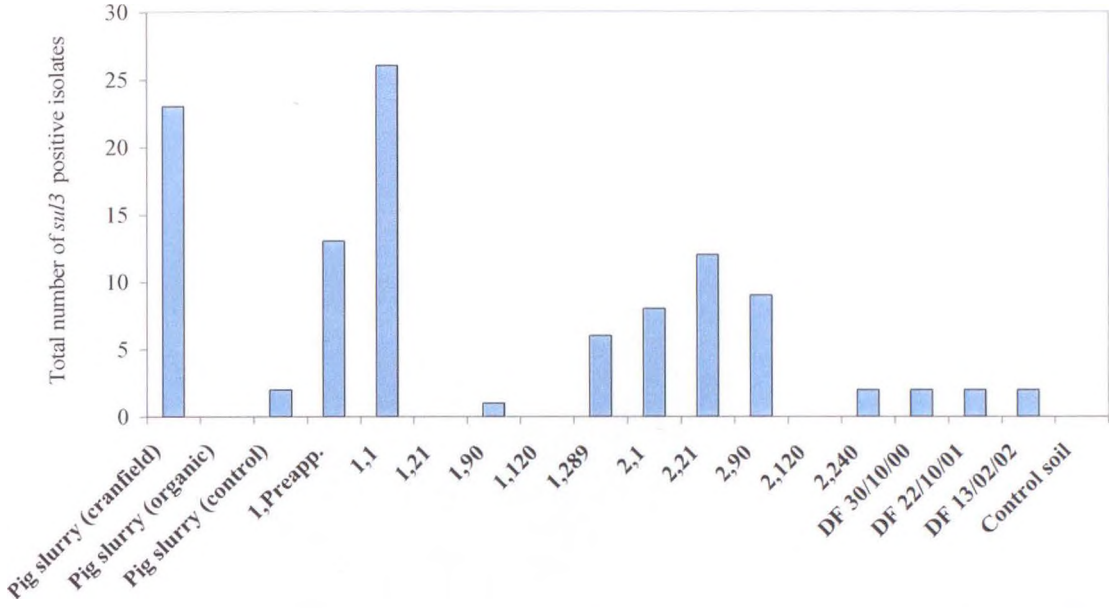


Table 4.15 Prevalence of isolates carrying *sul3* in each slurry and soil core sample.

Sample	Number of *sul3* positives	% prevalence of *sul3* positives	Total number of screened isolates from each sample
Pig slurry (Cranfield)	23	21.50	78
Pig slurry (organic)	0	0	32
Pig slurry (control)	2	1.87	18
control soil	0	0	15
1,P	13	12.15	79
1,1	26	24.3	71
1,21	0	0	20
1,90	1	0.93	39
1,120	0	0	1
1,289	6	5.6	41
2,1	7	6.54	45
2,21	12	11.21	39
2,90	9	8.41	41
2,120	0	0	4
2,240	2	1.87	29
DF 30/10/00	2	1.87	8
DF 22/10/01	2	1.87	10
DF 13/2/02	2	1.87	13
TOTAL	***107***	***100***	***583***

Fig. 4.15 Distributions of the *sul3* gene in isolates collected from selective plates containing different antibiotic concentrations.

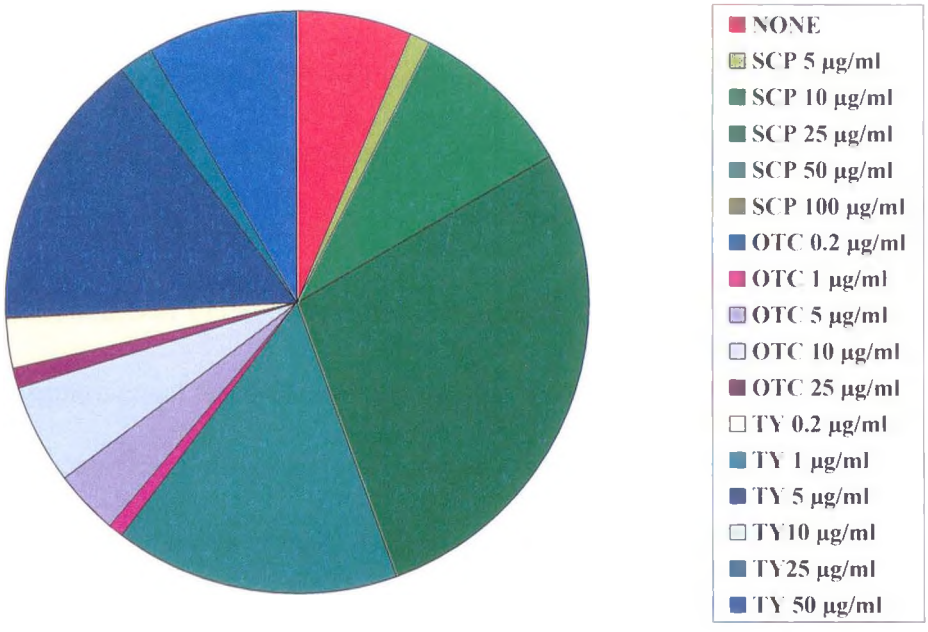


Table 4.16 Prevalence, on a number of selective plates containing antibiotics at different concentrations, for isolates PCR positive for the *sul3* gene.

Antibiotic concentration (µg/ml)	Number of *sul3* positive	% of *sul3* positives from each selective plate	Number of total isolates from each antibiotic selective plate
None	7	6.54	77
TY 0.2	3	2.8	3
TY 1	0	0	3
TY 5	17	15.88	40
TY 10	0	0	18
TY 25	2	1.87	16
TY 50	9	8.41	123
SCP 5	1	0.93	3
SCP 10	10	9.35	48
SCP 25	30	28.04	64
SCP 50	16	14.96	41
SCP100	0	0	10
OTC 0.2	0	0	29
OTC 1	1	0.93	21
OTC 5	4	3.74	36
OTC 10	6	5.61	23
OTC 25	1	0.93	30
TOTAL	***107***	***100***	***583***

4.3.4 Genotype combinations

The various combinations of genotypes displayed from the screened genes found in the total number of isolates collected in this study are given in Fig. 4.16 and Table 4.17. Of the 583 screened, 189 culturable bacterial isolates were negative for all of the screened genes and the genotypes observed at the highest frequencies were those containing a single sulphonamide resistance gene; *sulI* (16.98 %), *sulII* (14.24 %) and *sul3* (7.89 %).

4.3.5 Best fit association between the screened genes

Pearson's chi-square exact test was used to evaluate the association between each of the screened genes and the t-values are recorded in Table 4.18. An association between two genes can result in a positive result, indicating that the genes are found together in the same isolate or linked in some way. A negative result may signify that the genes are not located together or linked. There was no association found between *sulI* and *intI1*, *intI1* and *intI2*, *intI2* and *sulII*, *intI2* and *sul3*, *intI1* and *sulII*, nor between *sulI* and *sul3*. Prevalence data had suggested that the *sulII* gene may have become integrated onto a Class 1 integron replacing the *sulI* gene, but linkage analysis conflicts with this data and implies this is unlikely to have occurred. Association may occur between *intI2* and *sulI* suggesting the *sulI* gene has been found integrated onto a Class 2 integron and a link was also observed between *sulI* and *sulII*, and *sulII* and *sul3*, suggesting multiple sulphonamide resistance genes on a single plasmid or other mobile genetic element.

Fig. 4.16 Summary of genotypes containing screened genes found in the total culturable bacterial isolates collected in this study.

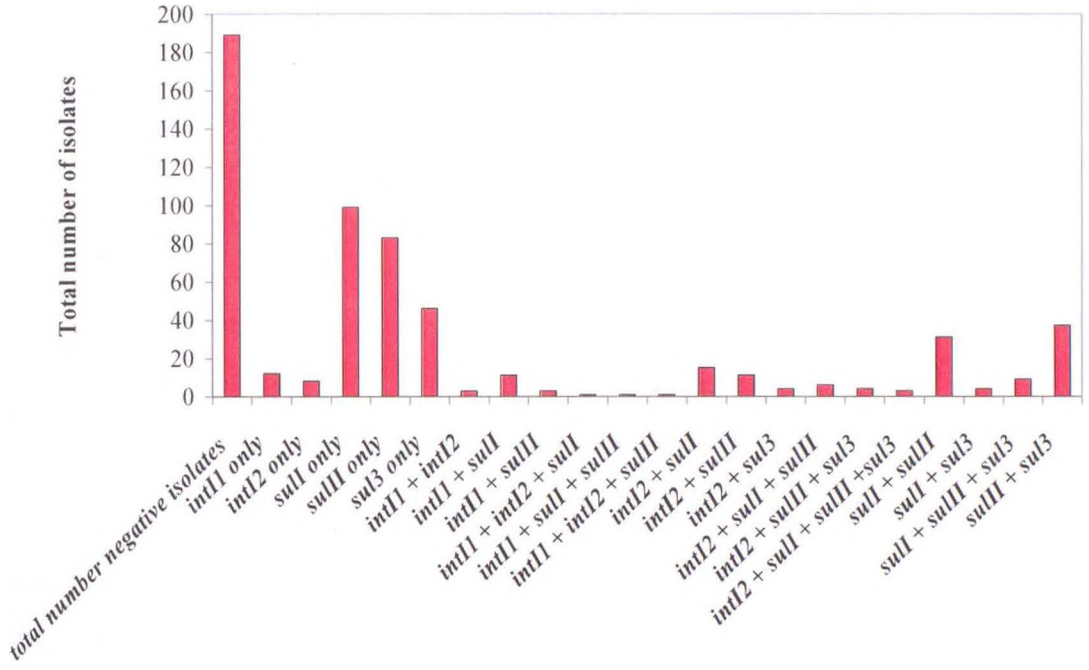


Table 4.17 Summary of frequencies of the genotypes containing the screened genes in the culturable bacterial isolates collected in this study

Genotype	No. isolates PCR positive for genotype	% from total number of collected isolates
Negative	189	32.08
intI1 only	12	2.06
intI2 only	8	1.37
sulI only	99	16.98
sulII only	83	14.24
sul3 only	46	7.89
intI1 + *intI2*	3	0.51
intI1 + *sulI*	11	1.89
intI1 + *intI2* + *sulI*	1	0.17
intI1 + *sulII*	3	0.51
intI1 + *intI2* + *sulII*	1	0.17
intI1 + *sulI* + *sulII*	2	0.34
intI1 + *sul3*	0	0
intI2 + *sulI*	16	2.74
intI2 + *sulII*	11	1.88
intI2 + *sul3*	4	0.68
intI2 + *sulI* + *sulII*	6	1.03
intI2 + *sulI* + *sulII* + *sul3*	3	0.51
intI2 + *sulII* + *sul3*	4	0.68
sulI + *sulII*	31	5.32
sulI + *sul3*	4	0.69
sulI + *sulII* + *sul3*	9	1.54
sulII + *sul3*	37	6.35
TOTAL	***583***	***100***

Table 4.18 Association between the screened genes, t-values of Pearson's chi-square exact test.

	intI2	*sulI*	*sulII*	*sul3*	*intI1*
intI2	-	0[1]	-6.18	-9.72	-5.61
intI1	-5.61	-7.58[2]	-14.48	-	-
sulI	0[1]	-	9.07[3]	-16.46	-7.58[2]
sulII	-6.18	9.07[3]	-	17.36[4]	-14.48
sul3	-9.72	-16.46	17.36[4]	-	-

[1]**indicate *intI2* and *sulI* could be linked**

[2]**no link between *sulI* and *intI1* in this study**

[3]***sulI* and *sulII* are linked**

[4]***sulII* and *sul3* are also linked in this study**

Using Pearson's chi-square exact test (Uitenbroek, 1997).

4.4 DISCUSSION

4.4.1 Prevalence of *intI1* gene

Of the bacteria isolated in this study, 5.64 % encoded the *intI1* gene, on its own or in a number of different combinations with other screened genes. Previous environmental investigations have reported similar frequencies, Gaze et al., (2005) recorded a prevalence of 7.98 % in isolates from a QAC-polluted environment and Rosser and Young (1999) reported 3.6 % of bacteria isolated in their study carried a Class 1 integron. A higher incidence of 24 % was observed in a report into bacteria isolated from an aquatic environment, the high prevalence may have been due to the selection of coliforms for screening (Park et al., 2003).

The highest genotype prevalence in the current study was *intI1* without any screened genes. The *intI1* gene has been characterised alone in other studies; Fonseca et al., (2005) reported that 34 % of their *P. aeruginosa* isolates carried *intI1* and no gene cassettes, Park et al., (2003) identified 12 out of 150 coliform isolates encoded *intI1* alone and 11 of 3000 Gram negative isolates in the study by Rosser and Young (1999) gave a much lower prevalence than this study.

The highest combined prevalence was the association of *intI1* with the *sulI* gene. This association was expected as *intI1* has been commonly found on Class 1 integrons with the *sulI* gene forming part of its conserved 3' backbone structure (Sundström et al., 1988). Further characterisation of these isolates determined whether both genes were present together on a Class 1 integron, although statistically there was no association between the two genes.

A second sulphonamide gene, *sulII* was present in isolates carrying the *intI1* gene. The *sulII* gene has not been observed as a gene cassette on a Class 1 integron but has

been found in other studies in isolates carrying the *intI1* gene (Maynard et al., 2003; Peirano et al., 2005; Sunde and Sørum, 2001). The best fit analysis displays no significant link between *intI1* and *sulII*, therefore, has probably not replaced *sulI* in isolates found in the current study on a Class1 integron.

Interestingly, in this study *intI1* was not located in the same isolates as the *sul3* gene. The *intI1* and *sul3* genes have been reported together in the same isolate in other studies, for example, Antunes et al., (2005) in an investigation of sulphonamide resistance genes in *S. enterica* strains. In this current study, no *intI1* in the same isolate as *sul3* may indicate an incompatibility with the mobile element encoding the latter gene. Little is known about the *sul3* gene and only a limited number of papers have been published since its identification by Perreten and Boerlin (2003) in which they mapped the *sul3* gene to a 54 Kb conjugative plasmid flanked by IS elements. No further studies have investigated the genetic elements encoding the *sul3* gene. The *intI1* and *sul3* prevalence could be the result of a lower distribution of the genotype in this environment, *sul3* was found at a 13 % lower frequency than the *sulI* and *II* genes. The PCR screen and the isolation method may also have been biased and not cultured *sul3* positive isolates or these isolates may have been unculturable.

The *intI1* gene was not characterised in isolates from the organic or control slurry possibly due to the lack of selective pressure since the pigs from the organic farm had not been exposed to any antibiotics to our knowledge and Class 1 integrons are not described as carrying tylosin resistance genes for co-selection in the case of the control slurry. Class 1 integrons have been most widely described in Enterobacteriacae (White et al., 2001), therefore the lower prevalence of *intI1* genes in these slurries may be the result of no bacteria from this group being isolated or a smaller number of isolates

cultured from these samples in comparison to the Cranfield slurry. The control and Cranfield slurries only differed in the addition of SCP and OTC spiking to the latter. This may have increased the selection of resistant bacteria in the slurry but we would have expected a high number of *intI1* genes present in the control and possibly a decrease in those numbers by the addition of antibiotics to the Cranfield slurry. This difference may again have been due to the lower numbers of isolated bacteria from these samples, 18 and 78 isolates respectively.

The highest prevalence of *intI1* gene positive isolates was in the pre-application soils in year 1, therefore demonstrating that the indigenous bacteria in this study carried the *intI1* genes before the application of slurry to the soil. The long-term continuous application of slurry from tylosin-fed pigs could be the source of the *intI1* gene in the soil bacterial population, supplementing the soils gene pool and the tylosin providing a selective pressure for horizontally transferred genes. No trend was exhibited in the data to propose that the application of Cranfield slurry to the pre-application soil did lead to an increase in the prevalence of the *intI1* gene and no trend was observed which indicated that the *intI1* gene was being transferred into the wider indigenous population. A control in which tylosin had not been applied would be necessary to check the environmental consequences of long-term tylosin exposure.

A link in this study between the *intI1* gene and tylosin resistance is proposed. Despite the higher numbers of total isolates from tylosin 50 µg/ml plates, the number of positive isolates is higher compared to isolates numbers from other antibiotic concentrations. This association may have been established due to the continual selection in the pig gut by tylosin in the feed. No published studies have reported an association between *intI1* and tylosin resistance.

4.4.2 Prevalence of the *intI2* gene

The *intI2* gene prevalence was higher in this study than the *intI1* and a number of published studies also observed the frequency of *intI2* to be higher than *intI 1*(Du et al., 2005; Gonzalez et al., 1998). The reports by Du et al., (2005) and Gonzalez et al., (1998) both investigated medically relevant bacteria indicating that the prevalence and distribution of integrons are similar in the diverse niches of the environment and clinical setting.

The *intI1* and *intI2* genes were carried together in 4 isolates, one with the *sulI* gene and another with the *sulII* gene and in two isolates they were alone. To date no environmental studies have screened for both the *intI1* and the *intI2* genes, but a number of studies into specific species have identified both genes at higher frequencies than those recorded in this study. A percentage of 4.7 % of *S. enterica* serovar *paratyphi* B isolates from poultry encoded both genes in a German study by Miko et al., (2003) and 2 out of 27 epidemic strains of *S. sonnei* also contained both genes (McIver et al., 2002). The presence of both classes of integrons in the same isolates may indicate a highly transferable pool of resistance genes in this current study environment and bacteria having twice the capability for HGT.

Unlike the *intI1* gene, *intI2* was identified in isolates with the *sul3* gene. This genotype may be possible in this environment because the *intI2* gene has different ancestry (Fig. 1.13) and is located on different plasmids (Rowe-Magnus et al., 2001). Neither *intI1* nor *intI2* have been characterised with the *sul3* gene in any published studies.

Sulphonamide genes were also characterised in the same isolate as *intI2* in this study but only the *sulI* gene has previously been reported on a Class 2 integron (Peirano et al.,

2005). Statistically *intI2* and *sulI* may be associated in this study and further characterisation of these isolates will reveal if the sulphonamide genes are on an integron or elsewhere in the genome. One question to be addressed is whether the *sulII* gene has replaced *sulI* on the integron structures and now acts as a gene cassette. The data suggests this not to be the case as there is no correlating relationship between *intI1* nor *intI2* with the *sulII* gene.

The results demonstrated that the *intI2* gene was most frequently located in samples from the Cranfield slurry and none were recorded in the organic/control slurries or control soils. This may be due to the same reasons detailed for the *intI1* gene, a lack of selection and bias sampling numbers. As with *intI1*, there was no increase in frequencies of *intI2* with the addition of pig slurry to pre-application soils in years 1 or 2. It can be concluded that the *intI2* gene positive isolate frequencies in soil cores from the agricultural field were not affected by the addition of pig slurry containing *intI2* positive isolates.

In contrast to the *intI1* gene, the highest frequencies of *intI2* gene positive isolates were cultured on OTC containing plates, therefore having no link with tylosin resistance. No published studies have reported a link between tetracycline resistance and the *intI2* gene, and there are no instances where a tetracycline resistance gene has been mapped as a gene cassette on a Class 2 integron. Tetracycline resistance genes have been identified on plasmids containing integrons; *tet(A)* and *tet(R)* genes on pTet33 in *C. glutamicum* but not encoded within the integron (Tauch et al., 2002). The high prevalence of the *intI2* gene from OTC containing plates may therefore indicate the mobility of a plasmid within this environment that co-selected for the Class 2 integron due to tetracycline resistance determinates.

4.4.3 Frequencies of sulphonamide resistance genes

The sulphonamide resistance genes were all characterised singularly in the isolates collected from this study and in combination with and without the integrase genes screened. A number of the *sulI* only positive isolates were re-screened to clarify that they did not carry the *intI1* gene and therefore were not linked to Class 1 integrons, also supported by the statistical data previously mentioned.

Of the total number of cultured isolates, 12 contained all three sulphonamide resistance genes, 2 of which carried the *intI2* gene. This genotype has been previously reported in *Salmonella* spp. (Antunes et al., 2005). Bacteria often carry more than one resistance determinant for the same antibiotic but they are often different genes conferring resistance with different mechanisms, for example; streptomycin resistance encoded by the *strA* gene (found on conjugative plasmids) and *aadA* genes located on integrons (Brenner Michael et al., 2005). The *sulI*, *sulII* and *sul3* genes all encode for alternative DHPS enzymes therefore the question arises as to why an individual bacterial isolate would carry all three genes. As previously mentioned the *sul* genes have been identified on different mobile elements and the three genes confer different degrees of resistance but no evidence supports the theory that encoding more than one sulphonamide resistance gene gives the bacterium an increased protection with a higher MIC (Perreten and Boerlin, 2003; Sköld, 2001). The carriage of these three genes also suggests a lack of a detrimental fitness cost without a selective pressure. The *sulII* gene was reported not to confer a fitness advantage over the carriage of *sulI* in *E. coli* strains (Enne et al., 2004). Do bacteria collect genes for future use in the environment or by random events? Previous studies have reported conflicting evidence for the collection and maintenance of antibiotic resistance genes; Andersson, (2003) discussed in a recent

review that investigations, both laboratory and epidemiological, conclude that a decrease in antibiotic use does not always lead to a decrease in resistance with several processes including compensation evolution, occurrence of cost-free resistances and co-selection between antibiotics leading to the long-term persistence of resistant bacteria and resistance genes. One such study investigated sulphonamide resistance in *E. coli* from human sources and concluded that despite a decrease in sulphonamide prescriptions, the prevalence of *sulI* and *sulII* increased between the years studied (Enne et al., 2001). In contrast, Danish researchers recorded a decrease in resistance to a number of antibiotics with a decrease in their tonnage use in veterinary medicines and growth promotion (Wegener, 2003).

Of the three sulphonamide resistance genes, *sulII* is recorded the most frequently in this study, followed by *sulI* and *sul3*. The differences in prevalence maybe due to the number of bacterial isolates collected, selection of one gene over the other through sulphonamide selection or a bias for bacterial species that carry those particular genes but it may represent a selection for *sulI* and *sulII* in this environment. The preferential selection of *sulII* in this environment, maybe due to; a fitness advantage, its presence on a successful and highly mobile plasmid or the genes' co-selection via linkage to one which is necessary for bacterial growth in this environment.

An association has been demonstrated between *sulI* and *sulII*, and *sulII* and *sul3*, but not *sul3* and *sulI*, indicating that there may a number of plasmids containing both the *sulII* and *sulI* genes but not *sul3*. There was no cross screening with the PCR primers for any of the *sull* genes (Fig. 7.2).

The *sulI* gene, as *intI1*, was predominantly identified in the pre-application soils, this maybe due a link with an element conferring tylosin resistance and its co-selection due

to continual tylosin use. Unlike *sulI*, *sulII* and *sul3* positive isolates were identified predominately in soils from year 1, day 1. The results suggest an increase in bacteria positive for *sulII* and *sul3* between soils from year 1, day 1 and pre-application soil cores due to the application of Cranfield slurry containing bacterial isolates positive for these genes. This effect was possibly due to the transient survival of enteric bacteria, the same effect was observed in year 2.

Bacterial isolates containing *sulII* and *sul3* were isolated in highest numbers from sulphonamide containing plates. This maybe because *sulII* and *sul3* have been reported to confer a higher resistance than the *sulI* gene although in all three cases the highest prevalence of sulphonamide containing isolates from sulphonamide containing plates was recorded on 25 μg/ml SCP. As most of the *sulII* and *sul3* isolates were collected from year1, day 1 these genes may confer a selective advantage to those bacteria and selection may have occurred in the slurry for sulphonamide resistance at sub-lethal levels.

4.5 Conclusions

This study demonstrated the presence of sulphonamide resistance genes and Class 1 and 2 integrons in agricultural soils where slurry has been applied and in the slurry itself. The study also indicates that the long-term application of tylosin-fed pig slurry onto agricultural soils has had a discernible effect on the prevalence and distribution of those antibiotic resistance genes through direct selection and as a result of co-selection, possible due to the presence of detected integron genes involved in HGT, as displayed by the lack of bacterial isolates PCR positive for the *intI1* and *intI2* genes in the organic and control slurries.

Sulphonamide application to soils via spiking the slurry may have selected for sulphonamide resistance gene containing bacteria both in the animal slurry and in the soil environment. This selection poses a biological environmental hazard that directly correlates with the chemical grading of the compound as a significant hazard despite its rapid temporal movement through the soil profile in comparison to tetracyclines.

4.6 FURTHER WORK

As mentioned in the discussion a number of areas need further characterisation and investigation. The identification of isolates would allow an investigation as to whether there is a presence of surviving enterics from the slurry after its application, whether sulphonamide resistance gene containing isolates are indigenous bacteria and would also provide an indication of transfer of these genes. Characterisation of the isolates would allow localization of the sulphonamide resistance genes. Integron characterisation would determine if *intI1* and *sulI* are on Class 1 integrons in this study and would allow genetic confirmation of the association analysis.

The isolate numbers vary significantly between antibiotic concentrations and samples. Logistics permitting these numbers should be balanced providing a clearer comparison of isolates between time points and selective plates.

A future study could be undertaken to investigate the link between *intI1* and tylosin resistance. Further work into this area may indicate if the long-term application of tylosin-fed pig slurry to the agricultural land in this study has had an effect. The study would benefit from a control experiment undertaken on agricultural soil where no antibiotics and specifically no tylosin had been applied. True environmental controls are difficult to acquire due to the widespread usage of antibiotics in agriculture,

veterinary and clinical settings and land may have direct or indirect contact with antibiotics through a number of routes including faecal and water contamination.

The unculturable pool of bacteria has not been investigated in this study; this could be addressed by the use of the soils for the extraction of total community DNA and molecular studies to screen for identical genes in the unculturable population. Newly emerging techniques such as metagenomic approaches and micro-arrays could further be used to interrogate the isolates and unculturable population, although not available to this study there are published micro-arrays which allow screening for the *sulI* and *sulII* genes but techniques such as micro-arrays and metagenomic approaches become increasing difficult when dealing with complex environmental bacteria reservoirs and where DNA has been extracted from soils rich in humic acids (Call et al., 2003).

CHAPTER 5:

CHARACTERISATION OF *intI1* GENE POSITIVE BACTERIAL ISOLATES AND THEIR INTEGRONS

Chapter 5: Characterisation of *intI1* gene positive isolates

5.1 INTRODUCTION

In Chapter 4, 33 (5.64 %) of the 583 bacterial isolates collected in this study were observed to be PCR positive for the *intI1* gene. This chapter will aim to characterise these isolates and their integrons in more detail.

An understanding of the distribution of integrons in the environment is relevant as these genetic elements have the potential for horizontal transfer and the integron structures encode and express gene cassettes which can also be horizontally transferred due to the nature of their integrase sites (Collis et al., 1993; Holmes et al., 2003; Partridge et al., 2000). A link between antibiotic resistance in humans and Class 1 integrons in the environmental bacterial reservoir has been documented in the cases of *E. coli* and *Salmonella* spp. which increases the concern over antibiotic release into the environment and the potential for selective pressures to force and maintain antibiotic resistant reservoirs of bacteria in the environment, a potential source for antibiotic resistance in clinically significant bacteria (Fluit and Schmitz, 2004).

5.1.1 Integron structure: brief overview

The integron structure has been discussed previously, but it is prudent to summarise briefly in the context of integron characterisation. A Class 1 integron is commonly based on a 5' conserved region containing an integrase gene, promoters and gene cassette integration sites (Stokes and Hall, 1989). The 3' conserved region may contain the *qacEΔ1* gene and the *sulI* gene (Rädström and Swedberg, 1988; Stokes and Hall, 1989). Integration of a gene cassette flanked by 59 bp elements which act as integration sites can occur between the 5' and 3' regions of the integron; the variable region (Collis et al., 1993; Partridge et al., 2000).

5.1.2 Environmental occurrence of integrons

Integrons, particularly those of the Class 1 type, have been found in a diverse number of bacterial genera and in varied environments including; apple orchards (Schnabel and Jones, 1999), fish farms (Schmidt et al., 2001a), estuarine environments (Rosser and Young, 1999) and polluted environmental sites (Gaze et al., 2005). Class 1 integrons are prevalent in the *Enterobacteriacae* (Sallen et al., 1995), but have also been found in *Acinetobacter spp.* (Gonzalez et al., 1998), Pseudomonads (Naas et al., 1999), *Bordetella bronchiseptica* (Kadlec et al., 2005), *H. pylori* (Crespo et al., 2005), *V. cholerae* (Mazel et al., 1998), *Aeromonas salmonicidia* (Schmidt et al., 2001b), *B. cepacia* (Crowley et al., 2002), *Stenotrophomonas maltophilia* (Chang et al., 2004), *C. jejuni* (Lee et al., 2002) and *Corynebacterium spp.* (Tauch et al., 2002).

Integron frequencies differ between the clinical and environmental studies. Frequencies are on average higher in the clinical setting, with the highest prevalence reported being 81 % in a study of ciprofloxacin-resistant *E. coli* isolates from Dutch hospitals, the average being approximately 40 % (Mooij et al., 2005; Mukherjee and Chakraborty, 2005; Singh et al., 2005; Zhao et al., 2005). The average prevalence of Class 1 integrons in environmental isolates is approximately 5 % in studies which do not focus on one specific bacterial genera, for example 3.6 % of isolates from an estuarine environment carried a Class 1 integron (Rosser and Young, 1999) , 7.98 % of isolates in a quaternary ammonium compound polluted environment (Gaze et al., 2005) and 24 % of coliform isolates from an aquatic environment also carried a Class 1 integron (Park et al., 2003). The 5.64 % of isolates found with the *intI1* gene in this study therefore correlates with these studies.

5.1.3 Tylosin resistance

The results in Chapter 4 indicated that there was a link between the incidence of the *intI1* in this study and the isolation of bacteria from tylosin containing plates. As detailed in Chapter 1, tylosin is a 16-membered ring macrolide antibiotic targeting the 23S ribosome (Baltz and Seno, 1988). Tylosin was commonly used in animal food production as a growth promoter until its European ban as such in July 1999 but can still be used therapeutically (EMEA, 1999).

Tylosin resistance is not necessarily determined by resistance genes conferring protection to the macrolide family of antibiotics, particularly those conferring resistance to the 14 and 15-membered rings. The biosynthetic gene cluster for tylosin contains 43 genes, 4 of which are resistance genes (Fouces et al., 1999; Stratigopoulos et al., 2004). The four resistance genes have not been found outside of the producer but share homology to the Erm family of methlytransferases, hence their re-naming in 1999 (Roberts et al., 1999). Mutations in the 23S rRNA commonly confer resistance to macrolides but only the A2062C mutation in Spirochetes has been reported to confer resistance to tylosin (Prapasarakul et al., 2003).

A further number methlytransferases have been observed to confer tylosin resistance; *ermB* and *ermX* identified from *Arcanobacteria pyogenes* (Jost et al., 2003; Jost et al., 2004), *ermC* gene in *S. aureus* strains (Lodder et al., 1997), the *ermT* gene was reported in *Lactobacillus reuteri* tylosin resistant strains (Whitehead and Cotta, 2001) and the *ermAMR* gene operon which was identified in *E. faecalis* (Oh et al., 1998).

Efflux pumps (Cagliero et al., 2005) and enzyme modification genes have also been reported to confer resistance to tylosin (Cagliero et al., 2005; Roberts et al., 1999; Singh et al., 2001; Taniguchi et al., 1999).

A number of reports have focused on tylosin resistance in isolates from pig herds where it was used as a growth promoter, observing resistance levels as high as 50 % (Kobayashi et al., 2005: Pringle et al., 2006). Since the ban of tylosin as a growth promoter in 1999, a number of investigation have monitored resistance after and comparing figures to those taken before the ban. Aarestrup et al., (2001) investigated tylosin resistance in *Enterococci* spp. from broilers and pigs between the years 1995 and 2000 concluding that with a decrease in usage a decrease in resistance was observed 46.7 % to 28.1 % with associations between tylosin resistance and glycopeptides, general macrolide resistance also decreasing from above 40 % to 6 % in 2000 (Aarestrup et al., 2001; Boerlin et al., 2001). This report and an investigation into sub-therapeutic use on farms and tylosin resistance in the bacterial soil population led to a conclusion that sub-therapeutic levels of antibiotics leads to an increase in numbers of resistant bacteria (Aarestrup et al., 2001; Onan and LaPara, 2003).

5.2 AIMS

The prevalence of the *intII* gene in this study has been determined as 5.64 % but it was unknown as to whether these genes were part of a Class 1 structure which may therefore be a vector for antibiotic resistance gene cassettes. Further characterisation of these isolates was needed to determine this.

This study's objectives were:

1. Screen, by PCR, for a number of specific genes which conferred resistance to the sulphonamide group of antibiotics; *sulI, sulII* and *sul3,* from the 33 culturable bacterial isolates obtained from environmental soil samples before and after the application of slurry and from bacterial isolates from the applied slurry, which contain the *intI1* gene;

2. To screen the same isolates as in 1, by PCR, for the *intI2*, this would indicate the presence of multiple compatible integrons and possible vectors for HGT and the mobilisation of the sulphonamide resistance genes;

3. To investigate the isolates which were collected from tylosin containing plates to determine whether there was a link between the *intI1* gene and tylosin resistance in this environment;

4. To characterise the isolates' Class 1 integrons by PCR and sequencing, determining which gene cassettes are present on the integrons.

5.3 RESULTS

5.3.1 16S rRNA identification of *intI1* positive bacteria isolates

As detailed in Chapter 4, 33 (5.64 %) of the 583 bacterial isolates collected in this study were PCR positive for the *intI1* gene. These bacterial isolates were identified with over 97 % similarity using the 16S rRNA gene (Table 5.1). The isolates were from 11 diverse bacterial genera; the majority of isolates identified as Pseudomonades (20 isolates), 3 *Acinetobacter* spp., 2 *Bacillus* spp., and a single strain of *Aerococcus*, *Shigella*, *Enterococcus*, *Arthrobacter*, *Carnobacterium*, *Psychrobacter*, *Klebsiella* and *Enterobacter*. The *Bacillus* spp., *Carnobacterium*, *Klebsiella*, an *Enterobacter* sp., and the *Psychrobacter* were all isolates collected from plates containing no antibiotic. Significantly, a number of the isolates identified were Gram positive bacteria, the *Arthrobacter* sp. and *Bacillus* spp.

Enteric, Gram positive and opportunistic bacteria were isolated from the Cranfield slurry, soil cores and drain-flow samples. For example, isolate PGS22 from the Cranfield slurry was identified, with 99 % similarity to *A. viridians*, isolate C145 from

Table 5.1 Characterisation of *intI1* positive bacterial isolates from this study, displaying isolate numbers, source, isolation plate antibiotic concentrations, 16S rRNA identification and *intI2* genotype.

ISOLATE NO.	SLURRY/ SOIL SAMPLE	ANTIBIOTIC CONC. (µg/ml) ISOLATED	16S rRNA identification	% similarity	*intI2*
PGS22	PS(CR)	TY 50	*Aerococcus viridians*	99	-
C519	PS(CR)	NONE	*Bacillus pumilus*	99	-
C209	1,P	TY 50	*Pseudomonas fluorescens*	99	-
C213	1,P	TY 50	*Pseudomonas fluorescens*	98	-
C250	1,P	TY 50	*Pseudomonas putida*	99	-
C284	1,P	TY 50	*Pseudomonas fluorescens*	97	-
C285	1,P	TY 50	*Pseudomonas putida*	97	-
C286	1,P	TY 50	*Pseudomonas fluorescens*	97	-
C405	1,1	TY 50	*Pseudomonas fluorescens*	97	-
C91	1,1	OTC 5	*Acinetobacter RUH1139*	98	-
C230	1,21	TY 50	*Pseudomonas putida*	97	+
C231	1,21	TY 50	*Pseudomonas putida*	97	+
C296	1,90	TY 50	*Pseudomonas putida*	97	-
C297	1,90	TY 50	*Pseudomonas putida*	97	-
C298	1,90	TY 50	*Pseudomonas putida*	97	-
C301	1,90	TY 50	*Pseudomonas fluorescens*	99	-
C548	1,289	NONE	*Bacillus sp. C213*	99	-
C361	1,289	SCP 5	*Arthrobacter arilaitensis*	99	-
C389	2,1	SCP 50	*Pseudomonas putida*	97	-
C147	2,21	SCP 5	*Acinetobacter haemolyticus*	97	-
C322	2,21	TY 50	*Pseudomonas sp. IBUN1402*	97	-
C419	2,21	OTC 0.2	*Pseudomonas sp. IBUN1402*	97	+
C257	2,90	TY 50	*Pseudomonas putida*	97	-
C258	2,90	TY 50	*Pseudomonas corrugara*	97	-
C259	2,90	TY 50	*Pseudomonas putida*	97	-
C260	2,90	TY 50	*Pseudomonas putida*	97	-
C506	DF13/02/02	OTC 1	*Shigella flexneri*	99	+
C507	DF13/02/02	OTC 1	*Enterococcus casseliflavus*	98	+
C508	DF13/02/02	OTC 1	*Bacillus silvestris*	99	+
C606	CS	NONE	*Carnobacterium maltaromaticum*	98	-
C608	CS	NONE	*Psychrobacter psychrophilus*	98	-
C609	CS	NONE	*Klebsiella sp. HPC157*	97	-
C610	CS	NONE	*Enterobacter hormaechi*	98	-

PS(CR), Cranfield slurry; 1,P, year 1 pre-application soils; 1,1, year1, day 1; DF, drain-flow runoff from a high rainfall event on 13/02/2002 and CS, Control soils. Colours indicate: blue, all tylosin 50 µg/ml isolated bacteria; red, enteric pathogenic bacteria; green, Gram positive bacteria; brown, indigenous soil bacteria and pink, opportunistic pathogens.

a year 2, day 21 soil core was identified with 97 % homology to an *A. haemolyticus* and a *S. flexneri* strain (C506) was identified from the drain-flow samples in year 2.

Isolates of particular interest as Gram positive bacterial species were the *Arthrobacter* sp. (C361) from the year 1, day 289 soils isolated on 5 µg/ml SCP, which has been identified as a significant indigenous soil bacterium and the *Bacillus* spp. isolates from the Cranfield slurry, year 1, day 289 soils and drain-flow samples (C519, C548 and C508). *Pseudomonas* spp. were isolated from all soil core samples collected and examined.

Of the isolates, 57 % were collected from plates containing 50 µg/ml tylosin and the other 43 % were collected from a range of SCP and OTC concentrations. There maybe a significant relationship between isolation on tylosin and *intI1* gene carriage, but 18 of the 19 isolates were identified as *Pseudomonas* spp. and 1 as an *Acinetobacter* sp. This possible link between *intI1* and tylosin resistance was investigated further by PCR screening of a number of *erm* genes (which potentially confer tylosin resistance) and MIC determinations of tylosin resistance.

5.3.2 Possible link between *intI1* and tylosin resistance

The isolates positive for the *intI1* gene were screened by PCR for the *ermA, B, C* and *X* genes, using separate primers for the *erm B* and *X* genes, whereas the *ermA* and *C* genes were screened using a multiplex system (Chapter 2, Table 2.6).

No isolates were PCR positive for the *ermA* gene, but 8 isolates were PCR positive for the *ermB* gene, C405 (*P. fluorescens*) was positive for the *ermB* and *ermC* genes (Table 5.2). One representative band of approximately 405 bp, amplified from isolate PGS22 (*A. viridians*) for *ermB*, was excised from its agarose gel and sequenced. The band had 99 % similarity to the *ermB* gene encoded on Tn*917* from *E. faecalis* strain DS16,

Table 5.2 Characterisation of *intI1* positive isolates, *erm* genotype and MIC for tylosin with (iMIC) and without induction (mg/l).

ISOLATE NO.	SLURRY / SOIL SAMPLE	16S rRNA identification	*erm* gene	TY MIC (mg/l)	TY iMIC (mg/l)
*PGS22	PS(CR)	*Aerococcus viridians*	B	>256	>256
C519	PS(CR)	*Bacillus pumilus*		0.5	1
*C209	1,P	*Pseudomonas fluorescens*	-	128	128
*C213	1,P	*Pseudomonas fluorescens*	-	128	128
*C250	1,P	*Pseudomonas putida*	-	128	128
*C284	1,P	*Pseudomonas fluorescens*	B	128	128
*C285	1,P	*Pseudomonas putida*	B	>128	>128
*C286	1,P	*Pseudomonas fluorescens*	-	>256	>256
*C405	1,1	*Pseudomonas fluorescens*	B,C	>128	>128
C91	1,1	*Acinetobacter RUH1139*	X?	>256	>256
*C230	1,21	*Pseudomonas putida*	-	>128	>128
*C231	1,21	*Pseudomonas putida*	-	128	128
*C296	1,90	*Pseudomonas putida*	-	>128	>128
*C297	1,90	*Pseudomonas putida*	-	>256	NG
*C298	1,90	*Pseudomonas putida*	-	>256	>256
*C301	1,90	*Pseudomonas fluorescens*	-	>256	NG
C548	1,289	*Bacillus sp. C213*		0.5	1
C361	1,289	*Arthrobacter arilaitensis*	-	1	1
C389	2,1	*Pseudomonas putida*	-	>256	>256
C147	2,21	*Acinetobacter haemolyticus*	-	128	128
*C322	2,21	*Pseudomonas sp. IBUN1402*	-	>256	>256
C419	2,21	*Pseudomonas sp. IBUN1402*	-	>128	>128
*C257	2,90	*Pseudomonas putida*	B	128	128
*C258	2,90	*Pseudomonas corrugara*	B	>128	>128
*C259	2,90	*Pseudomonas putida*	B	128	128
*C260	2,90	*Pseudomonas putida*	-	64	>128
C506	DF13/02/02	*Shigella flexneri*	-	>128	>128
C507	DF13/02/02	*Enterococcus casseliflavus*	B	16	32
C508	DF13/02/02	*Bacillus silvestris*	-	0.5	1
C606	CS	*C. maltaromaticum*	-	ND	ND
C608	CS	*Psychrobacter psychrophilus*	-	ND	ND
C609	CS	*Klebsiella sp. HPC157*	-	ND	ND
C610	CS	*Enterobacter hormaechi*	-	ND	ND

*** denotes isolates from TY 50 µg/ml containing plates.**

? PCR result not confirmed by sequencing.

(GenBank accession no. M11180) (App. A1a) (Shaw and Clewell, 1985). The *ermC* PCR fragment from C405 (*P. fluorescens*) had a 96 % similarity to the *ermC* gene from the *Staphylococcus hyicus* plasmid pSES21 (GenBank accession no. Y09003) (App. A1b) (Schwarz, 1996). One isolate, C91, identified as an *Acinetobacter* sp., was detected as being PCR positive for an *ermX* gene which could not be confirmed by sequencing.

Tylosin MIC measurements were determined with and without induction with tylosin. Induction was observed in the *Bacillus* spp. with tylosin MIC levels increasing from 0.5 to 1 mg/l where no *erm* genes were identified in these isolates (C519, C548 and C508). In the case of isolate C260, *P. putida*, the MIC for tylosin was increased from 64 to >128 mg/l with induction and from 16 to 32 mg/l in the case of an *E. casseliflavus* (C507), PCR positive for *ermB*, collected from the drain-flow samples.

There was no correlation between the carriage of an *erm* gene, increased resistance and high MIC figures. For example, in isolate C286, isolated from a tylosin 50 µg/ml plate, had a calculated tylosin MIC of >256 mg/l with no known *erm* gene. Isolate C405, PCR positive for *ermB* and *ermC*, produced an MIC of >128 mg/l. Both isolates; C286 and C405 were identified as *P. fluorescens* (Table 5.1). The *Bacillus* spp. (C519, C548 and C508) and *Arthrobacter* sp. (C361) were observed to be tylosin sensitive strains with no *erm* genes and none of these isolates were collected from plates containing tylosin.

5.3.3 PCR screening for the sulphonamide resistance genes

The *intI1* positive isolates were screened by PCR for the *sulI*, *sulII* and *sul3* genes and the MIC for SCP was determined in each isolate where growth was obtained (Table 5.3).

Table 5.3 Characterisation of *intI1* positive isolates, *sul* genotype and MIC for sulphachloropyridazine (mg/l).

ISOLATE NO.	SLURRY / SOIL SAMPLE	16S rRNA identification	*sulI*	*sulII*	*sul3*	SCP MIC (mg/l)
PGS22	PS(CR)	*Aerococcus viridians*	-	+	-	8
C519	PS(CR)	*Bacillus pumilus*	-	-	-	8
C209	1,P	*Pseudomonas fluorescens*	-	-	-	4
C213	1,P	*Pseudomonas fluorescens*	+	-	-	8
C250	1,P	*Pseudomonas putida*	+	-	-	4
C284	1,P	*Pseudomonas fluorescens*	+	-	-	8
C285	1,P	*Pseudomonas putida*	+	-	-	4
C286	1,P	*Pseudomonas fluorescens*	+	-	-	8
C405	1,1	*Pseudomonas fluorescens*	-	-	-	8
C91	1,1	*Acinetobacter RUH1139*	-	+	-	ND
C230	1,21	*Pseudomonas putida*	-	-	-	8
C231	1,21	*Pseudomonas putida*	+	+	-	8
C296	1,90	*Pseudomonas putida*	+	-	-	8
C297	1,90	*Pseudomonas putida*	-	-	-	4
C298	1,90	*Pseudomonas putida*	-	-	-	4
C301	1,90	*Pseudomonas fluorescens*	+	-	-	8
C548	1,289	*Bacillus sp. C213*	-	-	-	8
*C361	1,289	*Arthrobacter arilaitensis*	+	+	-	5
*C389	2,1	*Pseudomonas putida*	-	-	-	4
*C147	2,21	*Acinetobacter haemolyticus*	-	+	-	16
C322	2,21	*Pseudomonas sp. IBUN1402*	-	-	-	8
C419	2,21	*Pseudomonas sp. IBUN1402*	+	-	-	8
C257	2,90	*Pseudomonas putida*	+	-	-	8
C258	2,90	*Pseudomonas corrugara*	+	-	-	8
C259	2,90	*Pseudomonas putida*	+	-	-	8
C260	2,90	*Pseudomonas putida*	+	-	-	4
C506	DF13/02/02	*Shigella flexneri*	-	+	-	8
C507	DF13/02/02	*Enterococcus casseliflavus*	-	-	-	4
C508	DF13/02/02	*Bacillus silvestris*	-	-	-	8
C606	CS	*C. maltaromaticum*	-	-	-	ND
C608	CS	*Psychrobacter psychrophilus*	-	-	-	ND
C609	CS	*Klebsiella sp. HPC157*	-	-	-	ND
C610	CS	*Enterobacter hormaechi*	-	-	-	ND

*** denotes isolates from SCP containing plates.**

Of the 33 *intI1* positive isolates; 14 isolates (42 %) carried the *sulI* gene, 5 the *sulII* gene and 2 isolates encoded both the *sulI* and *sulII* genes; C231 identified by 16S rRNA as *P. putida*, and isolate C361, *Arthrobacter arilaitensis*.

No correlation was observed for the *intI1* positive isolates between *sul* gene carriage, the number of *sul* genes carried, SCP MIC values or isolate collection from SCP containing plates. Only 3 of the 33 *intI1* positive isolates were collected from SCP containing plates; 1 *Pseudomonas* sp., isolate number C389, containing no *sul* genes and a low MIC for SCP; isolate C361 (*A. arilaitensis*) which carried two *sul* genes, *I* and *II*, but also displayed a low SCP MIC and isolate C147, an *Acinetobacter* sp., carrying the *sulII* gene with one of the highest SCP MIC values observed for the 33 *intI1* positive isolates.

As stated and discussed in Chapter 4, no *intI1* positive isolates were PCR positive for the *sul3* gene.

5.3.4 Multiple antibiotic resistance of the *intI1* positive isolates

The 33 *intI1* positive isolates were screened for their resistance against a bank of 8 antibiotics excluding tylosin and SCP. A number of the isolates failed to be tested due to their lack of growth during the phenotyping (Table 5.4).

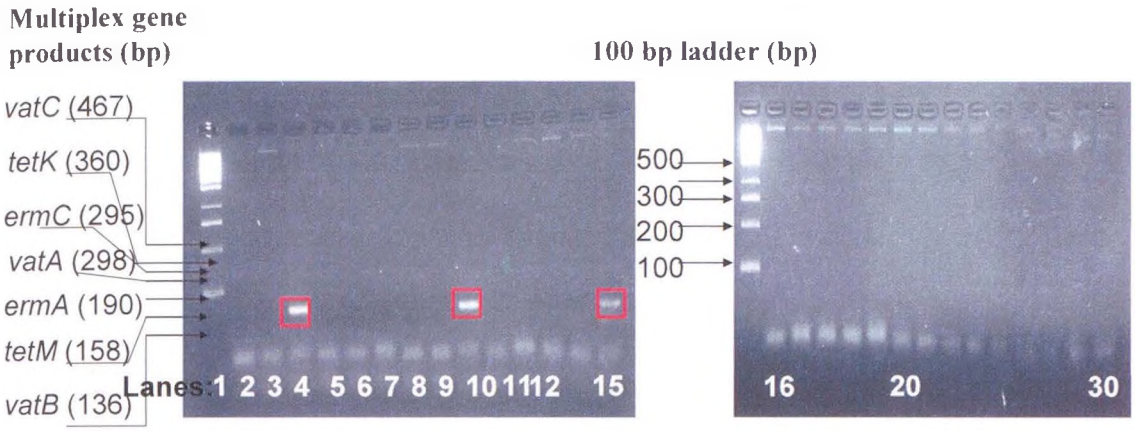
Of the isolates investigated; 2 strains, C260 (*P. putida*) and C506 (*S. flexneri*) were resistant to all the 8 antibiotics tested, a further 2 strains were also resistant to 7 of the 8 antibiotics, excluding kanamycin. The most antibiotic sensitive isolates were C507 (*E. casseliflavus*) resistant only to streptomycin and tetracycline, one isolate of *P. fluorescens* (C284) from the year 1, pre-application soils, displayed resistance to nalidixic acid, chloramphenicol and tetracycline and the *Bacillus* isolates; C519, C548

Table 5.4 Characterisation of *intI1* positive isolates' multiple antibiotic resistance phenotypes and PCR screening for further antibiotic resistance genes.

ISOLATE NO.	SAMPLE	16S rRNA identification	Antibiotic resistance	Genes
PGS22	PS(CR)	*Aerococcus viridians*	Sm^R, Nm^R, Nal^R, Cm^R, Tet^R	*tet(M)*
C519	PS(CR)	*Bacillus pumilus*	Sm^R, Nal^R,Cm^R, Tet^R	-
C209	1,P	*Pseudomonas fluorescens*	Nal^R, Amp^R, Cm^R, Tet^R, Tmp^R	-
C213	1,P	*Pseudomonas fluorescens*	NG	-
C250	1,P	*Pseudomonas putida*	NG	-
C284	1,P	*Pseudomonas fluorescens*	Nal^R, Cm^R, Tet^R	-
C285	1,P	*Pseudomonas putida*	Nal^R, Amp^R, Cm^R, Tet^R, Tmp^R	-
C286	1,P	*Pseudomonas fluorescens*	Nal^R, Amp^R, Cm^R, Tet^R, Tmp^R	-
C405	1,1	*Pseudomonas fluorescens*	Sm^R, Nm^R, Nal^R, Amp^R, Cm^R, Tet^R, Tmp^R	*tet(M)*
C91	1,1	*Acinetobacter RUH1139*	Sm^R, Nm^R, Nal^R, Amp^R, Cm^R, Tet^R, Tmp^R	-
C230	1,21	*Pseudomonas putida*	Nal^R, Amp^R, Cm^R, Tet^R, Tmp^R	-
C231	1,21	*Pseudomonas putida*	Nal^R, Amp^R, Cm^R, Tet^R, Tmp^R	-
C296	1,90	*Pseudomonas putida*	Sm^R, Nm^R, Nal^R, Cm^R, Tet^R	-
C297	1,90	*Pseudomonas putida*	NG	-
C298	1,90	*Pseudomonas putida*	NG	-
C301	1,90	*Pseudomonas fluorescens*	NG	-
C548	1,289	*Bacillus sp. C213*	Sm^R, Nal^R,Cm^R, Tet^R	-
C361	1,289	*Arthrobacter arilaitensis*	Sm^R, Nm^R, Nal^R,Cm^R, Tet^R	-
C389	2,1	*Pseudomonas putida*	Nal^R, Amp^R, Cm^R, Tet^R	-
C147	2,21	*Acinetobacter haemolyticus*	Sm^R, Nm^R, Nal^R, Cm^R, Tet^R, Tmp^R	-
C322	2,21	*Pseudomonas sp. IBUN1402*	Nal^R, Amp^R, Cm^R, Tet^R, Tmp^R	-
C419	2,21	*Pseudomonas sp. IBUN1402*	Nal^R, Amp^R, Cm^R, Tet^R, Tmp^R	-
C257	2,90	*Pseudomonas putida*	NG	-
C258	2,90	*Pseudomonas corrugara*	Nal^R, Amp^R, Cm^R, Tet^R, Tmp^R	-
C259	2,90	*Pseudomonas putida*	NG	-
C260	2,90	*Pseudomonas putida*	Sm^R, Nm^R, Nal^R, Km^R, Amp^R, Cm^R, Tet^R, Tmp^R	-
C506	DF13/02/02	*Shigella flexneri*	Sm^R, Nm^R, Nal^R, Km^R, Amp^R, Cm^R, Tet^R, Tmp^R	-
C507	DF13/02/02	*Enterococcus casseliflavus*	Sm^R, Tet^R	*tet(M)*
C508	DF13/02/02	*Bacillus silvestris*	Sm^R, Nal^R,Cm^R, Tet^R	-
C606	CS	*C. maltaromaticum*	ND	-
C608	CS	*Psychrobacter psychrophilus*	ND	-
C609	CS	*Klebsiella sp. HPC157*	ND	-
C610	CS	*Enterobacter hormaechi*	ND	-

Where; NG: no growth of isolate (therefore no phenotype determination), ND: Not done, Sm^R: resistance to Streptomycin 16 µg/ml, Amp^R: Ampicillin 16 µg/ml, Km^R: Kanamycin 16 µg/ml, Cm^R: Chloramphenicol 16 µg/ml, Tet^R: Tetracycline 8 µg/ml, Tmp^R: Trimethoprim 16 µg/ml, Nm^R: Neomycin 8 µg/ml, Nal^R: Nalidixic acid 16 µg/ml. Isolates were additionally PCR screened for the *tet(M)* and *(K)* genes and *vatA*, *B* and *C* genes, constitutive macrolide resistance genes in *Staphylococcus* spp.

Fig. 5.1 Multiplex PCR fragments for *intI1* positive isolates



Lanes:

1. 100 bp ladder	16. 100 bp ladder
2. No DNA control	17. C209
3. C389	18. C213
4. PGS22*	19. C230
5. C147	20. C231
6. C506	21. C257
7. C91	22. C258
8. C250	23. C259
9. C301	24. C260
10. C507*	25. C284
11. C508	26. C285
12. C419	27. C296
13. C361	28. C298
14. C286	29. C322
15. C405*	30. C297

Where; * /red square denotes PCR fragments sequenced and alignments displayed below. Multiplex PCR has the potential to amplify 6 genes, their approximate product sizes are denoted above.

and C508 which all exhibited resistance to streptomycin, nalidixic acid, chloramphenicol and tetracycline.

Resistance to kanamycin was only displayed in isolates C260 (*P. putida*) and C506 (*S. flexneri*), whereas all 22 of the screened isolates exhibited resistance to tetracycline, but in only 3 isolates was a tetracycline resistance determinant found, *tet(M)* in PGS22 (*A. viridians*), C507 (*E. casseliflavus*) and C405 (*P. fluorescens*) with 98 % homology to the *tet(M)* gene on Tn*1545* from *S. pneumoniae* (GenBank accession no. X04388) (Table 5.4, Fig. 5.1 and App. A2) (Martin et al., 1986; Perreten et al., 1997).

5.3.5 Integron annotation and characterisation

For characterisation of the integrons, isolates which were *intI1* PCR positive were also PCR screened for the *qacE* and *qacEΔ1* genes, revealing 3 isolates carrying *qacE*. The PCR fragment of isolate PGS22 (*A. viridians*) was sequenced and was calculated to have similarity of 98 % with the *qacE* gene from *K. aerogenes* (GenBank accession no. X68232 (Table 5.5 and App. A3a) (Paulsen et al., 1993). A further four isolates were identified which were PCR positive for the *qacEΔ1* gene, these isolates included C506 (*S. flexneri*), its' PCR fragment for the *qacEΔ1* gene identified with 96 % similarity to the *qacEΔ1* gene encoded within Tn*1696* on the plasmid R1033 from *P. aeruginosa* (GenBank accession no. U12338) (Table 5.5 and Fig. 5.2b) (Wohlleben et al., 1989).

In an attempt to characterise Class 1 integrons from the *intI1* positive isolates, a number of additional strategies were employed. Primer pairs were used which would potentially amplify the variable region of a Class 1 integron (Cass1/Cass2 or

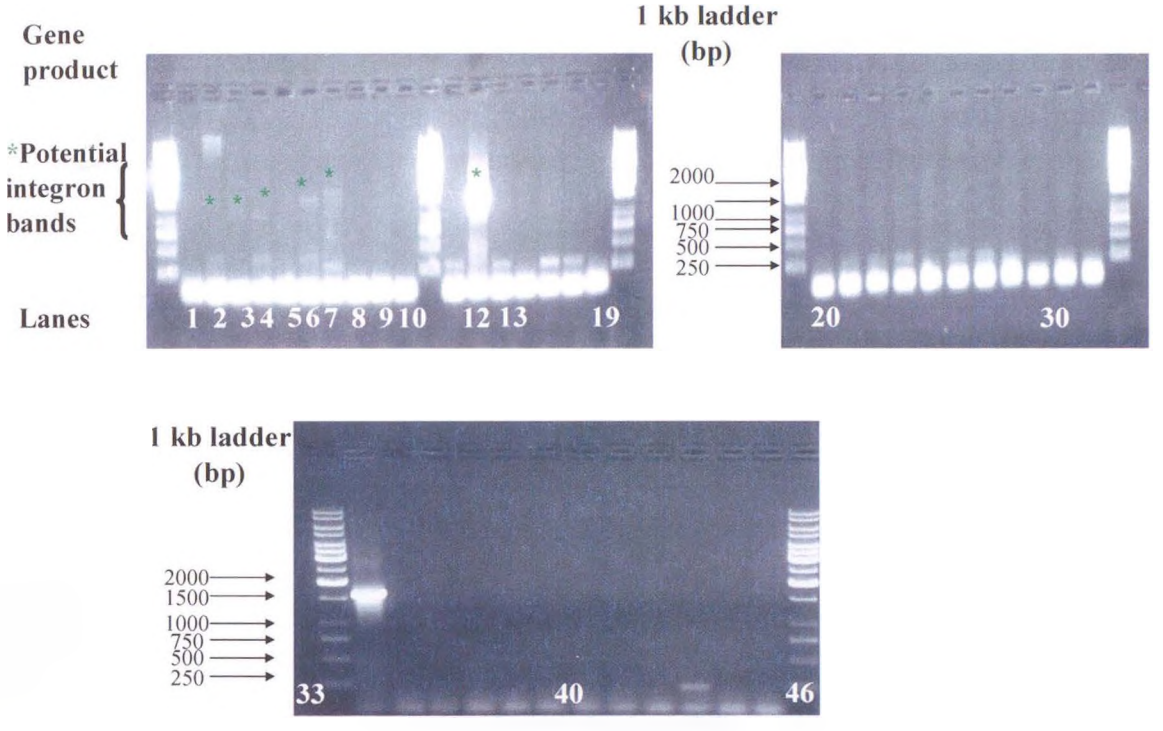
Table 5.5 Integron characterisation of the *intI1* PCR positive bacterial isolates by PCR screening for Class 1 integron variable regions, the *qacE* gene and the *qacEΔ1* gene.

ISOLATE NO.	SLURRY / SOIL SAMPLE	16S rRNA identification	Integron variable region	*qacE*	*qacEΔ1*
PGS22	PS(CR)	*Aerococcus viridians*	+?	+	-
C519	PS(CR)	*Bacillus pumilus*	-	-	-
C209	1,P	*P. fluorescens*	-	-	-
C213	1,P	*P. fluorescens*	-	-	-
C250	1,P	*P. putida*	+?	-	-
C284	1,P	*P. fluorescens*	-	-	-
C285	1,P	*P. putida*	-	-	-
C286	1,P	*P. fluorescens*	-	-	-
C405	1,1	*P. fluorescens*	-	-	+
C91	1,1	*A.sp. RUH1139*	+?	-	-
C230	1,21	*P. putida*	-	-	+
C231	1,21	*P. putida*	-	-	-
C296	1,90	*P. putida*	-	-	-
C297	1,90	*P. putida*	-	-	-
C298	1,90	*P. putida*	-	+	-
C301	1,90	*P. fluorescens*	-	-	-
C548	1,289	*Bacillus sp. C213*	-	-	-
C361	1,289	*A. arilaitensis*	+	-	+
C389	2,1	*P. putida*	-	-	-
C147	2,21	*A. haemolyticus*	+?	-	-
C322	2,21	*P. sp. IBUN1402*	-	-	-
C419	2,21	*P. sp. IBUN1402*	-	-	-
C257	2,90	*P. putida*	-	-	-
C258	2,90	*P. corrugara*	-	-	-
C259	2,90	*P. putida*	-	-	-
C260	2,90	*P. putida*	-	-	-
C506	DF13/02/02	*S. flexneri*	-	-	+
C507	DF13/02/02	*E. casseliflavus*	-	-	-
C508	DF13/02/02	*B. silvestris*	-	-	-
C606	CS	*C. maltaromaticum*	-	-	-
C608	CS	*P. psychrophilus*	-	+	-
C609	CS	*Klebsiella sp. HPC157*	-	-	-
C610	CS	*Enterobacter hormaechi*	-	-	-

? denotes PCR results unconfirmed by sequencing.

Fig. 5.2 PCR products for primer sets (a) Cass1/Cass2, (b) VarF/VarR and (c) IntI1F/SulIR to characterise the Class 1 integron variable regions from the *intI1* gene positive isolates.

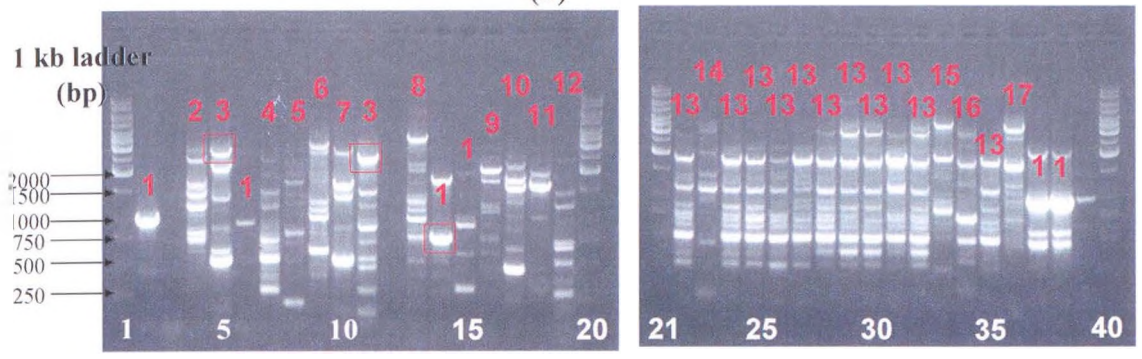
(a)



Lanes:

1. No DNA control
2. C389
3. PG22*
4. C147*
5. C506*
6. C91*
7. C250*
8. C301
9. C507
10. C508
11. 1 Kb ladder
12. C419
13. C361*
14. C286
15. C405
16. C209
17. C284
18. C213
19. 1 Kb ladder
20. 1 Kb ladder
21. C285
22. C230
23. C296
24. C231
25. C298
26. C257
27. C322
28. C258
29. C297
30. C259
31. C260
32. 1 Kb ladder
33. 1 Kb ladder
34. C361
35. No DNA control
36. C442
37. C35
38. C506
39. C36
40. C37
41. C060
42. C607
43. C608
44. C609
45. C610

(b)

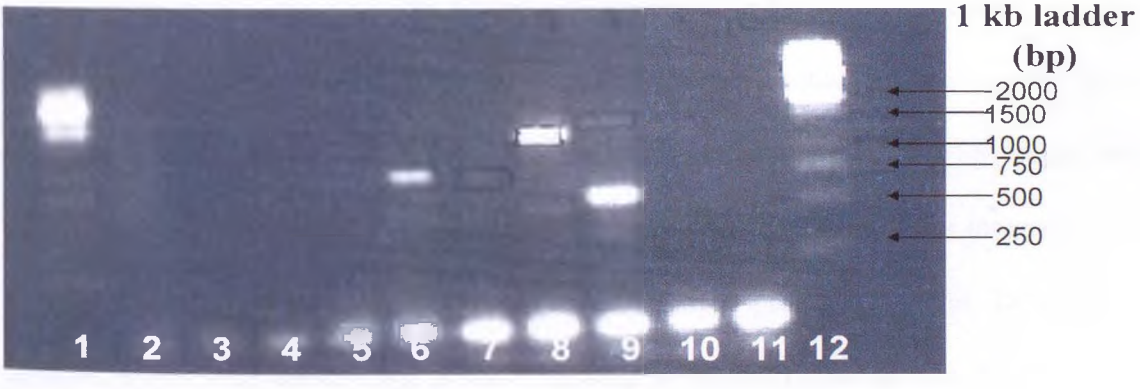


☐ **Denotes band sequenced, red numbers: denote identical banding patterns.**

Lanes:

1. 1 Kb ladder	11. C609	21. 1 Kb ladder	31. C257
2. C361	12. C298	22. C286	32. C322
3. No DNA control	13. C519	23. C405	33. C258
4. C147	14. C548	24. C209	34. C297
5. C506	15. C389	25. C284	35. C259
6. C91	16. C301	26. C213	36. C260
7. C250	17. C507	27. C285	37. C361
8. C606	18. C508	28. C230	38. C361
9. C608	19. C419	29. C296	39. No DNA control
10. C610	20. 1 Kb ladder	30. C231	40. 1 Kb ladder

c. IntIF/SulIR PCR gel



Lanes:

1. 1 Kb ladder	5. C147	9. C301*
2. No DNA control	6. C506*	10. C507
3. C389	7. C91*	11. C508
4. PG22	8. C250*	12. 1 Kb ladder

*** denotes bands sequenced but all sequencing failed for these products.**

VarF/VarR) or the region between the *intI1* gene and *sulI* gene (Int1F/SulIR) (Table 2.6). Isolates PGS22, C147, C91, C506, C250 and C361 gave positive bands with the Cass1/Cass2 primers (Fig. 5.2a). Isolate PGS22 (*A. viridians*) overall carried *sulII*, *tet(M)* and *qacE* genes but the integron fragment could not be amplified and sequenced.

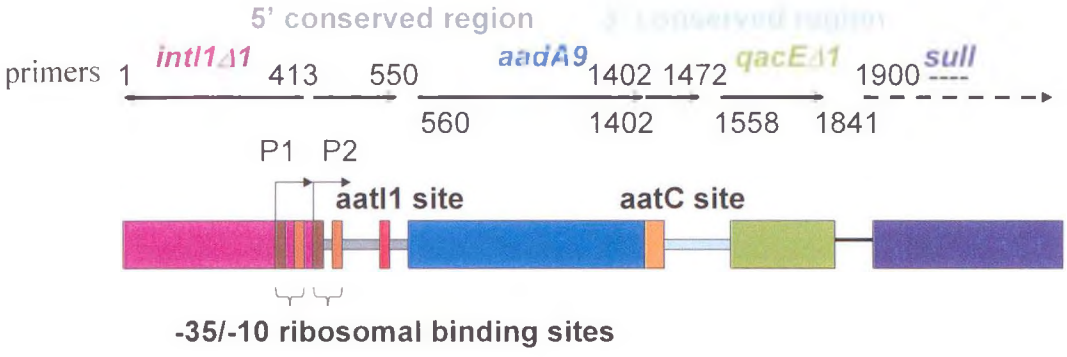
The PCR bands amplified by the VarF/VarR produced a multiple banding pattern, with similar band arrangements observed for isolates; C361/C91 and C548/C389. Isolate C389 (*P. putida*) had not produced a PCR product from the Cass1/Cass2 primer set (Fig. 5.2b). Banding pattern 3 was observed in isolates C506 (*S. flexneri*) and C609 (*Klebsiella* sp.), whereas banding pattern 13 occurred most frequently in 11 of the *intI1* positive isolates.

The IntIF/SulIR primer set produced a 1 Kb band from isolate C506 (*S. flexneri*) and an approximately 2 Kb band from C250 (*P. putida*), which had not given a band for any of the other primer sets (Fig. 5.2c). No sequence could be obtained for the C250 PCR fragment.

The sequences obtained from the C506 PCR fragment for the Class 1 integron variable region denoted the integration of an *aadA1* gene with 99 % homology to an *aadA1* gene on plasmid pHB01 from *K. pneumoniae* (GenBank accession no. AJ870988) (App. A4) (Decre et al., 2004) and a *qacEΔ1* gene in the 3' conserved region of the integron. No *sulI* gene was observed despite the amplification of a product with the IntIF/SulIR primers, which may indicate the primers binding to the *sulII* gene as a replacement for *sulI*, although no evidence for this was found through localisation.

Isolate C361 (*A. arilaitensis*) produced an approximately 2 Kb PCR product of its integron variable region using the Cass1/Cass2 primers (Table 5.5). An additional 5 isolates were PCR positive with the Cass1/Cass 2 primers but the products were not

Fig. 5.6 Schematic of the Class 1 integron sequenced from C361 (*A. arilaitensis*) using primer sets Cass1/Cass2 and Cass3, Cass4 and Cass5 sequencing primers.



This Class 1 integron had 99 % similarity with the pTET3 plasmid carried by *C. glutamicum* (GenBank accession no. AJ420012). The integron encoded an *intI1Δ1* gene with the P1 promoter and the -35 and -10 regions of the ribosomal binding site (Collis and Hall, 1995). Nucleotides 413 to 442 encode a P2 promoter and ribosomal binding sites followed by a 5' conserved region which includes the aatI1 site for integrase binding and gene cassette integration (Collis et al., 1993; Collis and Hall, 1995). Downstream of the aatI1 site was the codon for an *aadA9* gene cassette which confers streptomycin and spectinomycin resistance (adenyltransferase), the 3' end of which encodes the 59 bp element with an attC site for integration. The 3' conserved region of the integron starts at nucleotide, 1451, and encodes the *qacEΔ1* and the *sulI* genes.

able to be sequenced. The amplified integron variable region of C361 was sequenced and annotated according to known knowledge of integron structures and previously annotated sequences, a schematic of the variable region and 5' to 3' conserved regions

are displayed in Fig. 5.3 (annotated full sequence displayed in App. A5). This integron structure had 99 % similarity to the Class 1 integron found on the pTET3 plasmid in a *C. glutamicum* (GenBank accession no. AJ420012) (Tauch et al., 2002). The C361 integron also encoded an unusual *intI1* gene with a deletion (*intI1Δ1*), an *aadA9*, *qacEΔ1* and *sulI* genes. The sequence encoded within the 5' conserved region of the C361 Class I integron; two promoters and attI1 sites for gene cassette integration. The 3' conserved region containing the gene cassette 59 bp element and attC site for integration (Collis et al., 1993; Collis and Hall, 1992, 1995). No evidence for the location of the *sulII* gene was found.

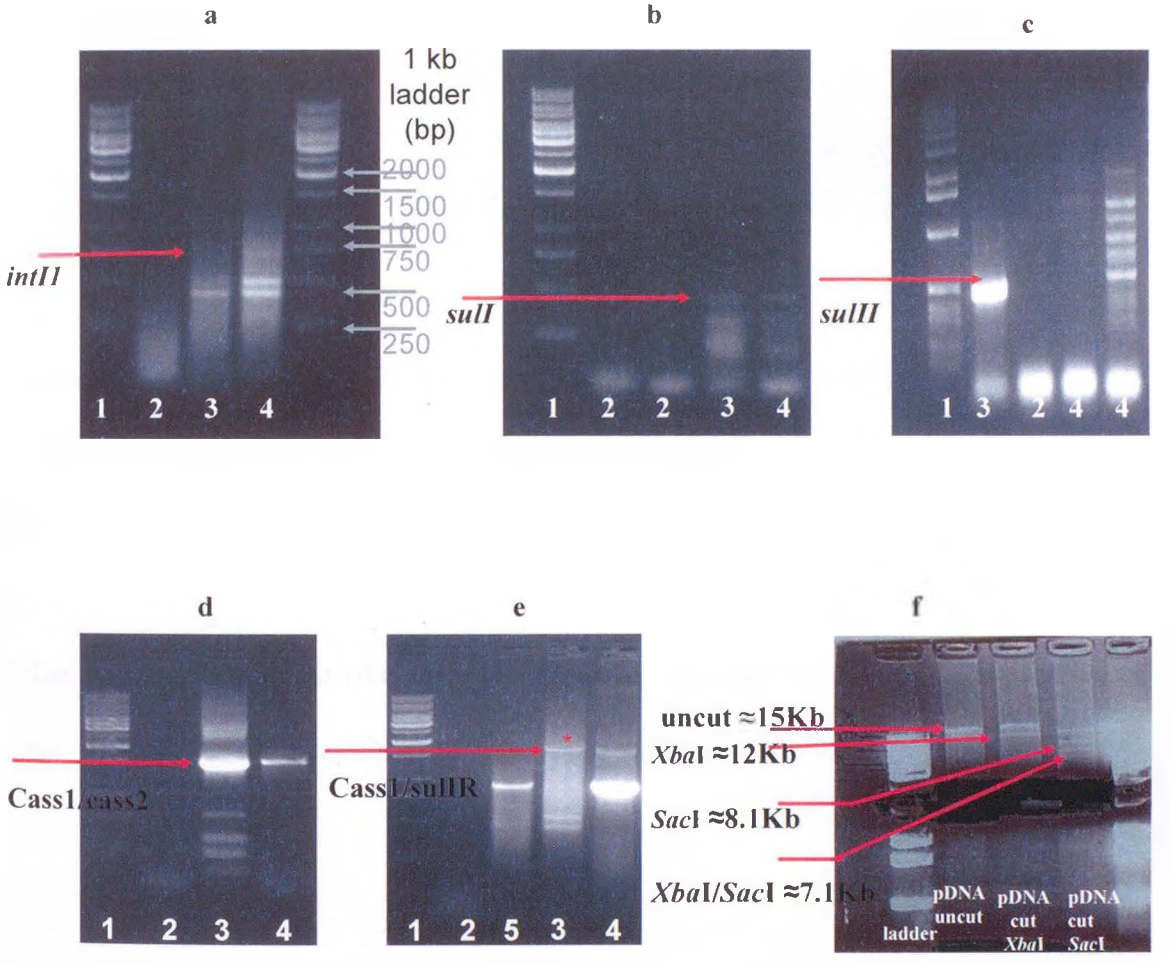
5.3.6 Further characterisation of isolate C361 (*A. arilaitensis*)

To locate the Class 1 integron, a plasmid and chromosomal extraction of DNA was performed on isolate C361 and the resulting preparations screened for *intI1*, *sulI*, *sulII*, and the variable and non-variable regions of the Class 1 integron (Fig. 5.4a-e). The resulting PCR products indicated that the complete Class 1 integron was located on a plasmid.

Although the Class 1 integron sequence was 99 % identical to the plasmid from *C. glutamicum* pTet3, a restriction digest of the chromosomal and plasmid DNA indicated the fragment profile was not identical and therefore not the complete plasmid (Fig. 5.4f) (Tauch et al., 2002). PCR screening did amplify *tet(A)* and *tet(R)* genes, which had 99 % homology to the same genes from pTet3 (Fig. 5.5a).

To further characterise C361, PCR screening was performed to identify more information on the *sulII* gene which was not located on the plasmid encoding the Class 1 integron, hence providing evidence for 2 plasmids carrying different sulphonamide resistance genes demonstrated by the lack of transfer of both genes at identical rates or

Fig. 5.4 Evidence for Class 1 integron carriage in *Arthrobacter arilaitensis* on a plasmid resembling pTet3 from *Corynebacterium glutamicum* (Tauch et al., 2002). PCR screening for the genes; (a) *intI1*, (b) *sulI*, (c) *sulII*, (d) the Class 1 integron variable region with Cass1/2 and (e) Cass1/sulI primer sets and (f) PFGE of plasmid DNA.

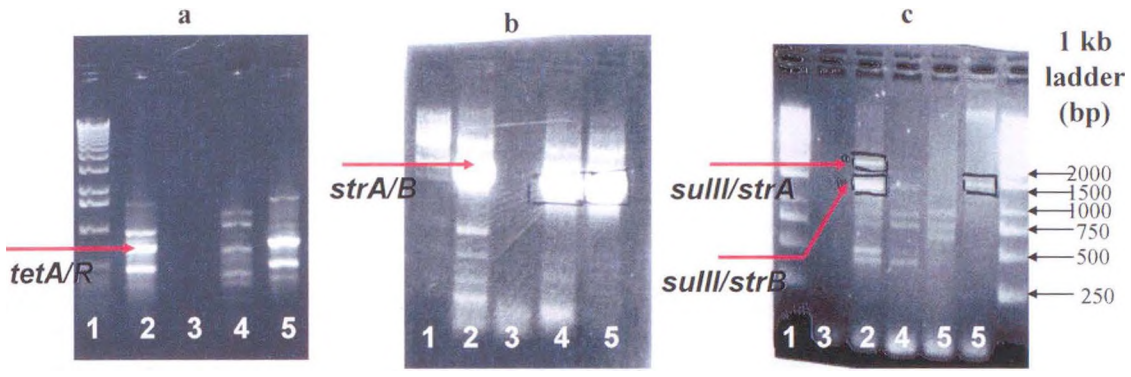


Lanes: 1. 1 Kb ladder 2. No DNA control 3. C361 chromosomal DNA

4. C361 plasmid DNA 5. Positive DNA control

*confirmed by sequencing

Fig. 5.5 **Additional characterisation of the genotype for isolate C361. PCR screening for the genes; (a) *tetA/R*, (b) *strA/B* (c) *sulI* through *strA/B*.**



Lanes: 1. 1 Kb ladder 2. Positive DNA control 3. No DNA control

4. C361 chromosomal DNA 5. C361 plasmid DNA

Table 5.6 Conjugal transfer rates (transconjugants per donor cell) of genes from C361 (*A. arilaitensis)* to recipient strains *P. putida* and *E.coli.*

Recipient	*intI*	*sulI*	*sulII*
E. coli K-12 CV601	3.71×10^{-3}	3.71×10^{-3}	4.79×10^{-3}
P. putida UWC1	2.98×10^{-3}	2.98×10^{-3}	1.4×10^{-4}

simultaneously (Table 5.6). The *sulII* gene was flanked on the 3' end by *strA* and *strB* gene (Figs. 5.5b and c). Conjugal transfer experiments also demonstrated that transfer

of the mobile elements containing the *sulI* and *sulII* genes were able to transfer into Gram negative recipients from a Gram positive host displaying a broad host range (Table 5.6).

5.4 DISCUSSION

5.4.1 16S rRNA identification of *intI1* positive bacteria isolates

Of the bacterial isolates collected in this study, 33 were *intI1* positive. These 33 isolates belonged to a low diversity of 11 bacterial genera, 5 or possibly 6 of these genera were soil bacteria and two were Gram positive.

There have only been a few reports in which *intI1* genes have been identified in Gram positive bacteria and the mobile elements in this study were also demonstrated to be capable of conjugal transfer from their Gram positive host into Gram negative recipients, *P. putida* and *E. coli*, indicating a broad host range for these potential plasmids. The Class 1 integron which encoded *intI1Δ1* and *sulI* was detected in isolate C361 (*A. arilaitensis*) and had high similarity to a Class 1 integron which was first identified in a *C. glutamicum* strain (Tauch et al., 2002) and Nandi et al, (2004) reported that Gram positive bacteria were the major reservoir of integrons in an investigation into poultry houses and litter. *Arthrobacter* are soil dwelling bacterium which are commercially significant due to their capacity for breakdown of potentially environmentally toxic compounds such as crude oils (Diaz-Ramirez et al., 2003), phenolic and benzolic compounds (Backman and Jansson, 2004). Class 1 integrons have been reported previously in this bacterial genus (Furukawa and Chakrabarty, 1982; Lu et al., 2003).

The frequency of integrons in the bacterial group of *Pseudomonas*, may be due to some of the members capabilities for natural transformation (Carlson et al., 1983), controlled internally by shifts in growth state, allowing for potentially high rates of transfer in the soil environment (Olsen and Shipley, 1973). *Acinetobacter* are also indigenous soil bacteria but have been increasingly implicated in hospital acquired infections and infections where bacteria with MD resistance have been observed commonly linked to integron carriage (Abbo et al., 2005; Chastre, 2003; Coelho et al., 2004). Like Pseudomonads, some species of *Acinetobacter* have also been reportedly capable of naturally transformation and thus responsible for interspecies transformation, providing evidence for a ubiquitous genus (Juni, 1972). The prevalence of uptake has been observed to be directly proportional to the size of the DNA fragments with typical transfer rates in *A. calcoaceticus* at 9.5×10^{-2} transconjugants per donor cell (Lorenz and Wackernagel, 1994). Natural transformation in *Acinetobacter* is also controlled internally. Maximum competence is gained with a shift from exponential growth to stationary phase, usually as a result of nutritional state or a state of unbalanced growth, such as those observed in the soil environment (Juni, 1972; Palmen et al., 1992). The state of natural competence has been reported to be preceded by the natural release of DNA during exponential growth displaying a mechanism for co-ordinated gene transfer, making transformation a biologically significant event (Palmen and Hellingwerf, 1995; Stewart and Carlson, 1986). This state of natural competence may also explain a number of *Acinetobacter* species ability for multiple antibiotic resistance, its problematic emergence as an opportunistic pathogen in the clinical setting, and the multiple resistance phenotypes in the bacterial isolates from this study (Abbo et al., 2005; Chastre, 2003).

The isolation of *Psychrobacter* spp. was unusual. This genus belongs to the family *Moraxellaceae*. They are commonly isolated from cold marine environments including water (Bowman et al., 1997; Shivaji et al., 2005), soils (Cavanagh et al., 1996) and sediments but have also been implicated in an ocular infection (Gini, 1990) and a case of meningitis (Lloyd-Puryear et al., 1991). Integrons have not been reported previously in this genus and their presence in these isolates indicates an opportunity for the bacterium to gain multiple antibiotic resistance genes.

C. maltaromaticum (isolate C606) was renamed from *Lactobacillus* in 2003 (Mora et al., 2003), a lactic acid bacteria associated with food spoilage, therefore it may have been shed by the pigs and survived in the soil as it has been reported in the permafrost of Alaska as a psychrotolerant bacteria (Pikuta et al., 2005). No Class 1 integrons have been observed previously in this genus.

The other 5 bacterial genera in which Class 1 integrons were reported in this study, were common enteric bacteria, either isolated directly from the Cranfield slurry or isolated as a result of the slurry application to the soils; these include *Aerococcus, Enterobacter, Shigella, Enterococcus* and *Klebsiella.* Of these genera several reports of Class 1 integrons have been observed in *Enterobacter* (Jeong et al., 2003), *Shigella* and *Klebsiella* (McIver et al., 2002), where the integrons are commonly linked to β-lactamase resistance. There has been to date one report of integrons in *Enterococcus* linked to lincosamide resistance (Heir et al., 2004), but no reports of Class 1 integrons in *Aerococcus* have been reported previously.

The *S. flexneri*, C506, was isolated from a drain-flow sample, its presence either due to runoff from the field after survival from the applications of slurries or a possible indication of faecal contamination of the river/stream due to release upstream from

either a farm or human sewage. If the *Shigella* isolate was from the slurry runoff it had survived in the field for at least 90 days, this exceeds reports of *Shigella* survival in the laboratory and in the aquatic environment where survival has been observed to be affected by physio-chemical characteristics such as pH and salinity as well as the temperature of both the water and the environment (Islam et al., 1996; Wait and Sobsey, 2001). Reports have characterised survival to be as long as 10 days at lower temperatures such as 6 °C (Wait and Sobsey, 2001).

In total, 3 of the 11 bacterial genera in which a Class 1 integron was observed in this study had been previously unreported, including *Psychrobacter*, *Carnobacterium* and *Aerococcus*.

5.4.2 Possible link between *intI1* and tylosin resistance

As stated previously in Chapter 4, no reports in the published literature have linked the *intI1* gene or Class 1 integrons with tylosin resistance and no genes conferring tylosin resistance have been found on Class 1 integrons. Despite the apparent link in this study between *intI1* and tylosin resistance, no correlation was found between the screened *erm* genes (*erm A, B, C* and *X* genes) and tylosin resistance. Without additional work there is still the possibility of genes linked to the *intI1* gene by their presence on a mobile element or resistance through mutations on the isolates 23S rRNA genes (Jost et al., 2003; Jost et al., 2004; Prapasarakul et al., 2003; Whitehead and Cotta, 2001). There is also the likelihood that the tylosin resistance was not linked to the *intI1* gene but was due to the intrinsic resistance of the *Pseudomonas* isolates collected in this study, as 18 out of the 19 isolates collected from plates containing 50 µg/ml tylosin and were *intI1* positive.

According to the BSAC breakpoints for other macrolides, tylosin is not listed, MIC breakpoints for *Pseudomonas* to tobramycin is 4 mg/l, to moxiflacin it is between 2-4 mg/l and to amikamycin it is 10 mg/l, all lower than the lowest MIC of 64 mg/l and highest of >256 mg/l observed in this study (BSAC, 2005). The BSAC breakpoints for *Acinetobacter* are 4 mg/l to tobramycin and 16 mg/l for amikamycin, again lower than the MIC for tylosin of between 128 mg/l and >256 mg/l in this study (BSAC, 2005). According to the BSAC breakpoints all the *Pseudomonas* and *Acinetobacter* spp. isolated in this study, which were *intI1* positive, were resistant to tylosin, even though isolated from plates not containing tylosin or PCR positive for any of the *erm* genes screened.

Pseudomonads have not been reported to be resistant to tylosin and studies into macrolide resistance commonly neglect the genetic background of resistance and hence the *mphA* gene is the only reported macrolide resistance conferring gene to have been observed in *P. aeruginosa* (Kastner and Guggenbichler, 2001; Noguchi and Katayama, 1998). The high tylosin resistance of the Pseudomonads isolated in this study may be due to the expression of MD efflux systems which are common both intrinsically and acquired in this genus or cell polysaccharide extrusion (Poole, 2004b).

A small induction of resistance was observed in the *Bacillus* strains which may have been due to experimental error including inoculum concentration which has been observed to effect resistance levels in *Acinetobacter* strains, an increased inoculum resulting in an increased resistance (Drusano et al., 2001). The *P. putida* strain, C260, displayed induction but no *erm* genes were recorded in this strain. This could also be experimental error but the induction level is relatively high, therefore a further inducible gene maybe present in this strain. The induction in strain C507 *(E. casseliflavus)* may

have been due to the *ermB* gene but the sequencing of the fragment began after the 5' leader peptide which would be necessary for induction of increased resistance by tylosin (Jost et al., 2003).

5.4.3 Frequencies of sulphonamide resistance genes in the *intI1* positive isolates

Under half of the *intI1* positive isolates contained an *intI1* and *sulI* genotype despite the common Class 1 integron structure containing a 3' conserved region encoding the *qacEΔ1* and *sulI* genes (Rädström and Swedberg, 1988). Exceptions to this structure have been observed in the presence of the *qacE* gene in which the 3' conserved region are commonly reported to lack the *sulI* gene and in structures with altered transposon evolution (Partridge et al., 2002; Rosser and Young, 1999). The only isolate in this study that was PCR positive for *intI1*, *sulI* and *qacEΔ1*genes, was isolate C361 (*A. arilaitensis*), in which the full integron was sequenced and annotated.

As in previous investigations, the presence of *sul* genes in this study, did not result in heightened resistance in all isolates as demonstrated by MIC levels (Sköld, 2000). The occurrence of the *sul* genes may also confer different levels of resistance in different bacterial genera, but little research has occurred in this area except in *E. coli* (Sköld, 2001). The MIC figures for all the *intI1* positive isolates in this study were lower than the resistance levels determined for *E. coli*; resistance levels of 2 mM (570 μg/ml) where a *sulI* gene was encoded and >4 mM with the *sulII* or *sul3* gene (Guerra et al., 2003; Sköld, 2000, 2001). The presence of a gene identified by PCR does not provide evidence for its transcription or translation and further work should be performed to investigate the transcription rates of these genes by RTPCR and QRTPCR. In the case of *sulI*, previous work has investigated the promotion and transcription rates for gene cassettes on integron structures. If the *sulI* gene had been located on a functional

integron it would have been under the transcriptional control of the P_{ant} and/or P2 promoters (Collis and Hall, 1995). Transcription levels of integron-associated gene cassettes and the 3' terminus genes are affected by their distance from the promoter and therefore the number of genes in between. The nearer a gene cassette to the promoter the higher its transcription levels due to premature transcription termination occurring between the gene cassettes, the 59 bp elements functioning as transcriptional terminators (Collis and Hall, 1995).

In this study, three *Acinetobacter* spp. were isolated that contained the *intI1* gene and the *sulII* gene. The identification of Class 1 integrons in *Acinetobacter* from the soil environment displays a mechanism for the integration of antibiotic resistance genes into its genomes. This integration has been observed in the medical environment where isolates have been accumulating resistance to a large number of antibiotic groups including the sulphonamides, resulting in *Acinetobacter* infections becoming a serious problem in ICUs (Chastre, 2003; Van Looveren and Goossens, 2004). Class 1 integrons were first identified in sulfamethoxazole resistant *Acinetobacter* spp. isolated from a Danish fish farm, whereas the *sulII* gene has been characterised only once in *A. baumanni*, flanked by insertion sequence IS_{ABA-1} on a transposon (Petersen et al., 2000; Segal et al., 2005). The low number of reports investigating the prevalence of the *sul* genes in *Acinetobacter* is because the majority of clinical reports only phenotypically identify antibiotic resistance and there are few genetic studies undertaken in the clinical environment into these organisms, with the exception of extended spectrum β-lactamases (Van Looveren and Goossens, 2004).

The *sulI* or *sulII* genes have not been identified previously in *Arthrobacter* or *Aerococcus* isolates, but the *sulI* gene and Class 1 integrons have been reported in

Pseudomonads adding to their increasing number of antibiotic resistance genes, integrons, transposons and plasmids already observed (Kazama et al., 1998b; Normark and Normark, 2002; Weldhagen, 2004). In *Shigella*, integrons which contain the *sulI* gene have been reported on a number of plasmids including the NR1 plasmid which contains a 17 Kb region for multiple antibiotic resistance encoding two Class 1 integrons and resistance for streptomycin, sulphonamides, chloramphenicol, ampicillin, trimethoprim and mercury resistance (McIver et al., 2002; Rajakumar et al., 1997).

5.4.4 Multiple antibiotic resistance of the *intI1* positive isolates

Variable antibiotic resistance patterns were observed in the *intI1* PCR positive isolates from this study with no correlation to genus or sample from which collected, where a number of bacteria displayed resistance to a large number of antibiotics.

Breakpoints for antibiotics differ between bacterial species but the concentrations used in this study tried to find a midpoint to gauge some resistance for all isolates. For example, the BSAC breakpoint for ampicillin resistance in *Enterobacteriacae/Acinetobacter* and *Pseudomonas* is 16 mg/l but in *Enterococci* it is 8 mg/l, whereas the breakpoint for streptomycin is 8 mg/l for *Enterobacteriacae/Acinetobacter*, 4 mg/l for *Pseudomonas* but 128 mg/l for *Enterococci* (BSAC, 2005). For more detailed information on each isolate the MIC determinations should be performed specifically for each isolate utilising specific breakpoints (BSAC, 2005).

The isolation of only two bacterial isolates resistant to kanamycin is unusual because of the high levels of cross resistance between this and other aminoglycoside antibiotics including streptomycin, gentamycin, neomycin and tobramycin (Onaolapo, 1994). The Pseudomonads were the genus identified in this study displaying resistance to the

largest range of antibiotics, this maybe due to intrinsic/acquired resistance genes or the expression of MD efflux systems which are common in this genus (Poole, 2004b). As stated previously, some Pseudomonads are capable of natural transformation and perhaps as a consequence carry plasmids with numerous antibiotic resistance genes (Olsen and Shipley, 1973). Commenting on the *P. aeruginosa* strain PAO1 which has been observed to be resistant to virtually every front-line drug, Lister stated, "we have only just started to unlock the resistance potential of this pathogen" (Lister, 2002; Stover et al., 2000). Capture and expression of cassette-associated genes was demonstrated in a chromosomal integron of *P. stutzeri* (Coleman and Holmes, 2005). The genetic basis of the resistance in *Pseudomonas* isolates observed in this study was not investigated but medical studies have reported common resistance genes to include those for extended spectrum β-lactamases and the *ant/aac* groups of genes which can confer resistance, depending on the specific enzyme, to kanamycin, streptomycin, spectinomycin, gentamycin (Sabtcheva et al., 2003; Sekiguchi et al., 2005) and a large number of medically significant antibiotics such as amikacin, tobramycin and dibekacin (Weldhagen, 2004). It must also be considered that the majority of reports into antibiotic resistance in Pseudomonads have investigated medical isolates of *P. aeruginosa*, although one environmental study found *Pseudomonas* isolates resistant to chloramphenicol and streptomycin in wheat root rhizospheres of mercury polluted soil (Smit et al., 1998). The streptomycin resistant *Pseudomonas* found in this study and the investigation by Smit et al., (1998), maybe observing this bacteria's defence mechanism against the natural production of streptomycin by soil dwelling bacteria (Olsen, 1999).

The *Shigella* strain, C506, isolated in this study also displayed a high level of resistance. The isolate was resistant to all the 8 antibiotics tested and also demonstrated

some resistance to sulphonamides. This resistance phenotype has been reported previously in *S. flexneri* with a chromosomal multiple resistance region similar to the *Shigella* R-plasmid NR1 encoding ampicillin, streptomycin, tetracycline and chloramphenicol resistance, and also in *S. flexneri* 2a which encoded a chromosomal multiple resistance region, termed the Shigella Resistance Locus (SRL) within a pathogenicity island resembling those from *Salmonella* sp. (Luck et al., 2001; Rajakumar et al., 1997). Further work to locate the resistance determinates and characterise the integron in isolate C506 may yield some interesting results.

Multiple antibiotic resistance in *Enterococcus* spp. has been widely studied due to this genera's involvement in human infection and its role as a foodborne pathogen (Hong and Davis, 1996; Teuber et al., 2003). The multiple resistance of *Enterococcus* strains isolated in food-animals was one of the precipitators behind the ban on antibiotics as growth promoters in livestock and a number of studies have followed the resistance of these bacteria since the ban (Aarestrup et al., 2001; Boerlin et al., 2001). The isolate C507, *E. casseliflavus*, carried a *tet(M)* gene for tetracycline resistance and displayed a resistance phenotype towards streptomycin, both not uncommon in *Enterococcus* spp. (Clark et al., 1999; Min et al., 2003).

Due to time constraints the only tetracycline resistance determinates screened by PCR in this study were *tet(M)* and *tet(K)*. The *tet(K)* gene encodes efflux protection against tetracyclines, commonly encoded on small transmissible plasmids but is relatively uncommon in the environment, whereas *tet(M)* encodes for ribosomal protection, more commonly located in Gram positive bacteria (Roberts, 1996). The *tet(M)* gene is commonly found in soils and waters near sites in which tetracyclines has been used (Agerso et al., 2004), but other genes such as *tet(B)* (commonly found in swine isolates

and has the widest host range of any of the Gram negative determinates) (Sunde and Sørum, 2001), *tet(A)* (inducible resistance via the repressor *tet(R)*, commonly found together on plasmids) (Hinrichs et al., 1994; Rhodes et al., 2000), the *otr* genes (due to slurry spiking with OTC) (Nikolakopoulou et al., 2005) and *tet(G)*, (*L*), (*O*) and (*T*) (which have all been reported in the soil environment) (Roberts, 1997) (pers. comm. E.M.H.Wellington) should be screened for in the future, giving an indication of whether the oxytetracycline addition to the slurry in this study was bio-available and therefore acting as a selective agent for tetracycline resistance in the slurry and indigenous bacteria.

The *Bacillus* isolates which all exhibited the same antibiotic resistance profile may all have had the same MDR efflux pumps, multiple resistance plasmid encoding members of the *tet* group of resistance genes (Safferling et al., 2003) and the *cat* or *cm* resistance genes for chloramphenicol resistance (Goldfarb et al., 1981; Laredo et al., 1988), or, also the same chromosomal mutation in the *gyrA* gene conferring resistance to the quinolone group of antibiotics (nalidixic acid) (Bast et al., 2004; Grohs et al., 2004). Further investigation of the integrons or plasmids which these *Bacillus* isolates may carry would provide additional information on multiple resistance strains of *Bacillus* in the environment.

5.4.5 Integron annotation and characterisation

As briefly discussed in Section 5.4.3, the only isolate in which the common Class 1 integron structure of *intI1*, *qacEΔ1* and *sulI* was sequenced was in the isolate C361 (*A. arilaitensis*). The decreased prevalence in this study compared to others, of the *intI1/qacEΔ1/sulI* Class 1 integron structure, may be an attribute of this studies soil environment, in that integrons in this situation may differ from those in the medical

setting of which a large majority of integron characterisation has proceeded and in which detergents are commonly used (Russell, 2002). The selection of the *qacEΔ1* gene may be decreased in the soil environment unless pollutants are present and integrons with integrated antibiotic or metal resistance factors where present in antibiotic perturbed soils would be selected for, as demonstrated in this study by the lack of bacterial isolates from the organic and control slurries and control soils which were PCR negative for the *intI1/2* genes (Gaze et al., 2005; Nwosu, 2001). Rosser and Young, (1999) characterised a number of integrons in cultured bacteria, isolated from a river estuary in Scotland, which contained structures with a *qacE* 3` conserved region not *qacEΔ1* and *sulI*, as well as the *int11* gene only in *Pseudomonas*-like, *Vibrio*-like and coliform isolates. In6 and two plasmids which conferred β-lactamase resistance in *E. coli* clinical isolates, were reported to have altered 3` conserved regions; In6 and pSAL-1, carrying the bla_{DHA-1} gene with 3` truncated *qacEΔ1* genes and the second plasmid pCMXR1, carrying the bla_{CMY-9} gene but lacked the *qacEΔ1* and *sulI* genes (Doi et al., 2002). These variations in integron structure have been observed where altered phenotypes would be selected for by the environment; those bacterial carriers from the Rosser and Young study were in direct contact with untreated domestic sewage carrying a whole host of detergents, antibiotics and medical waste (Rosser and Young, 1999). As the role of the integron is the capture and expression of antibacterial resistance genes, bacteria with diverse structures would be selected for in specific environments to suit the variations in compounds which come into contact with the bacterial cell.

A number of the PCR products, for example the PGS22 integron fragment, could not be amplified or sequenced. This may have been due to the presence of the *qacE* gene or

an altered Class 1 integron structure, the VarF/VarR and Cass1/Cass2 primers annealing to the junction between the *qacEΔ1* and *sulI* genes (Partridge et al., 2002; Rosser and Young, 1999). The integron characterisation of isolates C506 and C609 is confusing. Despite the lack of *sulI*, C506 produced a PCR product for Int1F/SulIR primer set and an identical banding pattern to C609 with the VarF/VarR primer set. For C609 this may indicate an empty integron, ie. a *intI1* gene encoded within the 5' conserved region with the *qacEΔ1* gene of the 3' conserved region, no integrated gene cassettes and a deletion of the *sulI* gene. Isolate C506 was previously mentioned to have phenotypic resistance similarity with the SRL of *S. flexneri* 2a and also encoded an *aadA1* gene. Amplification with the IntI1F/SulIR primers therefore may be due to annealing on a truncated or partially deleted *sulI* gene as a result of the insertion of an IS element, the use of these primers would have allowed the detection of such an arrangement whereas the primers for the whole *sulI* gene would not have (Table 2.5) (Decre et al., submitted 2004; Luck et al., 2001).

5.5 CONCLUSIONS

This work reports a 5.64 % prevalence of the *intI1* gene in culturable bacterial isolates from slurry and slurry amended soils. It reports the first observations of Class 1 integrons in the putative bacterial genera *Carnobacterium*, *Psychrobacter* and *Aerococcus* of which the later two bacterial genera also demonstrated the first reports of sulphonamide resistance conferred by a *sul* gene. The occurrence of Class 1 integrons in putative Gram positive bacteria, *Bacillus* spp. and *Arthrobacter* are investigated, the later integron was confirmed to transfer in conjugal matings to Gram negative recipients, *P. putida* and *E. coli*.

Variable levels of multiple antibiotic resistance were recorded phenotypically with a number of resistance genes being identified such as *tet(M)* and a number of *erm* genes. Despite *erm* genes conferring tylosin resistance, no correlation was observed between *erm* gene carriage and tylosin resistance in *intI1* positive isolates and did not support the hypothesis that there was a link between *intI1* carriage and tylosin resistance in this study.

A second report of a Class 1 integron encoding multiple antibiotic resistance with an *intI1Δ1* gene was demonstrated in an *A. arilaitensis* isolate, the first being in a *C. glutamicum* strain with a pTet3 plasmid.

This work observed and reiterates the fact that multiple antibiotic resistant bacteria are located in the soil and slurry environment. The carriage of integrons mirroring the carriage of resistance conferring genes, and the co-selection of these genes by drugs present in the soils.

5.6 FURTHER WORK

Integron characterisation in the soils unculturable bacterial population and in those bacterial isolates collected could be determined further by a number of additional techniques. Exogenous transfers could be utilised to capture mobile elements with inserted integrons conferring antibiotic resistance, directly from the soils or slurries (Smalla et al., 2000a). PCR-based techniques, both sequence-dependent and sequence-independent have been used to recover new integrase genes and new gene cassettes from the environment by utilising the conserved nature of the 59 bp element flanking gene cassettes and the conserved region of the integrase genes (Nield et al., 2001; Stokes et al., 2001a; Stokes et al., 1997). An attempt was made to establish these techniques in this study but time constraints prevented the amount of cloning necessary

to distinguish between the multiple PCR bands gained using the primers for these PCR-based techniques. An additional technique, utilising a marked empty integron, could have been beneficial in this study to 'fish' for gene cassettes in the unculturable bacterial population.

For a number of *intI1* positive isolates further investigation should be performed. The integron of isolate C506 was only partially sequenced and a repeat of the PCR using the IntIF/SulIR primer set or an alternative PCR such as those using the 59 bp elements could be performed. These may reveal the full sequence of the integron and whether the resistance profile of C506 was due in part or in full to the integron (Stokes et al., 2001a). Investigation of the isolate C260 may reveal an inducible tylosin resistance gene, other than those already screened for, to explain the induction of tylosin resistance in this strain and sequencing upstream of the *ermB* gene fragment in C507 may reveal a leader peptide which allows the induction of tylosin resistance in this strain (Jost et al., 2003). Focussing particular attention on the *Bacillus* isolates may yield further information on the carriage of integrons and multiple drug resistance in the environment in this genus.

RTPCR and QRTPCR are both techniques that would allow the investigation of regulation and transcription and have been used in numerous studies to investigate antibiotic resistance gene expression in bacterial cells and expression with relation to resistance levels. The expression of the *ampC*, *bla1* and *bla2* genes were investigated in relation to penicillin resistance in *E. coli* and *B. anthracis* (Chen et al., 2004; Tracz et al., 2005), and *vanA* expression has been recorded in vancomycin resistant *S. aureus* (Perichon and Courvalin, 2004). RTPCR is currently the most sensitive technique for mRNA detection and quantification of gene expression.

As stated in the discussion with respect to the MIC determinations, these should be repeated individually for a number of the isolates tailoring the breakpoints to the genus in question to receive more detailed information on each isolate and not just a general investigation of all isolates.

To supplement this study, excision, integration and cognate assays might be performed to deduce whether the integrase enzymes found in this study were capable of catalysing integration and investigations as to whether the integrase enzymes were Class, gene cassette or bacterial species specific and to demonstrate whether they were active in the soil environments in which they were isolated, possibly demonstrating gene transfer in a number of soils and slurries obtained in this study (Collis et al., 2002b; Holmes et al., 2003).

The tetracycline resistance has been neglected in this study despite the addition of OTC to the slurry before its application to the soil. Further screening for tetracycline resistance genes including those commonly found in the slurry and soil environments such as *tet(B), (A), (G), (L), (O), (T)* and the *otr* genes maybe investigated using a micro-array or multiplex PCR system which have been previously used to screen for a number of these genes but as yet have only been used on single, often clinical isolates and not environmental samples (Call et al., 2003; Ng et al., 2001; Perreten et al., 2005).

CHAPTER 6:

CHARACTERISATION OF *intI2* GENE POSITIVE BACTERIAL ISOLATES AND THEIR INTEGRONS

Chapter 6: Characterisation of *intI2* positive isolates

6.1 INTRODUCTION

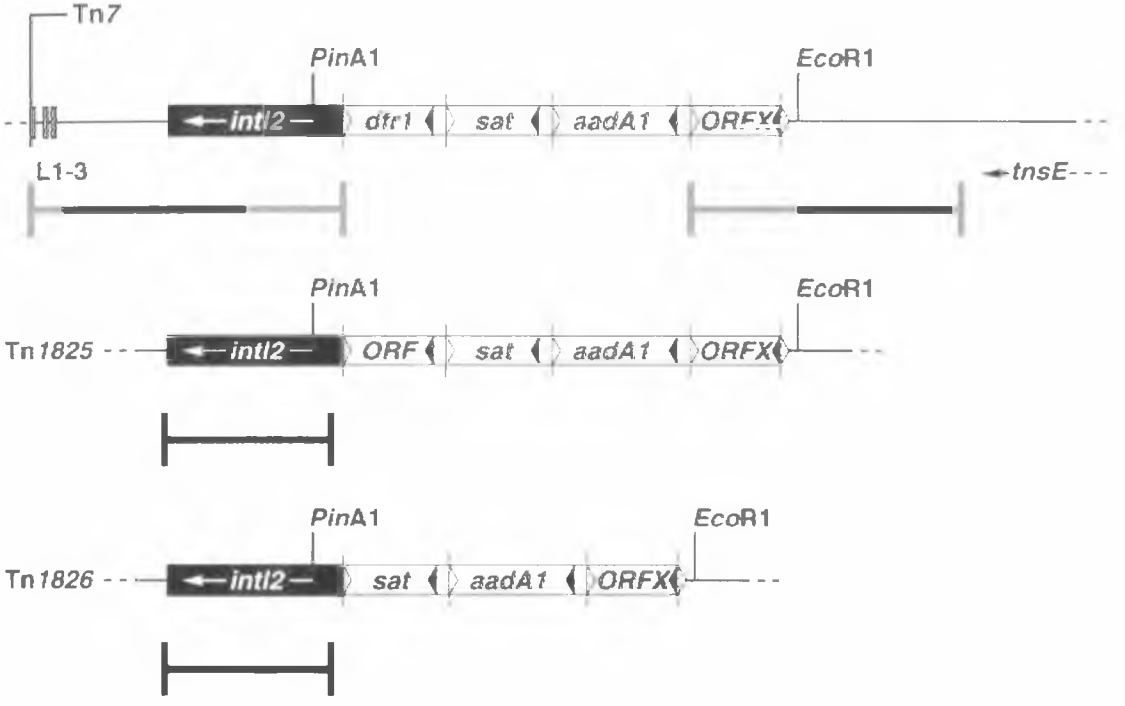
In Chapter 4, the prevalence of the *intI2* gene was determined by PCR to be higher than *intI1*, 57 (9.7 %) compared to 33 (5.64 %) of the bacterial isolates collected in this study.

Class 2 integrons are potentially mobile genetic elements, similar in structure to the Class 1 integrons, originating and often residing on the transposon Tn*7* (Fig. 1.10b) (Hall and Collis, 1995; Sundström et al., 1991). Investigations into integron structures will permit a better understanding of the gene pool in the soil and slurry environments as well as the potential for the movement and selection of these genes in diverse environments, especially in the presence of potential human pathogens.

6.1.1 Class 2 Integron structures

Compared to the Class 1 integron, fewer studies have been conducted, especially in the environment, into the frequencies, structures and mechanisms of a Class 2 integron. Whereas Class 1 integrons are typically borne on elements derived from Tn*21*, the Class 2 integrons are commonly derived from the non-replicate Tn*7* which preferentially inserts into specific domains in the chromosome (Hansson et al., 2002; Martinez and de la Cruz, 1990). The structural organisation of Class 2 integrons is similar to Class 1 integrons with a variation of cassette content, the former commonly encoding 3 cassettes, *dfrA1* (encoding trimethoprim resistance), *aadA1* and *sat* (streptothricin resistance) (Fig. 6.1) (Hansson et al., 2002; Tietze and Brevet, 1991). The *dfrA1* and *aadA1* gene cassettes are interchangeable with Class 1 integron structures due to the

Fig. 6.1 Backbone types of Class 2 integrons



The structures of the integron regions in Tn*7* and in related transposons Tn*1825* and Tn*1826*, where the black boxes indicate the conserved sequence of the integrase gene, *intI2* and cassettes are represented by white boxes. Left pointing arrowheads denote the 59 bp elements, and right pointing arrowheads denote attI/C (insertion) sites. Taken from Hansson et al. (2002).

similarity of their aatI/C integration sites (Hansson et al., 2002; Sundström and Sköld, 1990). A limited number of other gene cassettes have been reported on Class 2 integrons. The *catB2* gene was reported in a novel arrangement in *A. baumanni*, *ere(A)* and *estX* genes were sequenced on *E. coli* Class 2 integrons (Biskri and Mazel, 2003; Ramirez et al., 2005a) and a hybrid of a Class 1 and Class 2 integron carrying *dfrA1*, *sat*, *aadA1*, *qacEΔ1* and *sulI* was sequenced in a different *A. baumanni* strain (Ploy et al., 2000).

Unlike the nucleotide sequence of the *intI1* integrase gene, the *intI2* gene encodes an internal stop codon (TAA), potentially preventing integration of gene cassettes into Class 2 integrons via its attI/C sites (Simonsen et al., 1983). Investigations into the stop codon hypothesise that other integrases may bind to or bind over the DNA with the stop codon via helix-turn-helix motifs, recognising the motifs in the N-terminal moiety of the integrase protein, therefore giving complete integrase function, leading to the theory of an inhibitor polypeptide and the self regulation of an integrase (Hansson et al., 2002). The stop codon maybe the reason why the cassette structures of the Class 2 integron have been demonstrated to have less variation than the Class 1 integron although, it does not seem to have effected its distribution throughout the bacterial domains, possibly due to the action of other integrase proteins (Hansson et al., 2002). Hansson et al., (2002) investigated the cross-specificity of the IntI1 and IntI2 integrase proteins finding that only the IntI1 protein from Tn*21* was able to excise all cassettes from both Tn*21* and Tn*7* whereas the IntI2 protein from Tn*7* was not able to cross-excise in *E. coli*, even if the stop codon was mutated to a functional glutamic acid. The reason for this specificity maybe the nucleotide sequences of the integration sites (attI/C), which differ in each class of integron (Partridge et al., 2000). These results were further confirmed by Collis et al., (2002b) who also studied the specificity of the integrases encoded by the *intI1*, *intI2* and *intI3* genes for excision and integration at the varying attI/C sites displayed by the Classes of 1, 2 and 3 integrons.

6.1.2 Environmental occurrence of Class 2 integrons

As with the Class 1 integrons, epidemiological reports of Class 2 integrons commonly focus on a particular species and few environmental studies to date have screened for Class 2 integrons. Transposons, Tn*1825* (*sat1, aadA1*) and Tn*1826* (*sat2, aadA1*)

(Tietze et al., 1988), with integrated Class 2 integrons (Fig. 6.1) have been widely studied and mostly reported in Gram negative bacteria (Tschäpe et al., 1984) from a range of environments (Smalla et al., 1993) including a study investigating the transfer of the *sat2* genes via carriage on transposons from pig manure and gut flora into soils and river water (Götz and Smalla, 1997; Smalla et al., 2000a). The prevalence of Class 2 compared to Class 1 is variable between studies. Gonzalez et al., (1998) reported that the Class 2 integrons were the most prevalent in an investigation into Chilean *A. baumanni* isolates, a number of isolates carrying both Classes 1 and 2. Two further reports have also observed the Class 2 integron to be the most prevalent class in their isolates, 100 % of MD resistant *S. enterica* from poultry and a similar percentage in an investigation into epidemic *S. sonnei* strains in Australia (McIver et al., 2002; Miko et al., 2003). Alternatively, investigations have reported cases where the Class 1 integron is more prevalent (Roe et al., 2003a; Roe et al., 2003b; Seward, 1999). The two reports by Roe calculated Class 1 integron incidences higher than Class 2 in studies into total-community DNA from poultry carcasses and *E. coli* isolates from irrigation water and sediment collected from the Rio Grande, whereas Seward reported 1 out of 25 *Acinetobacter* isolates encoded a Class 2 integron compared to 17 Class 1 (Roe et al., 2003a; Roe et al., 2003b; Seward, 1999).

6.2 AIMS

The prevalence of the *intI2* gene in this study was determined as 9.7 % but it was unknown as to whether these genes were part of a Class 2 integron structure, a potential vector for antibiotic resistant gene cassettes. As few reports have investigated Class 2 integrons, additional characterisation of the *intI2* positive isolates would enhance

information on their distribution and cassette organisation in the soil environment. As with the *intI1* positive isolates, the objectives of this part of the study were to;

1. Screen, by PCR, for a number of specific genes which confer resistance to the sulphonamide group of antibiotics; *sulI, sulII* and *sul3,* from the 52 culturable bacterial isolates obtained from environmental soil samples before and after the application of slurry and from bacterial isolates from the applied slurry, which contained the *intI2* gene;

2. To screen the same isolates as in 1, by PCR, for the *intI1* gene, this would indicate the presence of multiple compatible integrons and possible vectors for HGT and the mobilisation of the sulphonamide resistance genes. The presence of a second integrase may also enable increased integration into Class 2 structures;

3. To identify the isolates carrying the *intI2* gene by 16S rRNA sequencing;

4. To determine whether there is a link between OTC resistance and the presence of the *intI2* gene;

5. To characterise the isolates' Class 2 integrons by PCR and sequencing, determining which gene cassettes are present on the integrons, and to;

6. Characterise the isolates multiple antibiotic profiles.

6.3 RESULTS

6.3.1 16S rRNA identification of *intI2* positive bacteria isolates

The 16S rRNA characterisation of the 52 *intI2* gene positive isolates displayed a biodiversity which spanned 10 bacterial genera, 4 of which are not commonly soil dwelling and one isolate was putatively identified with 97 % similarity to an uncultured bacteria (Table 6.1). These results also recorded 7 bacterial isolates observed

previously to carry the *intI1* gene in this study; such as *Pseudomonas* spp., *Bacillus* spp., a *Shigella* sp. and an *Enterococcus* sp. (Table 6.1). Isolate C485, was identified with 98 % similarity, as *Streptomyces verne*, selected from an OTC 5 µg/ml plate from year 2, day 1 soils.

Bacterial isolates positive for *intI2* were observed in all soil time points except the control and organic soils, and cultured from the Cranfield but not the organic slurries. There were 5 bacterial isolates from the 13/02/02 drain-flow sample which were PCR positive for the *intI2* gene; *S. flexneri*, *E. casseliflavus*, *B. silvestris* and two *A. lwoffi* strains.

Table 6.1 also recorded that a larger number (31) of the *intI2* positive isolates were cultured from soils or slurries on selective OTC plates containing from 0.2 to 25 µg/ml which were further investigated by multiplex PCR screening and OTC MIC plates.

6.3.2 PCR screening for a number of tetracycline resistance genes using a multiplex system

Table 6.2 displays the tetracycline genotypes of the *intI2* gene positive isolates. The *tet(M)* was located in 4 isolates including C507 (*E. casseliflavus),* two Pseudomonads (C402 and C471) and an *Acinetobacter* sp. (C4) (Table 6.2). The *tet(M)* encoding strains were isolated from the drain-flow samples, Cranfield slurries and year 2, day 1 soils. The majority, 3 of the 4 *tet(M)* positives isolates were collected from OTC selective plates. The isolates were screened for the *tet(M)* gene using a multiplex system which also allowed the amplification of the *tet(K)* gene, identified in 3 isolates, not collected from OTC selective plates. Isolate PGS63, a *Psychrobacter* sp., was PCR positive for the *tet(K)* gene.

Table 6.1 Characterisation of *intI2* positive bacterial isolates from this study, displaying isolate numbers, source, isolation plate antibiotic concentrations, 16S rRNA identification and *intI1* genotype.

ISOLATE NO.	SLURRY / SOIL SAMPLE	PLATE ANTIBIOTIC CONC. (µg/ml) ISOLATED	16S rRNA identification	% similarity	*intI1*
PGS21	PS(CR)	TY 50	*Aerococcus viridians*	99	-
PGS49	PS(CR)	TY 50	*Acinetobacter sp. An9*	97	-
PGS63	PS(CR)	TY 50	*Psychrobacter sp. A1-2*	97	-
PGS65	PS(CR)	TY 50	*Psychrobacter maritimus*	98	-
C3	PS(CR)	TY 5	*Acinetobacter lwoffi*	99	-
C4	PS(CR)	TY 5	*Acinetobacter lwoffi*	98	-
C5	PS(CR)	TY 5	*Pseudomonas borealis*	99	-
C15	PS(CR)	SCP 25	*Acinetobacter lwoffi*	99	-
C519	PS(CR)	NONE	*Bacillus pumilus*	99	+
PGS6	1,P	TY 5	*Pseudomonas lini*	99	-
PG20	1,P	SCP 25	*Psychrobacter ikaite*	97	-
C205	1,P	SCP 25	*Pseudomonas fluorescens*	99	-
C236	1,P	SCP 25	*Psychrobacter sp. A1-2*	98	-
C237	1,P	SCP 25	*Bacillus sphaericus*	98	-
C241	1,P	SCP 25	*Pseudomonas fluorescens*	99	-
C409	1,1	OTC 5	*Pseudomonas fluorescens*	98	-
C410	1,1	OTC 5	*Psychrobacter ikaite*	99	-
C411	1,1	OTC 5	*Bacillus pumilus*	99	-
C230	1,21	TY 50	*Pseudomonas putida*	97	+
C231	1,21	TY 50	*Pseudomonas putida*	97	+
C305	1,90	SCP 10	*Pseudomonas fluorescens*	99	-
C454	1,90	OTC 0.2	*Psychrobacter LB13*	98	-
C456	1,90	OTC 0.2	*Acinetobacter sp. RUH1139*	97	-
C457	1,90	OTC 0.2	*Acinetobacter N2*	97	-
C402	2,1	OTC 10	*Pseudomonas jessenii*	98	-
C471	2,1	OTC 5	*Pseudomonas putida*	98	-
C472	2,1	OTC 5	*Shigella sonnei*	99	-
C485	2,1	OTC 5	*Streptomyces verne*	98	-
C141	2,21	OTC 25	*Acinetobacter lwoffi*	98	-
C154	2,21	TY 50	*Acinetobacter N2*	97	-
C317	2,21	OTC 10	*Uncultured bacteria EBSCPSA-6117*	97	-
C418	2,21	OTC 0.2	*Acinetobacter N2*	97	-
C419	2,21	OTC 0.2	*Pseudomonas sp. S1402*	97	+
C420	2,21	OTC 0.2	*Arthrobacter protophormiae*	97	-
C422	2,21	OTC 0.2	*Stenotrophomonas CL3B1*	98	-
C423	2,21	OTC 0.2	*Pseudomonas jessenii*	99	-
C436	2,90	OTC 5	*Bacillus sp. NIPHLO9094 B1*	99	-
C437	2,90	OTC 1	*Bacillus subtilis*	99	-
C438	2,90	OTC 1	*Bacillus weihenstephanesis*	99	-

C439	2,90	OTC 1	*Acinetobacter N2*	97	-
C440	2,90	OTC 0.2	*Bacillus weihenstephanesis*	99	-
C121	2,240	TY 50	*Pseudomonas putida*	97	-
C131	2,240	SCP 25	*Pseudomonas syringae*	97	-
C431	2,240	OTC 0.2	*Acinetobacter lwoffi*	99	-
C432	2,240	OTC 0.2	*Acinetobacter lwoffi*	99	-
C433	2,240	OTC 0.2	*Acinetobacter lwoffi*	99	-
C435	2,240	OTC 0.2	*Acinetobacter lwoffi*	99	-
C506	DF13/02/02	OTC1	*Shigella flexneri*	98	+
C507	DF13/02/02	OTC 1	*Enterococcus casseliflavus*	98	+
C508	DF13/02/02	OTC 1	*Bacillus silvestris*	99	+
C509	DF13/02/02	OTC 1	*Acinetobacter lwoffi*	98	-
C511	DF13/02/02	OTC 1	*Acinetobacter lwoffi*	98	-

PS(CR), Cranfield slurry; 1,P, Year 1 pre-application soils; 1,1, Year1, day 1 soils; DF, Drain-flow runoff from a high rainfall event on 13/02/2002. Colours indicate: blue, all bacteria isolated on OTC; red, enteric pathogenic bacteria; green, Gram positive bacteria; brown, indigenous soil bacteria and pink, opportunistic pathogens.

The OTC MIC varied between isolates from 0.2 to 16 mg/l (Table 6.2). Isolates encoding the *tet(K)* gene, displayed elevated OTC MIC values of 16 and 2 mg/l but isolates negative for this gene were also identified as having MIC values of 16 mg/l, C439 (*Acinetobacter* sp.). No correlation was observed between OTC MIC values and the *tet(M)* gene. The isolate identified as a *Streptomyces* sp. (C485), was PCR negative for the *tet(M)* and (*K)* genes but displayed an OTC MIC of 16 mg/l. In a number of isolates from OTC selective plates, the displayed OTC MIC values were lower than the concentrations of OTC on which they were originally isolated.

6.3.3 PCR screening for the sulphonamide resistance genes

Only 9 out of the 52 *intI2* positive isolates were PCR negative for any of the *sul* genes, Table 6.3 recorded that the largest number of isolates (15) contained a *sulI* gene only, and 12 contained only the *sulII* gene singularly. The highest observed multiple *sul* gene combination was *sulI* and *sulII*, whereas no isolates contained a combination of the *sulI* and *sul3* genes. All three *sul* genes were observed in 3 isolates; C15 (*A. lwoffi*), PGS20 (*P. ikaite*) and C141 (*A. lwoffi*), and were characterised and discussed in more detail in Chapter 7. In accordance with Chapter 5 which investigated isolates *intI1* gene positive, the *intI2* positive isolates displayed no correlation between SCP MIC values and *sul* genotype or isolates collected originally from SCP selective plates.

6.3.4 Multiple resistance of the *intI2* positive isolates

The *intI2* positive isolates displayed a range of multiple antibiotic resistance phenotypes (Table 6.4). No correlation was observed between bacterial genus and resistance phenotypes but the large majority of both *Acinetobacter* and *Pseudomonas*

Table 6.2 Characterisation of *intI2* positive isolates' tetracycline resistance genes and oxytetracycline MIC (mg/l).

ISOLATE NO.	SLURRY / SOIL SAMPLE	16S rRNA identification	*tet* gene	OTC MIC (mg/l)
PGS21	PS(CR)	*Aerococcus viridians*	-	0.2
PGS49	PS(CR)	*Acinetobacter sp. An9*	K	16
PGS63	PS(CR)	*Psychrobacter sp. A1-2*	K	16
PGS65	PS(CR)	*Psychrobacter maritimus*	-	0.2
C3	PS(CR)	*Acinetobacter lwoffi*	-	0.2
C4	PS(CR)	*Acinetobacter lwoffi*	M	0.2
C5	PS(CR)	*Pseudomonas borealis*	-	0.2
C15	PS(CR)	*Acinetobacter lwoffi*	-	0.2
C519	PS(CR)	*Bacillus pumilus*	-	0.2
PSG6	1,P	*Pseudomonas lini*	-	0.2
PGS20	1,P	*Psychrobacter ikaite*	-	1
C205	1,P	*Pseudomonas fluorescens*	K	2
C236	1,P	*Psychrobacter sp. A1-2*	-	0.2
C237	1,P	*Bacillus sphaericus*	-	0.2
C241	1,P	*Pseudomonas fluorescens*	-	0.2
*C409	1,1	*Pseudomonas fluorescens*	-	0.2
*C410	1,1	*Psychrobacter ikaite*	-	0.2
*C411	1,1	*Bacillus pumilius*	-	0.2
C230	1,21	*Pseudomonas putida*	-	0.2
C231	1,21	*Pseudomonas putida*	-	0.2
C305	1,90	*Pseudomonas fluorescens*	-	0.2
*C454	1,90	*Psychrobacter LB13*	-	0.2
*C456	1,90	*Acinetobacter sp. RUH1139*	-	0.2
*C457	1,90	*Acinetobacter N2*	-	0.2
*C402	2,1	*Pseudomonas jessenii*	M	16
*C471	2,1	*Pseudomonas putida*	M	0.2
*C472	2,1	*Shigella sonnei*	-	0.2
*C485	2,1	*Streptomyces verne*	-	16
*C141	2,21	*Acinetobacter lwoffi*	-	1
C154	2,21	*Acinetobacter N2*	-	0.5
*C317	2,21	*Uncultured bacteria EBSCPSA-6117*	-	0.5
*C418	2,21	*Acinetobacter N2*	-	8
*C419	2,21	*Pseudomonas sp. S1402*	-	0.2
*C420	2,21	*Arthrobacter protophormiae*	-	0.2
*C422	2,21	*Stenotrophomonas CL3B1*	-	4
*C423	2,21	*Pseudomonas jessenii*	-	0.2
*C436	2,90	*Bacillus sp. NIPHLO9094 B1*	-	0.2

*C437	2,90	*Bacillus subtilis*	-	0.2
*C438	2,90	*Bacillus weihenstephanesis*	-	0.2
*C439	2,90	*Acinetobacter N2*	-	0.2
*C440	2,90	*Bacillus weihenstephanesis*	-	16
C121	2,240	*Pseudomonas putida*	-	0.2
C131	2,240	*Pseudomonas syringae*	-	0.2
*C431	2,240	*Acinetobacter lwoffi*	-	0.5
*C432	2,240	*Acinetobacter lwoffi*	-	0.2
*C433	2,2,40	*Acinetobacter lwoffi*	-	0.5
*C435	2,240	*Acinetobacter lwoffi*	-	0.5
*C506	DF13/02/02	*Shigella flexneri*	-	0.2
*C507	DF13/02/02	*Enterococcus casseliflavus*	*M*	0.2
*C508	DF13/02/02	*Bacillus silvestris*	-	0.2
*C509	DF13/02/02	*Acinetobacter lwoffi*	-	0.2
*C511	DF13/02/02	*Acinetobacter lwoffi*	-	0.2

*** denotes isolates from OTC containing plates.**

PCR results not confirmed by sequencing.

Table 6.3 Characterisation of *intI1* positive isolates, *sul* genotype and MIC for sulphachloropyridazine (mg/l)

ISOLATE NO.	SLURRY / SOIL SAMPLE	16S rRNA identification	*sulI*	*sulII*	*sul3*	SCP MIC (mg/l)
PGS21	PS(CR)	*Aerococcus viridians*	-	+	-	32
PGS49	PS(CR)	*Acinetobacter sp. An9*	-	+	+	16
PGS63	PS(CR)	*Psychrobacter sp. M1-2*	-	+	+	1
PGS65	PS(CR)	*Psychrobacter maritimus*	-	+	-	1
C3	PS(CR)	*Acinetobacter lwoffi*	+	-	-	1
C4	PS(CR)	*Acinetobacter lwoffi*	-	-	-	1
C5	PS(CR)	*Pseudomonas borealis*	-	-	+	1
*C15	PS(CR)	*Acinetobacter lwoffi*	+	+	+	5
C519	PS(CR)	*Bacillus pumilus*	-	-	+	1
PGS6	1,P	*Pseudomonas lini*	-	+	-	1
*PGS20	1,P	*Psychrobacter ikaite*	+	+	+	8
*C205	1,P	*Pseudomonas fluorescens*	+	-	-	1
*C236	1,P	*Psychrobacter sp. M1-2*	-	+	-	1
*C237	1,P	*Bacillus sphaericus*	+	-	-	1
*C241	1,P	*Pseudomonas fluorescens*	+	-	-	1
C409	1,1	*Pseudomonas fluorescens*	+	-	-	1
C410	1,1	*Psychrobacter ikaite*	+	+	-	16
C411	1,1	*Bacillus pumilius*	+	+	-	1
C230	1,21	*Pseudomonas putida*	-	-	-	8
C231	1,21	*Pseudomonas putida*	+	+	-	8
*C305	1,90	*Pseudomonas fluorescens*	-	-	-	1
C454	1,90	*Psychrobacter LB13*	+	-	-	1
C456	1,90	*Acinetobacter sp. RUH1139*	+	-	-	16
C457	1,90	*Acinetobacter N2*	-	-	-	1
C402	2,1	*Pseudomonas jessenii*	+	+	-	1
C471	2,1	*Pseudomonas putida*	+	-	-	4
C472	2,1	*Shigella sonnei*	-	+	-	1
C485	2,1	*Streptomyces verne*	-	+	-	1
C141	2,21	*Acinetobacter lwoffi*	+	+	+	6
C154	2,21	*Acinetobacter N2*	-	+	-	1
C317	2,21	*Uncultured bacteria EBSCPSA-6117*	+	+	-	1
C418	2,21	*Acinetobacter N2*	+	-	-	1
C419	2,21	*Pseudomonas sp. S1402*	+	-	-	1
C420	2,21	*Arthrobacter protophormiae*	-	-	-	1
C422	2,21	*Stenotrophomonas CL3B1*	+	-	-	1
C423	2,21	*Pseudomonas jessenii*	+	-	-	32
C436	2,90	*Bacillus sp. NIPHLO9094 B1*	-	+	-	1

C437	2,90	*Bacillus subtilis*	+	+	-	32
C438	2,90	*Bacillus weihenstephanesis*	+	+	-	1
C439	2,90	*Acinetobacter N2*	-	+	-	16
C440	2,90	*Bacillus weihenstephanesis*	+	+	-	32
C121	2,240	*Pseudomonas putida*	+	-	-	1
*C131	2,240	*Pseudomonas syringae*	+	-	-	32
C431	2,240	*Acinetobacter lwoffi*	+	-	-	16
C432	2,240	*Acinetobacter lwoffi*	-	-	-	2
C433	2,2,40	*Acinetobacter lwoffi*	-	+	-	1
C435	2,240	*Acinetobacter lwoffi*	-	-	-	1
C506	DF13/02/02	*Shigella flexneri*	-	+	-	8
C507	DF13/02/02	*Enterococcus casseliflavus*	-	-	-	32
C508	DF13/02/02	*Bacillus silvestris*	-	-	-	8
C509	DF13/02/02	*Acinetobacter lwoffi*	+	+	-	32
C511	DF13/02/02	*Acinetobacter lwoffi*	-	+	-	1

*denotes isolates collected from SCP selective plates.

Table 6.4 Characterisation of *intI2* positive isolates' multiple antibiotic resistance phenotypes

ISOLATE NO.	SLURRY / SOIL SAMPLE	16S rRNA identification	Antibiotic resistance
PGS21	PS(CR)	*Aerococcus viridians*	Cm^R Tet^R
PGS49	PS(CR)	*Acinetobacter sp. An9*	Sm^R Nal^R Tet^R
PGS63	PS(CR)	*Psychrobacter sp. AI-2*	Sm^R Km^R Tet^R Nal^R
PGS65	PS(CR)	*Psychrobacter maritimus*	Sm^R Km^R Nal^R
C3	PS(CR)	*Acinetobacter lwoffi*	NG
C4	PS(CR)	*Acinetobacter lwoffi*	NG
C5	PS(CR)	*Pseudomonas borealis*	Cm^R Tet^R
C15	PS(CR)	*Acinetobacter lwoffi*	Sm^R Cm^R Tet^R Tmp^R Nal^R
C519	PS(CR)	*Bacillus pumilus*	Sm^R Nal^R Cm^R Tet^R
PSG6	1,P	*Pseudomonas lini*	Sm^R Tmp^R Nal^R Amp^R
PGS20	1,P	*Psychrobacter ikaite*	Sm^R Cm^R Tet^R Tmp^R Nal^R
C205	1,P	*Pseudomonas fluorescens*	Sm^R Km^R Tmp^R Nal^R
C236	1,P	*Psychrobacter sp. A1-2*	Sm^R Tmp^R Nal^R
C237	1,P	*Bacillus sphaericus*	Sm^R Tmp^R Nal^R
C241	1,P	*Pseudomonas fluorescens*	NG
C409	1,1	*Pseudomonas fluorescens*	Tet^R Amp^R
C410	1,1	*Psychrobacter ikaite*	Tet^R
C411	1,1	*Bacillus pumilius*	Km^R Amp^R
C230	1,21	*Pseudomonas putida*	Nal^R, Amp^R, Cm^R, Tet^R, Tmp^R
C231	1,21	*Pseudomonas putida*	Nal^R, Amp^R, Cm^R, Tet^R, Tmp^R
C305	1,90	*Pseudomonas fluorescens*	Cm^R Tet^R Amp^R
C454	1,90	*Psychrobacter LB13*	Sm^R Cm^R Nal^R
C456	1,90	*Acinetobacter sp. RUH1139*	Cm^R Tet^R
C457	1,90	*Acinetobacter N2*	Sm^R Tmp^R Nal^R
C402	2,1	*Pseudomonas jessenii*	Sm^R Tet^R Tmp^R Nal^R
C471	2,1	*Pseudomonas putida*	Sm^R Cm^R Tet^R Tmp^R Nal^R
C472	2,1	*Shigella sonnei*	Sm^R Cm^R Tet^R
C485	2,1	*Streptomyces verne*	Amp^R
C141	2,21	*Acinetobacter lwoffi*	Sm^R Km^R Cm^R Tet^R Tmp^R Nm^R Nal^R Amp^R
C154	2,21	*Acinetobacter N2*	Cm^R Tmp^R Nal^R
C317	2,21	*Uncultured bacteria EBSCPSA-6117*	Sm^R Tmp^R Nal^R
C418	2,21	*Acinetobacter N2*	Sm^R Tet^R Amp^R
C419	2,21	*Pseudomonas sp. S1402*	Nal^R, Amp^R, Cm^R, Tet^R, Tmp^R
C420	2,21	*Arthrobacter protophormiae*	Amp^R
C422	2,21	*Stenotrophomonas CL3B1*	Sm^R
C423	2,21	*Pseudomonas jessenii*	Sm^R Cm^R Tet^R
C436	2,90	*Bacillus sp. NIPHLO9094 B1*	NG

C437	2,90	*Bacillus subtilis*	Sm^R Tet^R
C438	2,90	*Bacillus weihenstephanesis*	NG
C439	2,90	*Acinetobacter N2*	Sm^R Tet^R Tmp^R Nal^R
C440	2,90	*Bacillus weihenstephanesis*	Sm^R Cm^R Tet^R Amp^R
C121	2,240	*Pseudomonas putida*	NG
C131	2,240	*Pseudomonas syringae*	Tet^R
C431	2,240	*Acinetobacter lwoffi*	Nm^R Cm^R Tet^R
C432	2,240	*Acinetobacter lwoffi*	Sm^R
C433	2,2,40	*Acinetobacter lwoffi*	Sm^R Tet^R Nal^R
C435	2,240	*Acinetobacter lwoffi*	Cm^R
C506	DF13/02/02	*Shigella flexneri*	Sm^R, Nm^R, Nal^R, Km^R, Amp^R, Cm^R, Tet^R, Tmp^R
C507	DF13/02/02	*Enterococcus casseliflavus*	Sm^R, Tet^R
C508	DF13/02/02	*Bacillus silvestris*	Sm^R, Nal^R,Cm^R, Tet^R
C509	DF13/02/02	*Acinetobacter lwoffi*	Nm^R Cm^R Tet^R
C511	DF13/02/02	*Acinetobacter lwoffi*	Sm^R Nal^R

NG: no growth of isolate (therefore no phenotype determination), ND: Not done, Sm^R: resistance to Streptomycin 16 µg/ml, Amp^R: Ampicillin 16 µg/ml, Km^R: Kanamycin 16 µg/ml, Cm^R: Chloramphenicol 16 µg/ml, Tet^R: Tetracycline 8 µg/ml, Tmp^R: Trimethoprim 16 µg/ml, Nm^R: Neomycin 8 µg/ml, Nal^R: Nalidixic acid 16 µg/ml.

isolates were resistant to a large number of antibiotics. Isolate C485, the *Streptomyces* sp., was only phenotypically resistant to ampicillin at 16 μg/ml. The only displays of resistant towards neomycin were conferred by *Acinetobacter* spp. The *Psychrobacter* displayed multiple resistant phenotypes, differing between isolates.

6.3.5 Integron annotation and characterisation

In an attempt to characterise Class 2 integrons from the *intI2* positive isolates a set of primer pairs (Int2VarF/Int2VarR) were used which would potentially amplify the variable region between the *intI2* and the *orfX* genes if present (Table 2.6). On production of a sequence fragment using the Int2VarF primer a second forward sequencing primer was designed from the 3' end of this fragment to fill in the portion of sequence between Int2VarF and Int2VarR primer sequences (Table 2.6).

The results from the Int2VarF/Int2VarR PCR enabled the *intI2* positive isolates' Class 2 integrons to be separated into 4 groups; those with an *intI2* gene but failed the variable PCR; those which gave a 550 bp band product from the PCR therefore carrying an *intI2* gene with the 59 base element of the *orfX* gene; a third group: which gave an 1560 bp product from the PCR, sequenced or inferred from its size to carry a Type 1 Class 2 integron structure and a fourth group: the largest of the four which gave a 2300 bp product, sequenced or inferred to carry a Type 2 Class 2 integron structure (Table 6.5 and Figs. 6.2 a-c).

There were 10 isolates which fitted into the first group of Class 2 integrons characterised in this study, with no apparent link between the isolates. A fewer number of isolates, 8, were designated Group 2 Class 2 integrons. Isolate C141 (*Acinetobacter* sp.) belonged to this latter set also encoding the three known *sul* genes.

Of the isolates collected in this study; 5 were identified to carry a Type 1 Class 2 integron (Group 3) which encoded a *sat1* or *sat 2* gene, an *aadA1* gene and an *orfX* (Fig. 6.3a). Two of these integrons were inferred from size, but three were fully sequenced, those from isolates PGS20 (*P. ikaite*), C15 (*A. lwoffi*) and PGS21 (*A. viridians*) (complete and annotated sequences in App. A6-A8). The fragments from PGS20 and C15 displayed 100 % similarity to each other and gave 99 % similarity to the Class 2 integron on pIP100 from *E .coli* (GenBank accession no. AY183453), whereas the fragment from PGS21 displayed a lower similarity of 96 % to the fragments of PGS20 and C15, with 98 % identity to the Class 2 integron from *E. coli* ABR881 (GenBank accession no. DQ286458) differing in the *aadA1* sequence (App. A9) (Barlow et al., 2004; Biskri and Mazel, 2003). Both isolates PGS20 and C15 also encoded the three known *sul* genes.

The Type 2 Class 2 integrons (Group 4) identified in this study encoded either a *dhfr1* or a *dfrA1* gene, a *sat1* or in one case a *sat2* gene, an *aadA1* gene and an *orfX* (Fig. 6.3b). This was the largest group of characterised integrons in this study, with 29 isolates producing a 2300 bp product with the Int2VarF/Int2VarR primer set. Group 4 encompassed a diverse range of bacterial genera from *Pseudomonas* spp. and *Enterobacteriacae* to *Bacillus* spp. with no apparent other correlation such as the same *sul* genes. Of the 29 isolates in Group 4, only 9 Class 2 integron PCR fragments were sequenced, the remaining 20 were inferred from their size due to the extent of sequencing necessary (complete annotated sequences in App. A10-A18). A sequence alignment of the 9 sequence fragments was performed, with similarity ranging from between 100 % and 95 % between isolates (App. A19). Those Class 2 integrons which differed from the archetypal Type 2 were PGS49 (*Acinetobacter* sp. An9) which

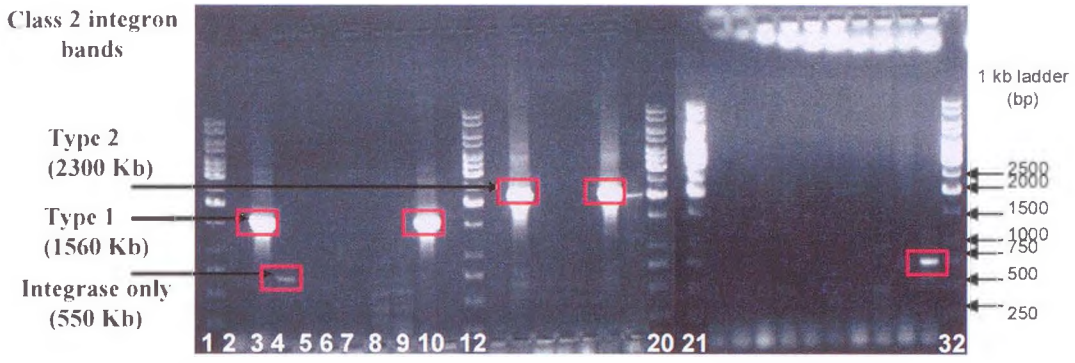
contained a GAT nucleotide insertion (which would encode for an aspartic acid residue) in the *dhfr1* gene, also found in a number of other isolates including C402 (*P. jessenii*) (App. A9 and A19). PGS49 displayed 98 % similarity to the Class 2 integron from *Proteus mirabilis* (GenBank accession no. AY736324) (Kim et al., 2005).

Isolates PGS63 (*Psychrobacter* sp. AI-2), PGS65 (*P. maritimus*), C519 (*B. pumilus*), C317 (Uncultured bacteria) and C506 (*S. flexneri*) encoded the *dfrA1* gene where the *dhfr1* gene is commonly inserted and isolate C317 encoded *sat2*, replacing a *sat1* gene (Fig. 6.3 and App. A16). Isolate C317, an *A. baumanni* strain displayed 97 % similarity to the Class 2 integron on a Tn*7* from *A. baumanni* AB300 (GenBank accession no. DQ176451) aligning with 98 % similarity to the sequence fragment from isolate PGS49, differing only in the *sat2* gene (Ramirez et al., 2005a).

No *sul* genes were found on any of the sequenced Class 2 integrons found in this study and no new genes or altered structures were recorded.

Fig. 6.2 PCR gels for primer set Int2VarF/VarR to characterise the Class 2 integron variable regions from the *intI2* gene positive isolates.

(a)

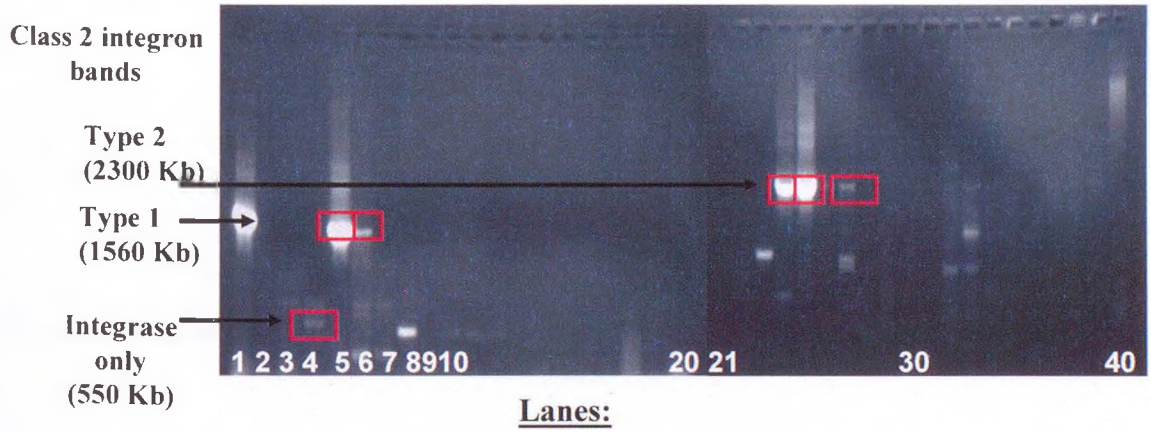


Lanes:

1. 1 Kb ladder	9. C333	17. PGS62	25. C4
2. –ve DNA control	10. PGS21*	18. PGS63*	26. C5
3. PGS20*	11. PGS23	19. PGS65*	27. C22
4. C141*	12. 1 Kb ladder	20. 1 Kb ladder	28. C121
5. C305	13. PGS48	21. 1 Kb ladder	29. C122
6. C15	14. PGS49*	22. PGS66	30. C154*
7. C105	15. PGS50	23. C1	31. C131*
8. C167	16. PGS5	24. C3	32. 1 Kb ladder

*** denotes PCR bands sequenced.**

(b)



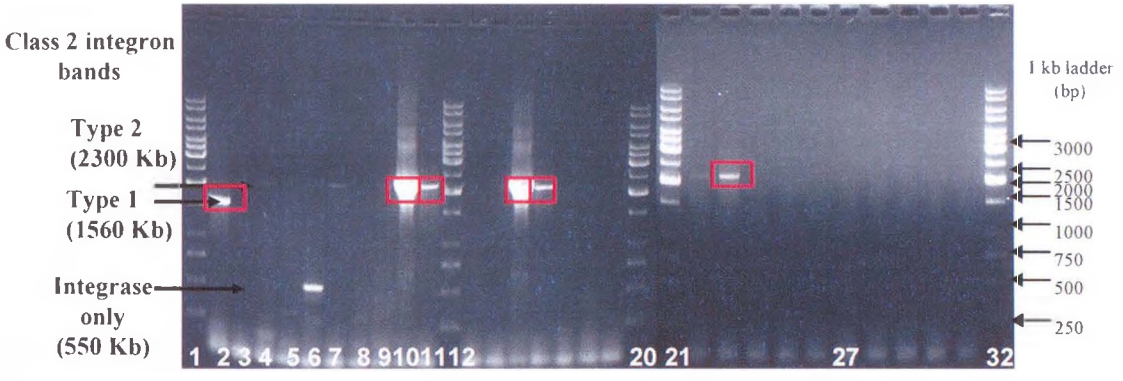
Lanes:

1. PG20*	11. C231	21. C426	31. C420
2. No DNA control	12. C257	22. C286	32. C422
3. PGS61*	13. C323	23. C514 #	33. C423

4. C205	14. C339	24. C317*	34. C431
5. C236*	15. C340	25. C402*	35. C432
6. C237*	16. C342	26. C409	36. C433
7. C291	17. C348	27. C410*	37. C435
8. C241	18. C372	28. C411	38. C436
9. C212	19. C377	29. C418	39. C437
10. C230	20. C388	30. C419	40. C438

*** bands sequenced; # band sequenced and identified as *ureC* gene, non-specific amplification**

(c)



Lanes:

1. 1 Kb ladder	9. C471*	17. C508	25. C524
2. PGS20*	10. C472*	18. C509	26. C531
3. No DNA control	11. C485	19. C511	27. C551
4. C439	12. 1 Kb ladder	20. 1 Kb ladder	28. C552
5. C440	13. C490	21. 1 Kb ladder	29. C565
6. C454	14. C491	22. PGS6	30. C582
7. C456	15. C506*	23. C519*	31. C583
8. C457	16. C507*	24. C521	32. 1 Kb ladder

*** denotes bands sequenced.**

Table 6.5 Characterisation of the *intI2* positive isolates Class 2 integrons, whether a PCR product was obtained from the Int2VarF/Int2VarR primer set, the band size and integron type according to sequencing data and band size.

ISOLATE NO.	SLURRY / SOIL SAMPLE	16S rRNA identification	Band from Int2Var primers	Band size (Kb)	Type of Class 2 integron	Similarity to seq. in databases	Similarity to PGS20(T1) PGS49(T2)
PGS21	PS(CR)	*Aerococcus viridians*	+	1560	T1	98 % 2	96 %
PGS49	PS(CR)	*Acinetobacter sp. An9*	+	2300	T2	98 % 3	-
PGS63	PS(CR)	*Psychrobacter sp. A1-2*	+	2300	T2	97 % 3	98 %
PGS65	PS(CR)	*Psychrobacter maritimus*	+	2300	T2	96 % 3	97 %
C3	PS(CR)	*Acinetobacter lwoffi*	-	-	Integrase only	-	-
C4	PS(CR)	*Acinetobacter lwoffi*	-	-	Integrase only	-	-
C5	PS(CR)	*Pseudomonas borealis*	-	-	Integrase only	-	-
C15	PS(CR)	*Acinetobacter lwoffi*	-	-	T1	99 % 1	100 %
C519	PS(CR)	*Bacillus pumilus*	+	2300	T2	96 % 2	95 %
PSG6	1,P	*Pseudomonas lini*	+	2300	T2	inferred	-
PGS20	1,P	*Psychrobacter ikaite*	+	1560	T1	99 % 1	-
C205	1,P	*Pseudomonas fluorescens*	+	550	Integrase/orfX	-	-
C236	1,P	*Psychrobacter sp. A1-2*	+	1560	T1	inferred	-
C237	1,P	*Bacillus sphaericus*	+	1560	T1	inferred	-
C241	1,P	*Pseudomonas fluorescens*	+	550	Integrase/orfX	-	-
C409	1,1	*Pseudomonas fluorescens*	+	2300	T2	inferred	-
C410	1,1	*Psychrobacter ikaite*	+	2300	T2	97 % 4	98 %
C411	1,1	*Bacillus pumilius*	-	-	Integrase only	-	-
C230	1,21	*Pseudomonas putida*	+	550	Integrase/orfX	-	-
C231	1,21	*Pseudomonas putida*	+	550	Integrase/orfX	-	-
C305	1,90	*Pseudomonas fluorescens*	-	-	Integrase only	-	-
C454	1,90	*Psychrobacter LB13*	+	2300	T2	inferred	-
C456	1,90	*Acinetobacter sp. RUH1139*	+	2300	T2	inferred	-
C457	1,90	*Acinetobacter N2*	+	2300	T2	inferred	-
C402	2,1	*Pseudomonas jessenii*	+	2300	T2	97 % 4	98 %
C471	2,1	*Pseudomonas putida*	+	2300	T2	inferred	-
C472	2,1	*Shigella sonnei*	+	2300	T2	99 % 5	98 %
C485	2,1	*Streptomyces verne*	+	2300	T2	inferred	-
C141	2,21	*Acinetobacter lwoffi*	+	550	Integrase/orfX	-	-
C154	2,21	*Acinetobacter N2*	+	550	Integrase/orfX	-	-
C317	2,21	*Uncultured bacteria EBSCPSA-6117*	+	2300	T2	97 % 6	98 %
C418	2,21	*Acinetobacter N2*	-	-	Integrase only	-	-
C419	2,21	*Pseudomonas sp. S1402*	-	-	Integrase only	-	-
C420	2,21	*Arthrobacter protophormiae*	-	-	Integrase only	-	-
C422	2,21	*S. sp.CL3B1*	+	2300	T2	inferred	-

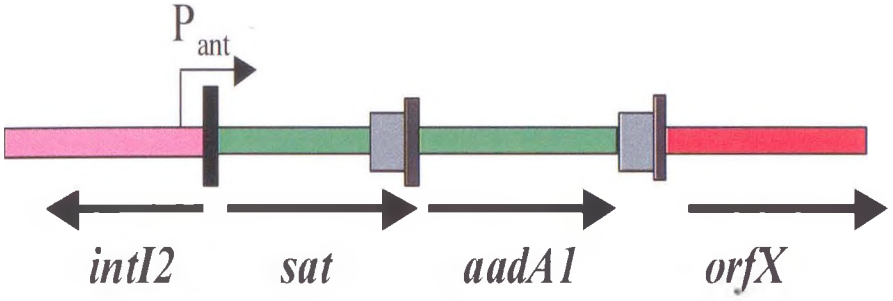
C423	2,21	*Pseudomonas jessenii*	+	2300	T2	inferred	-
C436	2,90	*Bacillus sp. NIPIII.O9094 B1*	+	2300	T2	inferred	-
C437	2,90	*Bacillus subtilis*	+	2300	T2	inferred	-
C438	2,90	*Bacillus weihenstephanesis*	+	550	Integrase/orfX	-	-
C439	2,90	*Acinetobacter N2*	+	2300	T2	inferred	-
C440	2,90	*Bacillus weihenstephanesis*	+	2300	T2	inferred	-
C121	2,240	*Pseudomonas putida*	-	-	?	-	-
C131	2,240	*Pseudomonas syringae*	+	550	Integrase/orfX	-	-
C431	2,240	*Acinetobacter lwoffi*	+	2300	T2	inferred	-
C432	2,240	*Acinetobacter lwoffi*	+	2300	T2	inferred	-
C433	2,2,40	*Acinetobacter lwoffi*	-	-	Integrase only	-	-
C435	2,240	*Acinetobacter lwoffi*	+	2300	T2	inferred	-
C506	DF	*Shigella flexneri*	+	2300	T2	98 % 7	98 %
C507	DF	*Enterococcus casseliflavus*	+	2300	T2	inferred	-
C508	DF	*Bacillus silvestris*	+	2300	T2	inferred	-
C509	DF	*Acinetobacter lwoffi*	+	2300	T2	inferred	-
C511	DF	*Acinetobacter lwoffi*	+	2300	T2	inferred	-

Where; the presence of the *intI2* gene only was designated integrase only; *intI2* and *orfX* 59 bp element but no cassette was designated integrase/orfX; Type1 Class 2 integrons contain *intI2*, *sat*, *aadA1* and *orfX*; Type 2 Class 2 integrons contain *intI2*, *dhfr1*, *sat*, *aadA1* and *orfX*.

Similarity to Class 2 integron on 1: pIP1100 from *E.coli* (GenBank accession no. AY183453) (Biskri and Mazel, 2003), 2: *E. coli* ABR881 (GenBank accession no. DQ286458) (Barlow et al., 2004), 3: *P. mirabilis* (GenBank accession no. AY736324) (Kim et al., 2005), 4: *Morganella morganii* (GenBank accession no. AJ938161) (Power et al., 2005), 5: chromosomal insertion sequence IS911 on *S. sonnei* (GenBank accession no. AY639870) (Ahmed et al., 2005), 6: Tn*7* in *A. baumanni* (GenBank accession no. DQ176451) (Ramirez et al., 2005a), 7: *S. flexneri* (GenBank accession no. AB234886) (Ahmed et al., 2005).

Fig. 6.3 Diagrammatic representation of the Class 2 integron fragments from a number of *intI2* positive isolates.

Type 1



This Class 2 integron structure was Tn*7* derived encoding; *intI2*, an *sat1* gene cassette for streptothricin resistance (streptothricin acetyltransferase), an *aadA1* gene cassette and an *orfX* which encodes a gene of unknown function. Grey boxes represent the *attc* sites which include the 59 bp regions and *attI* sites are indicated by black rectangles; pink rectangles represent integrase genes; red the integron backbone genes and green represents gene cassettes.

Type 2

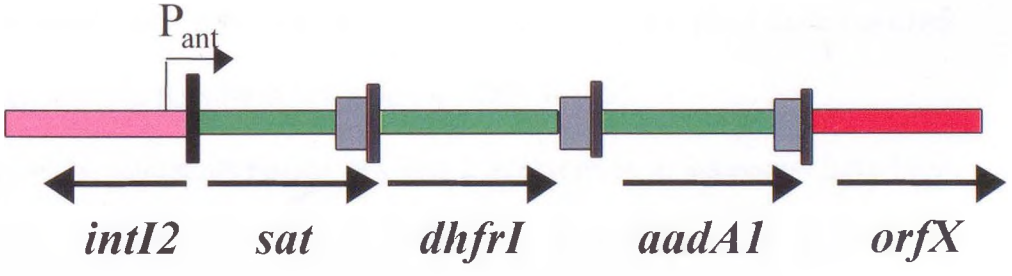


Figure legend as for Type 1, but this integron structure has the additional insertion of a *dhfrI* or *dfr1* gene cassette.

6.4 DISCUSSION

6.4.1 16S rRNA identification of *intI2* positive bacteria isolates

To date, there were 21 Class 2 integron sequences and 19 *intI2* sequences in the public databases spanning 12 separate genera. There has only been, for example, one database submission for Class 2 integrons in *B. cenocepacia* (Ramirez et al., 2005b), *Raoutella terrigena* (GenBank accession no. DQ275532), *E. cloacae* (GenBank accession no. DQ268533) and *M. morganii* (GenBank accession no. AJ938161), but a number of reports have been made for *A. baumannii* (Gonzalez et al., 1998; Ramirez et al., 2005a), *E. coli* (Roe et al., 2003b), *S. sonnei* and *S. flexneri* (McIver et al., 2002), as well as for *S. enterica* (Miko et al., 2003) and *S. typhimurium* (Ahmed et al., 2005). The majority of reports into Class 2 integrons have investigated members of the *Enterobacteriacae* and *Acinetobacter* (Gonzalez et al., 1998; Ploy et al., 2000; Seward, 1999) which may account for the first characterisations of Class 2 integrons in the genera of *Psychrobacter*, *Aerococcus*, *Bacillus, Streptomyces* and *Enterococcus* in this study. Apart from the Class 2 integron presented in this study, only one other has been reported for Pseudomonads, encoding *dfrA1*, *sat2*, *aadA1* and the *ybeA* gene cassettes on a Tn*7* in *P. aeruginosa* (GenBank accession no. DQ176869).

As stated previously, numerous reports of Class 2 integrons in *A. baumanni* have been published. One *Acinetobacter* Class 2 integron was characterised with a novel re-arrangement of gene cassettes (*sat2-aadB- dfrA1-catB2-dfrA1-sat2-aadA1*) and another identified as a hybrid Class 1/2 integron encoding *dfrA1*, *sat*, *aadA1*, *qacEΔ1* and *sulI* gene cassettes (Ploy et al., 2000; Ramirez et al., 2005a). Class 2 integrons have been recorded in world-wide strains of *A. baumanni*, one report observed the highest prevalence in the *A. baumanni* biotype which was most frequent (biotype 9) and no

integrons in scarce biotypes (Gonzalez et al., 1998; Seward, 1999). Gonzalez et al., (1998) also characterised the *intI1* and *intI2* genes together in the same isolates in 52.6 % of biotype 9 isolates, no gene cassette characterisation was performed in this investigation.

Numerous reports have also investigated the prevalence and genetic content of Class 2 integrons in *Shigella* spp. and a number of the *intI2* isolates in this study were identified as either *S. sonnei* or *S. flexneri* (Gassama-Sow et al., 2006; Peirano et al., 2005). These bacteria are commonly the causative agent of gastroenteritis and therefore not soil dwelling bacteria, the culturing of these strains from the soils after the application of slurry (year 2, day 1) and in the drain-flow samples suggests their survival for at least a short period in the environment and thus the opportunity for the uptake and transfer of antibiotic resistance gene cassettes to their integrons, which have been suggested as the causative agents for treatment failure of Shigellosis in a number of studies (McIver et al., 2002; Oh et al., 2003). *E. casseliflavus* was also identified as a surviving pathogen from the drain-flow samples. The presence of these surviving enterics may either have been due to the active wash-off from the soil environment and active transferring gene pool or it may have been present in the river water from an upstream source. Interestingly, the only indigenous soil bacteria to be isolated from the drain-flow samples were those belonging to the *Bacillus* genus, questioning whether *Bacillus* have poorer mechanisms of soil attachment than other indigenous soil bacteria which have mechanisms such as cell surface hydrophobicity as displayed by *P. fluorescens* (Jackson and Breitenbeck, 2000; Jana et al., 2000). The lack of run-off with indigenous bacteria may also have been due to the high clay content of the soil, studies have reported that the higher the clay content of the soil, the higher the sorption of the bacteria to the soil,

although this may have, to a small degree, been countered by the presence of manure in the soil (Guber et al., 2005; Hagedorn et al., 1978). Guber et al., (2005) studied the effects of manure on *E. coli* attachment to soil and observed a linear decrease in attachment with an increase in manure content.

The results stated that bacterial isolates positive for *intI2* were isolated from all soil time points except the control and organic, this maybe due to a lack of antibiotic selection for bacteria with integrons or antibiotic resistance genes, the presence of such in their genomes carrying too high a fitness cost in an relatively antibiotic free environment (Andersson, 2003). The same reason may explain the lack of *intI2* positive isolates in the organic slurries, although in contrast, persistence of such resistance or maintenance of additional genetic elements in the absence or decay of antibiotics in the soils has been observed in a number of other studies, compensatory evolution has been reported to ameliorate the cost of resistance by the occurrence of cost-free resistances and genetic linkage between non-selected and selected resistances to stabilize and maintain the resistant bacteria (Andersson, 2003; Normark and Normark, 2002).

6.4.2 Is there a link between oxytetracycline resistance and the *intI2* gene?

In the results it was displayed that, a number of the *intI2* positive isolates also encoded tetracycline resistance genes. These genes were not located on the Class 2 integron sequences identified in this study and no correlation was observed between the OTC MIC and presence of tetracycline resistance genes. No published link to date has been reported between Class 2 integrons and tetracycline resistance, and no tetracycline genes have been reported as cassettes on Class 2 integrons. Tetracycline resistance genes have been characterised in isolates with Class 2 integrons but were thought to be either integrated on the chromosome or on conjugative plasmids (Toro et al., 2005).

OTC resistance genes, *otr*(A), *otr*(B) and *otr*(C) have been reported in OTC producers, *S. rimosus* (Chopra and Roberts, 2001), in non-producers (Nikolakopoulou et al., 2005) and in *Mycobacterium* (Chopra and Roberts, 2001) but not in Gram negative bacteria, therefore these genes were not screened for in this study.

The *tet(M)* gene, which encodes for ribosomal protection from tetracyclines, was identified in isolate C507, *E. casseliflavus*, as discussed in Chapter 5, and has been reported previously in 21 bacterial genera including *Acinetobacter* and Pseudomonades (Roberts, 2005). Roberts (2005) pointed out in a recent update of tetracycline resistance genes that previously, multiple tetracycline resistant genes were commonly observed only in Gram positive bacteria but this trend had shifted to the Gram negative bacteria over a 4 year period of monitoring between 2001 and 2005. The *tet(M)* gene has been reported previously in the environment; Kim et al., (2004) identified the gene in tetracycline resistant isolates from fish and seawater at coastal aquaculture sites in Japan and Korea. Agerso et al., (2004) located the *tet(M)* gene in soils from Danish farmland which had been treated with pig slurry. Although tetracycline resistance has been investigated numerous times in different environments, OTC reports are fewer, centring on aquaculture which uses this antibiotic as a prophylactic (Esiobu et al., 2002; Huys et al., 2000; Nikolakopoulou et al., 2005; Sengelov et al., 2003). Huys et al. (2000) reported that from an investigation into aquatic environments, including hospital effluents and fish farms; 27 % of their OTC resistant isolates were *Acinetobacter* spp.

The OTC MIC did not vary between isolates and the MIC's were not comparable against commercial breakpoints because these are not available for isolates such as those belonging to the genera *Acinetobacter*, *Pseudomonas* or *Psychrobacter* as tetracycline is now only used for the treatment of Gram positive bacterial human

diseases such as *Streptococcus* and *Staphylococcus* infections, *Neisseria*, *Mycoplasma* and *H. influenza* or OTC for the treatment of livestock and fish (BSAC, 2005).

6.4.3 Frequencies of sulphonamide resistance genes in the *intI2* positive isolates and sulphachloropyridazine MIC values

The results observed in this study recorded that a higher number of *intI2* positive isolates carrying the *sulI* gene, which correlates with the linkage data presented in Chapter 4 in which a physical link was predicted between *intI2* and *sulI*. No further physical evidence for this link has been established either in this study or in the literature to date, and investigation of this link by further work may reveal the two genes on the same plasmid or transposable element, allowing transfer of this gene to a broad range of bacterial genera as is also observed in this study.

No correlation was observed between the bacterial species and *sul* gene carriage although a single gene was more frequently carried in a Pseudomonad strains than other bacterial species characterised.

6.4.4 Integron annotation and characterisation

As with studies into Class 1 integrons, there are few investigations which focus on Class 2 integrons in the environment, commonly they focus on specific bacterial species and not the integrons themselves. From the results into the variable regions of Class 2 integrons observed in this study, these genetic elements encoded within the *intI2* positive isolates were divided into four groups. These four groups have been reported separately in a number of studies into Class 2 integrons; Group 3 (Type 1) in Gram negative bacteria from the environment (Tschäpe et al., 1984), *E. coli* from meat and meat products in Norway as part of the Norm-Vet programme (Sunde, 2005) and Group

4 (Type 2) in *P. mirabilis* from U.S. meat (Kim et al., 2005). In an examination of *S. sonnei* isolates from adult patients with diarrhoea in Dakar, India by Gassama-Sow et al., (2006), 3 out of the 4 groups were recorded, 57.5 % of isolates contained the Group 3 (Type 1) Class 2 integron found in this study, where a total of 93 % of their isolates collected encoded Class 2 integrons.

The empty Class 2 integrons of Groups 1 and 2 maybe widespread in the bacterial community but reports either focus on the presence of the *intI2* gene, negating integron characterisation or they focus on the characterisation of full integrons (Gonzalez et al., 1998; McIver et al., 2002; Roe et al., 2003a; Seward, 1999). Probes are available which could potentially hybridise to Class 2 integrons containing the *sat1-3* genes, but these would also not detect empty integrons (Smalla and van Elsas, 1995; Tietze and Golubev, 1990). In a further investigation conducted by N. Abdouslam, a large number of empty integrons were also characterised in a QAC-contaminated reed bed (per. comm.), these were Class 1 integrons encoding the *intI1* and *qacE* genes. The presence of an empty Class 2 integron maybe due to the encoding of a truncated *intI2* gene, an internal stop codon (TAA), potentially preventing integration of gene cassettes into the Class 2 integrons via its attI/C sites (Simonsen et al., 1983). This prevention of integration maybe overcome by the activity of other integrases (Hansson et al., 2002). In this study, the presence of a Class 1 integron with a *intI1* integrase has not allowed increased integration of gene cassettes into Class 2 integrons; isolates C230 and C231 (*P. putida*) were characterised as having empty integrons with the 59 bp element of *orfX* and the *intI2* gene whereas isolates C519 (*B. pumilus*), C419 (*Pseudomonas sp.*), C506 (*S. flexneri*), C507 (*E. casseliflavus*) and C509 (*A. lwoffi*) all encode 3 gene cassettes of the Type 2 variety and the *intI1* gene. The presence of other integrases in close

proximity to the cell maybe sufficient for aiding integration although a further 24 isolates contained Type 2 Class 2 integron cassette structures and 5 with the Type 1 cassette structures but with no *intI1* gene transcribed in the cell. There may also be a second integrase in these isolates that has not been screened for in this study, such as *intI3*, although the presence of a Class 3 integron is unlikely due to its low prevalence in the environment but it has been reported in a *Pseudomonas* sp. (Senda et al., 1996). The presence of unclassified or unknown integrase proteins can also not be ruled out as Nield et al., (2001) reported the sequencing of numerous new integrases from the environment.

The Type 1 Class 2 integron sequences (Group 3) identified in this study varied in only 4 % from each other and matched with high similarity to those Class 2 integron sequences already in the public databases, differing only in the *aadA1* gene sequence homology (Altschul et al., 1990; Barlow et al., 2004; Biskri and Mazel, 2003). These results identify Class 2 integrons in 2 new bacteria genera; *Psychrobacter* and *Aerococcus*, the antibiotic genes also being unique in the former genus. Isolates PGS20 and C15 which were identified to encode all three known *sul* genes will be characterised and discussed in more detail in Chapter 7.

The sequencing of Type 2 Class 2 structures allowed the identification of the first Class 2 integrons in the genera of *Enterococcus* and *Bacillus*. The Type 2 Class 2 integron sequences (Group 4) identified in this study varied by 5 % from each other, also having high homology with those Class 2 sequences already in the public databases although with a number of differences observed in the genes and gene sequences (Altschul et al., 1990). The *dhfr1* gene was replaced in a number of integrons by a *dfrA1* gene (isolates PGS49 and C506). Both 'dhfr' and 'dfr' are interchangeable

nomenclature for genes encoding alterative dihydrofolate reductases (DHFR) which confers trimethoprim resistance (Sköld, 2001). A second substitution for genes in the integron structures occurred in isolate C317, *sat1* was replaced by *sat2*. Both genes encode for streptothricin resistance and have 95 % sequence similarity to one another. *sat2* was identified in a novel Class 2 integron encoded in *A. baumanni* (Ramirez et al., 2005a).

The GAT nucleotide insertion of isolate PGS63 (*Psychrobacter sp.)* may have been a sequencing error in the original sequence used for comparison as it is observed in a number of sequences in this study (Kim et al., 2005). The nucleotides would encode for an aspartic acid residue, leaving the protein in-frame but possibly within an altered DHFR protein structure. This acidic amino acid is known to play important roles in enzyme active centres, as well as in maintaining the solubility and ionic character of proteins so the GAT codon may not be an insertion but a sequencing error originally.

The diversity of gene cassettes and cassette structures was low in this study, this may have been due to the truncated IntI2 allowing less variation than the IntI1 integrase although it does not seem to have effected its distribution throughout the bacterial domains, possibly due to the action of other integrase protein, as observed in this and other studies (Collis et al., 2002b; Hansson et al., 2002). Another reason for the low diversity in Class 2 integron structure maybe its stability, its presence on transposable elements may allow for its rapid and diverse movement, while the stability and small size of its cassette number may allow maximum promotion of the gene cassette transcripts and a higher resistance for each antibiotic it provides protection from.

6.5 CONCLUSION

This study reports Class 2 integrons and the *intI2* gene in a number of Gram positive bacteria in soils and slurries perturbed with antibiotics but not in organic or control slurries or soils.

Low structural variation in the Class 2 integron sequences found in this study and others may have a pronounced effect on its success in the soil environment, especially since this study recorded its presence in 5 new bacterial genera, demonstrating stability and successful selection in new recipients. The presence of these mobile genetic elements in the soil environment and the lack of an effect due to antibiotic presence for selection provide supporting evidence for continuous HGT of these structures. The knowledge that HGT is an ongoing process in these environments and the application of slurries spiked with antibiotics may not have had as a dramatic detrimental effect as first thought, may result in the initial selection of bacteria gaining antibiotic resistance genes but not support their persistence long-term.

6.6 FURTHER WORK

As discussed in Chapter 5, further Class 2 integron characterisation in the soils unculturable bacterial population could be undertaken, again focussing particular attention on the *Bacillus* isolates may yield further information on the carriage of integrons and MD resistance in the environment. Additional studies could utilise real-time PCR, RTPCR and QRTPCR for investigations into the regulation and transcription of the *sul* and *intI2* genes. Real-time PCR has now been used effectively on environmental DNA from samples such as bovine and swine manures, composts and

swine effluent for rapid, quantitative, cultivation-independent measurements in ecological studies (Yu et al., 2005).

The question of aiding integrases for the *intI2* gene from Classes known and unknown could be addressed using PCR-based techniques both sequence-dependent and sequence-independent for the recovery of new integrase genes utilising the conserved nature of the 59 base pair element flanking the gene cassettes and the conserved region of the integrase genes (Nield et al., 2001; Stokes et al., 2001a; Stokes et al., 1997).

Further screening for tetracycline resistance genes to find a link between tetracycline resistance and the *intI2* gene could be broadened to include those genes commonly found in slurry and soil environments such as *tet(B), A, G, L, O, T*, possibly using microarray or multiplex-PCR systems which have been previously applied to screen for a number of these genes but as yet have only been used on single, often clinical isolates and not environmental samples (Call et al., 2003; Ng et al., 2001; Perreten et al., 2005).

Widening the sequencing from the Class 2 integrons to their flanking regions would allow additional information about their genetic environments and may locate *sul* genes on their potential transposon or plasmid hosts.

CHAPTER 7:

CHARACTERISATION OF ISOLATES ENCODING THE GENOTYPE *SULI*, *SULII* AND *SUL3*

Chapter 7: Characterisation of isolates encoding *sulI*, *II* and *3*

7.1 INTRODUCTION

In Chapter 4 the genotype of *sulI*, *II* and *3* was characterised in 12 (2.06 %) of isolates from this study. This genotype was first reported by Antunes et al., (2005) in sulphonamide resistant *S. enterica* strains from Portugal. This chapter reports the characterisation of these 12 isolates containing the genotype of *sulI*, *II* and *3*.

7.1.1 Multiple antibiotic resistance to the same antibiotic in the same bacterial cell

There are a number of reports which observe the occurrence of bacteria that are capable of encoding multiple genes for resistance within the same group of antibiotics, commonly, but not exclusively, with different resistant mechanisms, for example to streptomycin (Bischoff et al., 2005; Cizman, 2003; Kehrenberg et al., 2004; Werner et al., 2003). Investigations have been performed which determined whether multiple resistance genes to the same antibiotic equates to increased resistance and cell MIC. Sunde and Norström (2005) characterised *E. coli* strains from domestic animals (poultry, pork, cattle and sheep) during 2000-2003 from isolates taken for the Norway monitoring programmes of antimicrobial resistance. A number of strains were characterised which encoded, the *strA/B* genes, *aadA* gene cassettes or *strA/B* and *aadA*. The *strA* and *strB* genes confer resistance to streptomycin through the inactivation of the antibiotic by two phosphotransferase enzymes, aminoglycoside-3"-phosphotransferase and aminoglycoside-6"-phosphotransferase. Whereas, the closely related *aadA* gene cassettes confer resistance by an aminoglycoside adenyltransferase, which inactivates both streptomycin and spectinomycin (Recchia and Hall, 1997). The

MIC determinations of these strains revealed the combination of streptomycin resistance genes, *strA-B* and *aadA* conferred a higher MIC when in combination (>128 mg/l) than either the *strA-B* genes (MIC_{50} 128 mg/l) or *aadA* gene cassette (MIC_{50} 16 mg/l) singularly in a strain (Sunde and Norström, 2005). The *strA-strB* gene partnership is commonly reported, flanked by the *sulII* gene on their 5' side, on plasmids such as RSF1010, an IncQ plasmid, frequently isolated from Gram negative bacteria (Schölz et al., 1989).

7.1.2 Biological fitness costs of multiple antibiotic resistance genotypes

The encoding of plasmid-borne antibiotic resistance or chromosomal mutations, especially where there are multiple mutations or multiple genes, has been observed to incur a biological cost to the bacterial host, the cost increasing in the absence of a selective pressure (Breines et al., 1997; Friedman et al., 2001; Heisig, 1996). However, recent contradicting studies have demonstrated that host cells can evolve in such a way that ameliorates the fitness cost significantly. Lindgren et al., (2005) demonstrated that in *E. coli* strains, implicated in urinary tract infections (UTIs), an increased number of chromosomal resistance mutations to fluoroquinolones increased the levels of resistance to this antibiotic but, at the same time were associated with an increased reduction in biological fitness, (mouse UTI infection models). The same effect on fluoroquinolone resistance in *P. aeruginosa* was observed by Kugelberg et al., (2005), who recorded higher levels of resistance (<256 mg/l) in strains with double mutations in the *gyrA*, *parC*, *E* and *nfxB* genes than those strains with single mutations (4-48 mg/l).

Fitness costs resulting from the carriage of MD resistance plasmids were observed by Dahlberg and Chao, (2003) (2003)in *E. coli* strains carrying R1 (IncFII group) and RP4 (IncPα group), as well as by Lenski et al., (1994), who investigated decreased fitness in

E. coli K12 carrying a tetracycline resistance plasmid, in the presence of a selective pressure. The decrease in fitness due to the presence of a plasmid may be associated with the resistance functions disturbing bacterial growth through altered enzymes or efflux pumps or disturbances due to altered plasmid regulation either of plasmid replication or gene expression (Andersson, 2003). Due to the fitness cost borne by the host in the absence of an antibiotic pressure, the amelioration of the plasmid would be expected either on replication or via conjugation or segregation. This does not appear to be the case and plasmids are maintained despite a lack of antibiotic selection, but the cells seem to compensate in some way for the plasmids (Lenski et al., 1994).

7.1.3 Sulphonamide resistance, fitness costs and the occurrence of the genotype *sulI, II* and *3*

Only one report has investigated the biological cost of a host cell carrying a plasmid encoding sulphonamide resistance (Enne et al., 2004). The results of this investigation observed a fitness advantage when an *E. coli* strain carried the plasmid, p9123, encoding the *sulII* gene. The fitness advantage reported was in direct contrast to other studies but for other antibiotics (Dahlberg and Chao, 2003; Lenski et al., 1994).

As stated previously, the genotype of *sulI, II* and *3* was first reported by Antunes et al., (2005) in 3 % of their sulphonamide resistant *S. enterica* strains isolated from human clinical isolates, swine products and sea water collected from a beach. The 6 isolates, identified by Antunes, were also characterised as carrying Class 1 integrons encoding the *intI1*, *dfrA12*, *aadA2*, *qacEΔ1* and *sulI* genes, as well as the additional *sulII* and *sul3* genes. This is the only report currently that identifies the same isolate encoding all three known sulphonamide resistance genes and no further investigations reveal whether the presence of the three genes increased the host MIC for sulphonamides, or whether

their presence conferred a fitness cost on the host. The presence of *sulI*, *sulII* and *sul3* in a genome does not always confer resistance to an isolate and the MIC for *E. coli* susceptible to sulphonamides is <0.01mM , increasing to 2 mM (570 μg/ml) with the encoding of a *sulI* gene and >4 mM with the *sulII* or *sul3* gene (Guerra et al., 2003; Sköld, 2000, 2001).

7.2 AIMS

Isolates with the genotype containing the three known alternative DHPS enzymes conferring sulphonamide resistance were identified in Chapter 4. This genotype has only been reported once previously and no studies have characterised further these strains with respect to MIC, fitness costs or linkage between the 3 genes. The objectives were further characterisation of these isolates by;

1. Establishing whether the primers for PCR specifically identified the three different genes by sequencing and comparison/alignments of products;
2. Identifying the bacterial isolates by 16S rRNA sequencing;
3. To calculate the sulphonamide MIC in these isolates and investigated whether the MIC were affected due to the presence of the three genes and whether they conferred a fitness costs on the cells;
4. Investigate the mobility of the genes from their hosts and determine the rate of transfer into different recipients;
5. and to investigate whether the MIC values were effected in the soil environment by the presence of the three genes.

7.3 RESULTS

7.3.1 Designation of isolates containing the genotype *sulI, II* and *3*.

The majority of the 12 *sulI, II* and *3* isolates were collected from the pre-application soils, a number from the Cranfield slurry, and a number from the year 2 soils (Table 7.1). None of these isolates were collected from the control soils, control slurries or after day 90 in either year.

Of the isolates, 11 out of the 12 were selected from plates containing SCP, the remainder were from plates containing OTC.

7.3.2 *sulI, II* and *3* gene PCR product sequencing and sequence alignments

To confirm that the isolates encoded the three genes, PCRs were performed for each isolate and for each gene, all PCR band products were sequenced. The PCRs confirmed each isolate encoded the three-gene mosaic (Fig. 7.1). A non-specific lower band appeared in the *sulI* PCR and sequencing confirmed it was not a *sul* gene.

The sequence alignments determine that the *sulI*, *sulII* and *sul3* PCR fragments aligned with 99 % to 97 % similarity (App. A20).

7.3.3 16S rRNA identification of *sulI, II* and *3* isolates

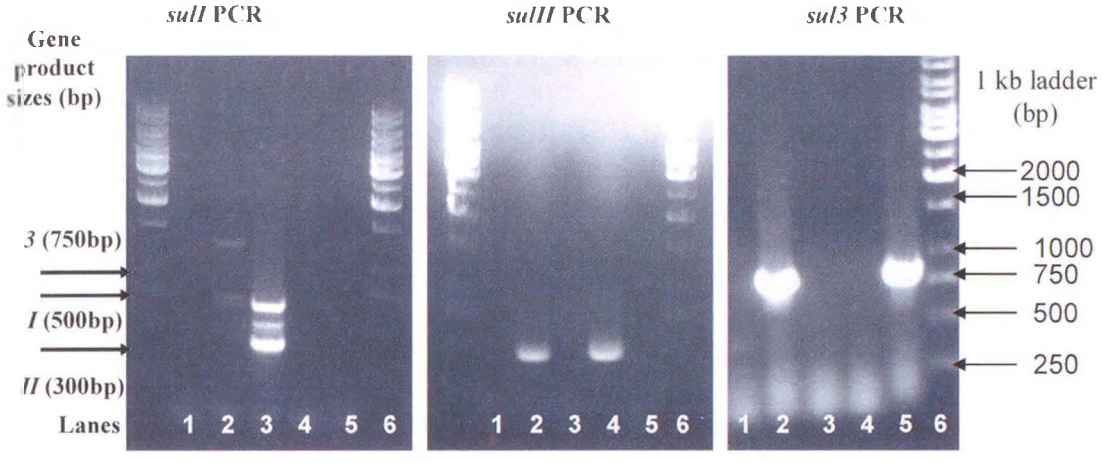
The 12 isolates are identified, by PCR for the 16S rRNA gene using the full length product from the PA/PH primers (Edwards et al., 1989), as 3 different genera; *Psychrobacter*, *Acinetobacter* and *Bacillus* (Table 7.1). These isolates have low diversity and 7 out of the 12 belong to the *Acinetobacter* genus.

Table 7.1 MIC determinations for sulphachloropyridazine and multiple resistance phenotypes of the bacterial isolates containing the genotype *sulI*, *II* and *3*.

ISOLATE	SOURCE	16S rRNA IDENTIFICATION (% similarity)	MULTIPLE RESISTANCE PROFILE	SELECTIVE PLATE (µg/ml)	SCP MIC (mg/l)
PGS16	1,P	*Psychrobacter DY9-2* (97 %)	Sm^R Tet^R Tmp^R Nal^R	SCP25	8
PGS17	1,P	*Psychrobacter frigidicola* (97 %)	Sm^R Tet^R Tmp^R Nal^R	SCP25	8
PGS20	1,P	*Psychrobacter ikaite* (97 %)	Sm^R Cm^R Tet^R Tmp^R Nal^R	SCP25	8
C15	PS	*Acinetobacter lwoffi* (99 %)	Sm^R Cm^R Tet^R Tmp^R Nal^R	SCP25	5
C20	PS	*Psychrobacter ikaite* (98 %)	Sm^R Cm^R Tet^R Tmp^R Nm^R Nal^R	SCP25	5
C35	1,P	*Acinetobacter calcoaceticus* (97 %)	Sm^R Cm^R Tet^R Tmp^R Nal^R	SCP25	8
C36	1,P	*A. calcoaceticus* (97 %)	Sm^R Cm^R Tet^R Tmp^R Nal^R	SCP25	8
C37	1,P	*A. lwoffi* (97 %)	Sm^R Cm^R Tet^R Tmp^R Nal^R	SCP25	8
C44	1,1	*A. rhizosphaerae* (98 %)	Sm^R Cm^R Tet^R Tmp^R Nm^R Nal^R	SCP25	5
C141	2,21	*Acinetobacter lwoffi* (98 %)	Sm^R Km^R Cm^R Tet^R Tmp^R Nm^R Nal^R Amp^R	OTC25	6
C328	2,21	*Bacillus psychrodurans* (98 %)	Sm^R Km^R Cm^R Tet^R Nm^R Nal^R	SCP25	8
C442	2,90	*Acinetobacter baumanni* (99 %)	Cm^R Tet^R Nal^R	SCP25	8

Percentage similarity calculated using the Blast programme to sequences in the public databases. NG: no growth of isolate, Sm^R: resistance to streptomycin 16 µg/ml, Amp^R: ampicillin 16 µg/ml, Km^R: kanamycin 16 µg/ml, Cm^R: chloramphenicol 16 µg/ml, Tet^R: tetracycline 8 µg/ml, Tmp^R: trimethoprim 16 µg/ml, Nm^R: neomycin 8 µg/ml, Nal^R: nalidixic acid 16 µg/ml. Where; PS, Cranfield slurry; 1,P, year 1, pre-application soils; 1,1, year1, day 1 soils; 2,21, year 2, day 21 soils and 2,90, year 2, day 90 soils.

Fig. 7.1 Comparison of *sulI, II* and *3* gene PCR gels displaying specific bands and sizes for each gene.



Lanes:

1. Negative blank
2. PG20 (*sulI,II* and *3*)
3. C352 (*sulI*)
4. C340 (*sulII*)
5. C100 (*sul3*)
6. 1kb DNA ladder

7.3.4 Sulphachloropyridazine MIC determinations corresponding to *sul* genotypes and multiple resistance phenotypes

SCP MIC determinations were performed on all 12 of the isolates and antibiotic resistance was tested against a further eight antibiotics (Table 7.1). The majority of isolates (8) had an MIC for SCP of 8 mg/l. This was the highest MIC observed for the *sulI, II* and *3* isolates. The lowest MIC of 5 mg/l was recorded in three isolates; *A. lwoffi* (C15), *P. ikaite* (C20) and *A. rhizosphaerae* (C44). In the case of *A. lwoffi* (C15), its' recorded MIC (5 mg/l) differed from the other isolate identified as *A. lwoffi* (C37) which had an identical antibiotic resistance profile (Table 7.1).

All isolates were resistant to tetracycline and nalidixic acid. The *Bacillus* strain (C328) had the lowest resistance, protected against tetracycline, nalidixic acid and chloramphenicol. C442 (*A. baumanni*) and PGS17 (*P. frigidicola*) displayed sensitivity to streptomycin and chloramphenicol respectively. Only four strains displayed resistance to neomycin; C20 (*Psychrobacter*), C44 (*Acinetobacter*), the *Bacillus* (C328) and C141 (*Acinetobacter*). The *P. ikaite* (C20) and *A. rhizosphaerae* (C44) differed in their phenotype from that of isolates also identified as the same species, PGS20 and C35 respectively, by their resistance to neomycin.

Additional experiments were performed on isolates, identified as *Acinetobacter* spp. from this study, which contained different combinations of the *sulI, II* and *3* genes, to determine their SCP MIC values. This investigation was to observe whether the three genes conferred different resistance levels and whether the genes' effects were cumulative in a cell when one or more were present (Table 7.2).

Table 7.2 Sulphachloropyridazine MIC values in bacterial isolates identified as *Acinetobacter* spp. containing different combinations of the *sul* genes.

SAMPLE NUMBER	*Sul* Genotype	16S rRNA IDENTIFICATION AND % SIMILARITY	SCP MIC (mg/l)
C86	-	*Acinetobacter lwoffi* (97 %)	8
PGS59	*I*	*Acinetobacter lwoffi* (99 %)	>16
C147	*II*	*Acinetobacter haemolyticus* (97 %)	16
C91	*II*	*Acinetobacter sp. RUHI1139* (98 %)	>16
C366	*3*	*Acinetobacter lwoffi* (98 %)	<2
C216	*I + II*	*Acinetobacter lwoffi* (98 %)	>16
C145	*I + 3*	*Acinetobacter lwoffi* (98 %)	>16
C49	*II + 3*	*Acinetobacter lwoffi* (98 %)	>16
C44	*I + II + 3*	*Acinetobacter lwoffi* (98 %)	8
C442	*I + II + 3*	*Acinetobacter baumanni* (99 %)	8

Percentage similarity was calculated by the Blast programme (Altschul et al., 1990).

The investigation revealed variable MIC levels for the different isolates. Lower MIC levels were recorded with the isolates carrying all three genes (C442, 8 mg/l) compared to isolates carrying just one gene (C91, *sulII*, >16 mg/l).

7.3.5 Presence of other resistance genes and integron characterisation

The isolates containing *sulI, II* and *3* were screened for the presence of the *intI1* and *intI2* genes to determine whether the isolates carried a mobile element which may have encoded the *sulI*, *sulII* or *sul3* genes (Table 7.3 and Fig. 7.2). The isolates were also additionally screened, by PCR, for the *ermC* gene; to investigate linkage between tylosin resistance and the *intI2* gene, and the *strA/B* genes to further characterise the genotypic environment of the *sulII* gene (Tables 7.3, 7.4 and Fig. 7.2).

Isolate PGS20 (*P. ikaite*) was positive by PCR for an *intI2* gene, an *ermC* gene (also identified in C15) and a *strA* gene (Table 7.3). Further characterisation of the Class 2 integron was performed and it was observed to encode *sat* and *aadA1* gene cassettes and an *orfX* gene with 99 % similarity to these genes in pIP1100 from *E. coli*, as determined in Chapter 6. Additional characterisation of MIC values for tylosin in PGS20, revealed high resistance of >128 mg/l (Table 7.4).

PCR screening also gave positive results for the *intI2* gene in isolates C15 (*A. lwoffi*) and C141 (*Acinetobacter* sp.) (Fig. 7.2). The Class 2 integrons of C15 and C141 were additionally characterised in Chapter 6, where sequencing revealed that C141 encoded the *intI2* gene but not cassette genes or an *orfX* gene at the 3' end. No *sul* genes were reported to be encoded on the Class 2 integron structures of the *sulI, II* and *3* isolates.

All isolates produced a positive PCR result for the *strA* gene. Further investigations were performed to determine whether the isolates encoded *sulII-strA-strB* linked genes. The isolates were screened using the strA-BFR, sulIF-strAR and strAF-sulIIR primer

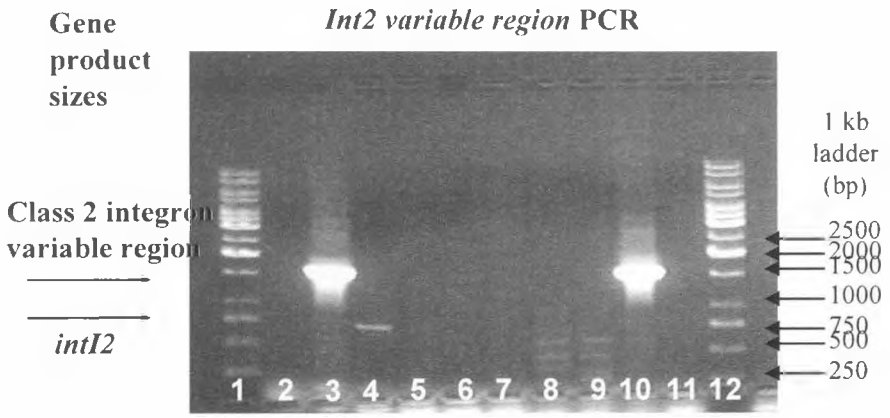
sets to determine if the genes were present and in which orientation. Table 7.3 and Fig. 7.3 identified that isolates C15, C20, C37 and C44 encoded both the *strA* and *strB* genes but only C20 (*P. ikaite*) produced a product with the sulIIF/strAR primers. The PCR results and sequencing indicated that in C20 the *sulII* gene was flanked on its 3' terminus by *strA* and possibly *strB* (with 99 % similarity to the p9123 *E. coli* plasmid, GenBank accession no. AY360321) (Figs. 7.3 and 7.4) (sequence in App. A20) (Enne et al., 2004).

Table 7.3 Bacterial isolates containing the genotype *sulI*, *II* and *3* PCR screened for other antibiotic resistance genes, gene linkages and the presence of integrons.

ISOLATE NUMBER	16S rRNA IDENTIFICATION	*intI1*	*intI2*	*ermC*	*strA*	*strA/B*	*strAF/IIR*	*IIF/strAR*
PGS16	*P. sp. DY9-2*	-	-	-	+	-	-	-
PGS17	*P. frigidicola*	-	-	-	ND	ND	ND	ND
PGS20	*P. ikaite*	-	+*	+	+	-	-	-
C15	*A. lwoffi*	-	+*	+	+	+	-	-
C20	*P. ikaite*	-	-	-	+	+	-	+*
C35	*A. calcoaceticus*	-	-	-	+	-	-	-
C36	*A. calcoaceticus*	-	-	-	ND	ND	ND	ND
C37	*A. lwoffi*	-	-	-	+	+	-	-
C44	*A. rhizosphaerae*	-	-	-	+	+	-	-
C141	*A. lwoffi*	-	+*	-	+	-	?*	-
C328	*B. psychrodurans*	-	-	-	+	-	?*	-
C442	*A. baumanni*	-	-	-	+	+	-	-

Where, ND: not done due to lack of DNA for additional characterisation. +*: positive PCR fragments were sequenced and further characterised where possible. ?*: positive band detected on PCR (Fig. 7.2) but sequencing failed.

Fig. 7.2 PCR gels depicting products for the *sulI, II* and *3* isolates for the Class 2 integron variable region.



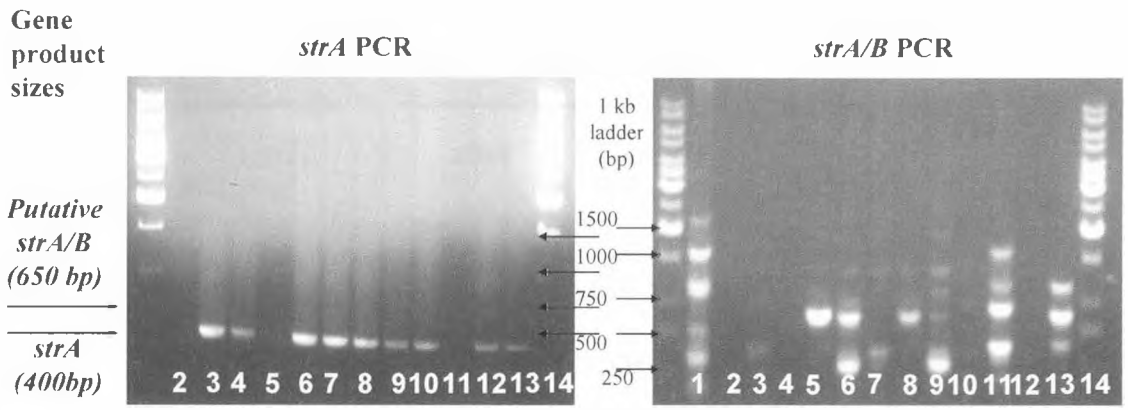
Lanes: 1. 1 Kb ladder
2. –ve DNA control
3. PGS20
4. C141
5. C328
6. C101
7. C105
8. C167
9. C333
10. C15
11. PGS23
12. 1 Kb ladder

Table 7.4 Tylosin MIC determinations for the bacterial isolates containing the genotype *sulI*, *II* and *3* and *ermC* gene.

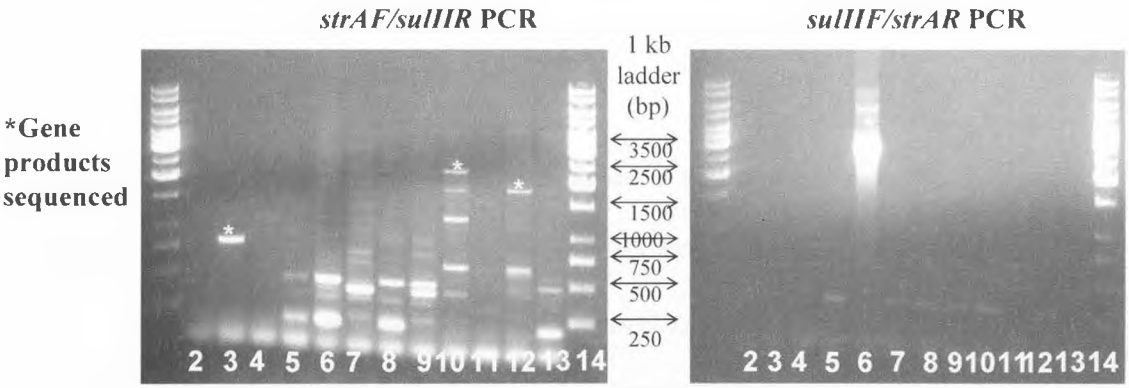
SAMPLE NUMBER	16S rRNA IDENTIFICATION	*ermC*	TY MIC (mg/l)
PG16	*P. sp. DY9-2*	-	32
PG17	*P. frigidicola*	-	32
PG20	*P. ikaite*	+	>128
C15	*A. lwoffi*	+	>128
C20	*P. ikaite*	-	32
C35	*A. calcoaceticus*	-	ND
C36	*A. calcoaceticus*	-	64
C37	*A. lwoffi*	-	128
C44	*A. rhizosphaerae*	-	128
C141	*A. lwoffi*	-	>128
C328	*B. psychrodurans*	-	ND
C442	*A. baumanni*	-	ND

Fig. 7.3 PCR products of the *sulI, II* and *3* isolates for (a) *strA* gene and *strA/B* genes and (b) strAF/sulIIR and sulIIF/strAR primers sets.

(a)



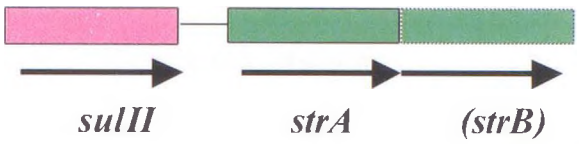
(b)



Lanes

1. +ve DNA control (p9123 Enne et al., 2004)
2. –ve No DNA control
3. PGS16
4. PGS20
5. C15
6. C20
7. C35
8. C37
9. C44
10. C141
11. C285 (-ve DNA control)
12. C328
13. C442
14. 1 Kb ladder

Fig. 7.4 Schematic of the PCR fragment from isolate C20 (*Psychrobacter ikaite*) which encoded the *sulII* and *strA* genes.



(inferred from PCR)

The *sulII* gene encodes for sulphonamide (an alternative DHPS) and *strA* gene encodes for streptomycin resistance (streptomycin phosphotransferase) (Annotated sequence in App. A21). The PCR fragment was not localised.

7.3.6 Conjugal transfers of the three *sul* genes from the host isolates encoding all three *sul* genes

Conjugal matings were performed to investigate if and at what rate the *sulI*, *sulII* or *sul3* genes would transfer from their bacterial hosts into *P. putida* UWC1 or *E. coli* K12 recipients (Table 7.5). In all cases where the genes transferred into *P. putida*, transfer was observed into *E. coli* but at a lower rate. In the majority of strains, the *sulI* and *sulII* genes transferred separately at different rates, with the exception of isolate C141, an *Acinetobacter* sp., from which both genes transferred at a rate of 3.44×10^{-3} into *P. putida* and 4.22×10^{-4} into the *E. coli* recipients. The *sulI* and *sulII* genes failed to transfer from isolates PGS17, C20, C37 and C328 into either *P. putida* or *E .coli* recipients. The *sul3* gene did not transfer into the any of the recipients used in this study.

Table 7.5 Conjugal transfer rates of the *sulI*, *sulII* and *sul3* genes from *sul* mosaic containing bacterial host strains into a *P. putida* UWC1 or *E. coli* K12 recipients.

Recipient + Host Isolate Mating	Transfer rates (transconjugants per donor cell)		
	sulI	***sulII***	***sul3***
P. putida + PGS16 (*Psychrobacter* sp. DY9-2)	7.28×10^{-3}	0	0
E. coli + PGS16 (*Psychrobacter* sp. DY9-2)	4.3×10^{-4}	0	0
P. putida + PGS17 (*P. frigidicola*)	0	0	0
E. coli + PGS17(*P. frigidicola*)	0	0	0
P. putida + PGS20 (*P. ikaite*)	1.89×10^{-3}	0	0
E. coli + PGS20(*P. ikaite*)	6.45×10^{-5}	0	0
P. putida + C15 (*A. lwoffi*)	7.47×10^{-3}	2.49×10^{-3}	0
E. coli + C15 (*A. lwoffi*)	2.64×10^{-4}	7.24×10^{-5}	0
P. putida + C20 (*P. ikaite*)	0	0	0
E. coli + C20 (*P. ikaite*)	0	0	0
P. putida + C35 (*A. calcoaceticus*)	1.07×10^{-3}	1.88×10^{-3}	0
E. coli + C35 (*A. calcoaceticus*)	9.48×10^{-4}	7.47×10^{-4}	0
P. putida + C37 (*A. lwoffi*)	0	0	0
E. coli + C37 (*A. lwoffi*)	0	0	0
P. putida + C44 (*A. rhizosphaerae*)	2.37×10^{-2}	7.1×10^{-2}	0
E. coli + C44 (*A. rhizosphaerae*)	9.02×10^{-4}	2.48×10^{-4}	0
P. putida + C141 (*A. lwoffi*)	3.43×10^{-3}	3.43×10^{-3}	0
E. coli + C141 (*A. lwoffi*)	4.22×10^{-4}	4.22×10^{-4}	0
P. putida + C328 (*B. psychrodurans*)	0	0	0
E. coli + C328 (*B. psychrodurans*)	0	0	0
P. putida + C442 (*A. baumanni*)	1.7×10^{-3}	0	0
E. coli + C442 (*A. baumanni*)	3.45×10^{-4}	0	0

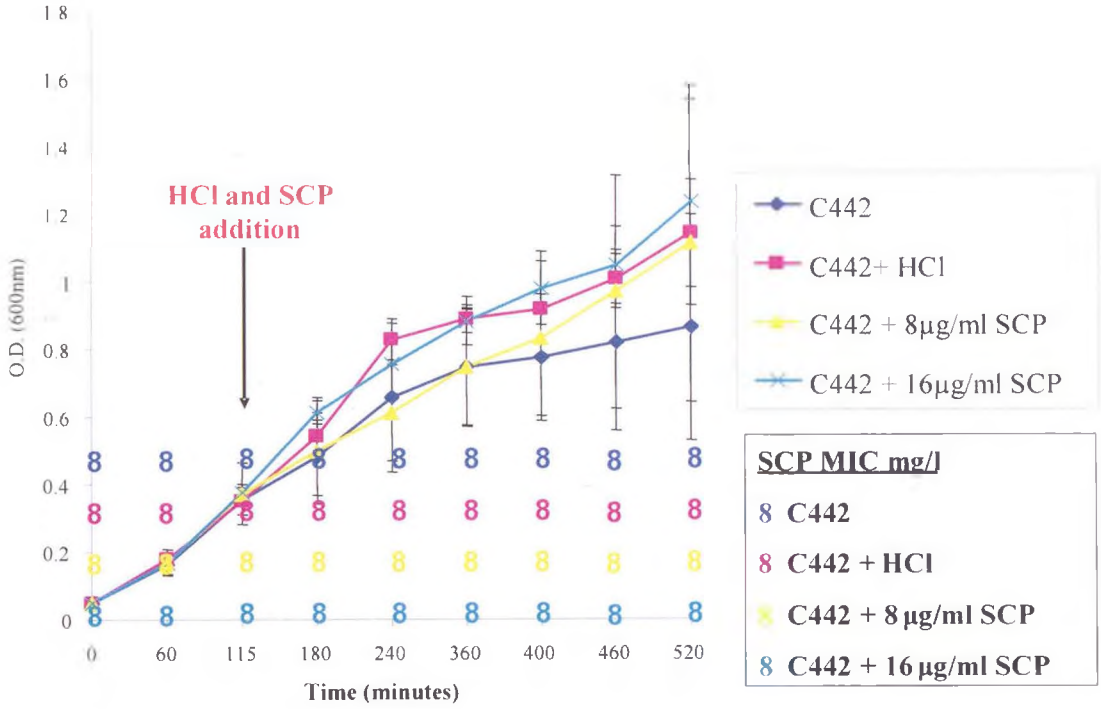
7.3.7 MIC phenomenon

SCP MIC determinations for the *sulI, II* and *sul3* isolates revealed, that in all but one isolate, the MIC was lower than the original concentrations of SCP used for isolate collection. For example, isolate C442 (*A. baumanni*) was collected from a plate containing 50 μg/ml SCP but its SCP MIC was calculated to be 8 mg/l (Table 7.1).

To investigate sulphonamide resistance induction, growth curves were performed for C442 with and without the addition of SCP and no difference was observed in the SCP MIC when tested at each time point (Fig. 7.5).

In an attempt to characterise this MIC phenomena, the effect of passage through soils on the SCP resistance for isolate C442 was investigated using microcosms. The results indicated that after 4 weeks in soils and in the presence of SCP, an elevated SCP MIC for C442 and altered resistance patterns for tetracycline and tylosin were observed (Fig. 7.6 and Table 7.6). Tylosin resistance decreased with passage through soils (from growth at 32 mg/l to no growth at 8 mg/l) and tetracycline resistance was increased with passage through soils to growth at 32 mg/l.

Fig. 7.5 Growth curves (600nm) for C442 (*Acinetobacter baumanni*), with and without sulphachloropyridazine additions to liquid growth media, investigating sulphachloropyridazine resistance induction and MIC determinations .



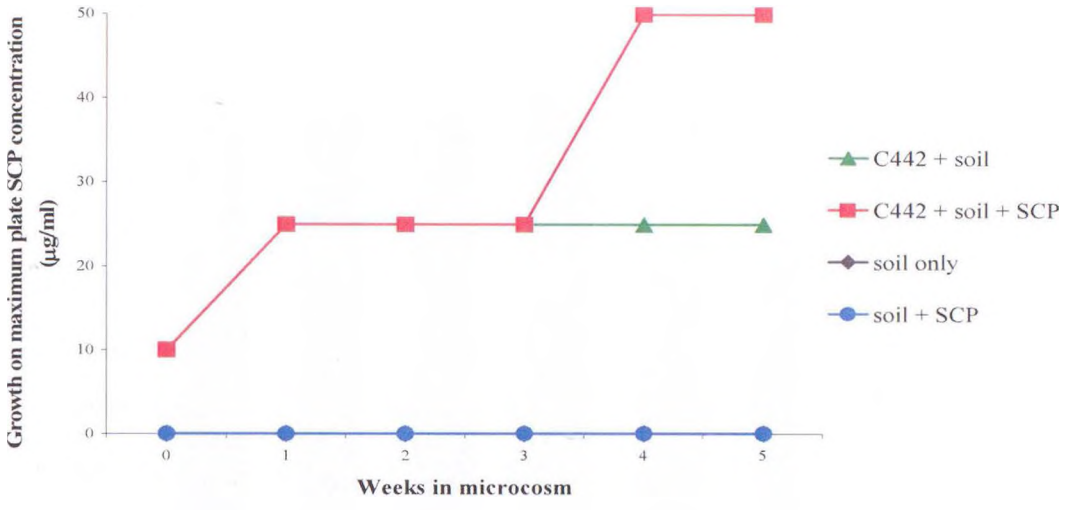
All cells were observed to maintain an SCP MIC of 8 µg/ml.

Table 7.6 Resistance profiles for C442 before and after its passage through soil microcosms for 6 weeks.

Antibiotic	Antibiotic concentration (mg/l)	Amount of colony growth for C442 after soil passage	Amount of colony growth for C442 from culture
Erythromycin	32	+++	+++
	64	+++	+++
	128	++	+++
	256	No growth	No growth
Tetracycline	32	+++	No growth
	64	No growth	No growth
	128	No growth	No growth
	256	No growth	No growth
SCP	16	+++	No growth
	32	+++	No growth
	64	+++	No growth
	128	No growth	No growth
Tylosin	8	+	+++
	16	No growth	+++
	32	No growth	+

Where growth; +:1-10 colonies, ++: 10-100 colonies and +++: 100-confluent growth.

Fig. 7.6 Soil microcosm experiments investigating sulphachloropyridazine resistance over time for C442 with and without sulphachloropyridazine additions to the soils.



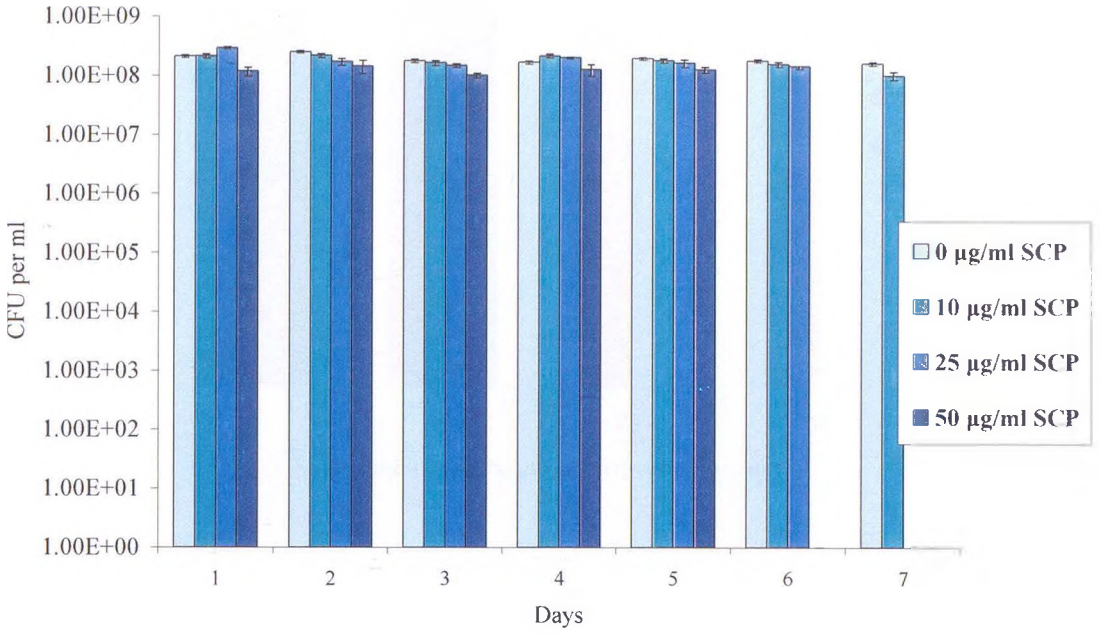
7.3.8 Stability of MIC after soil passage

An investigation was performed to observe the stability of heightened SCP MIC values in C442 after its passage through soil microcosms and by its continual subbing on solid and liquid media in the laboratory. After passage through microcosms containing SCP for 6 weeks, C442 was calculated to display increased MIC and grew in agar and liquid media containing 50 µg/ml SCP. After six repeated platings onto solid media with 24 hours growth between, C442 grew on solid media containing 50 µg/ml SCP (Fig. 7.7a). In liquid media, C442 required only three subbings to force a decrease in growth with additions of 25 and 50 µg/ml SCP to the media (Fig. 7.7b). In both cases, the *sulI, II* and *sul3* genes were still present in the genotype of the isolate (Fig. 7.8).

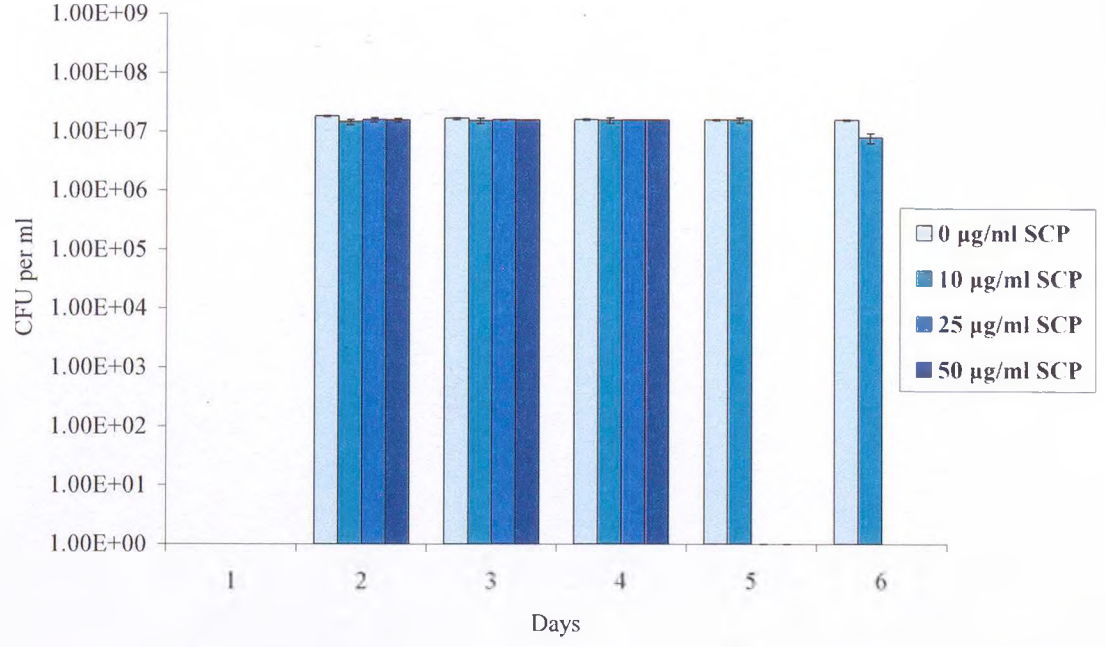
A loss in antibiotic resistance for SCP was observed after a freeze/thaw event when the soil from a microcosm containing C442 and 0.02 µg/kg SCP was frozen overnight in

Fig. 7.7 Stability of sulphonamide resistance in isolate C442 after passage through soil microcosms and multiple subbings on (a) agar and (b) liquid media over 7 days and (c) a freeze/thaw event

(a)



(b)



(c)

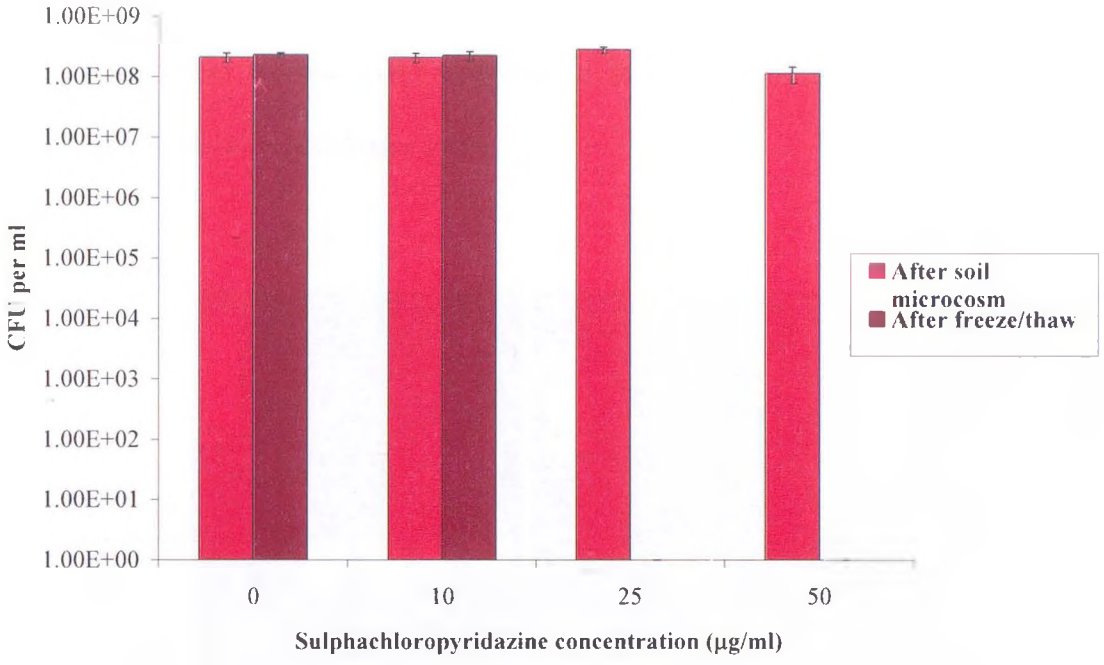
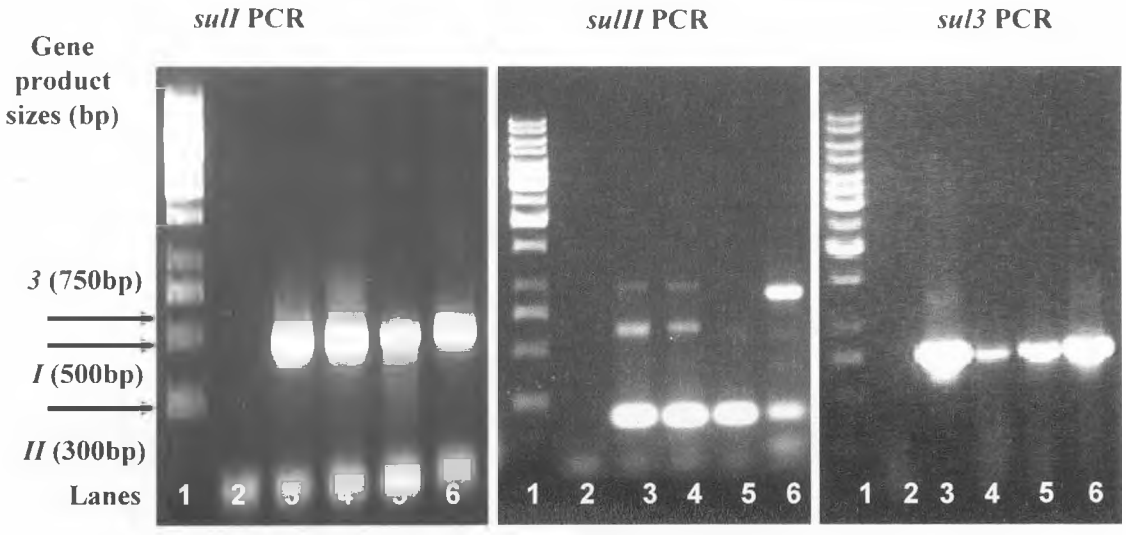

1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
CFU per ml
0
10
25
50
Sulphachloropyridazine concentration (µg/ml)
After soil microcosm
After freeze/thaw

Fig. 7.8 Confirmation of the presence of *sulI, II* and *3* gene PCR products for C442 before and after 6 weeks in a microcosm and after a microcosm and freeze/thaw event to ensure the decrease in SCP MIC could not be attributed to loss of sulphonamide resistance genes.



Lanes:

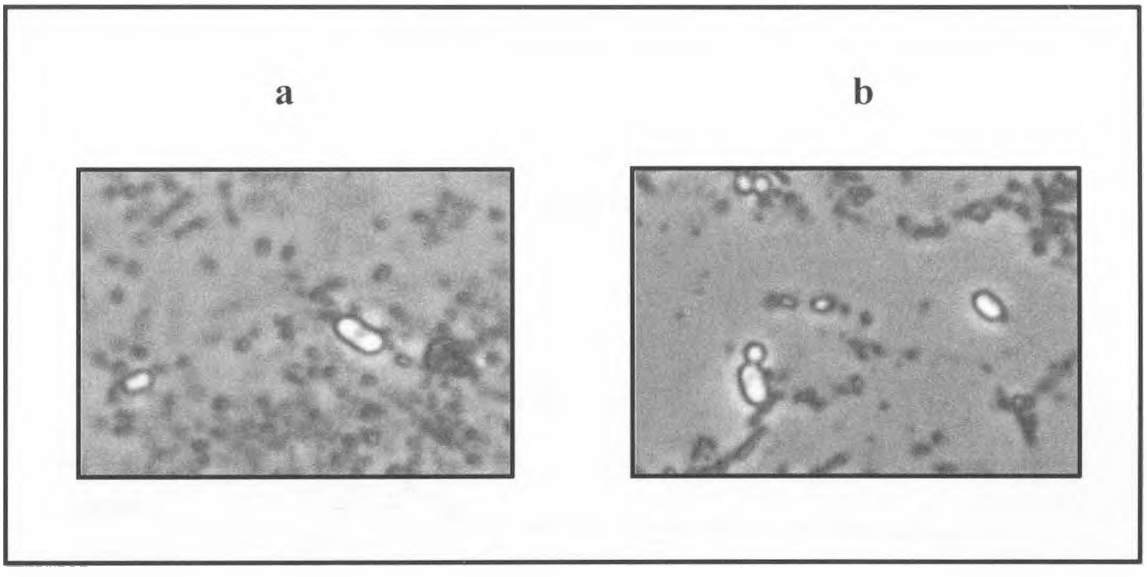
1. 1kb DNA ladder
2. Negative blank
3. Positive DNA control
4. C442 before microcosm
5. C442 after 6 weeks in microcosm
6. C442 after microcosm and freeze/thaw

glycerol and viable counts taken after defrosting, no loss of sulphonamide resistance genes were observed (Figs. 7.7c and 7.8).

7.3.9 Microscopy of soil and plate C442 cultures

Staining for evidence of polysaccharide or mucoid layers after passage through the soils or growth on plates did not reveal any positive results (Fig. 7.9).

Fig. 7.9 Polysaccharide layer stain of C442; a) control, from plate, (b) isolate after passage through soil microcosm



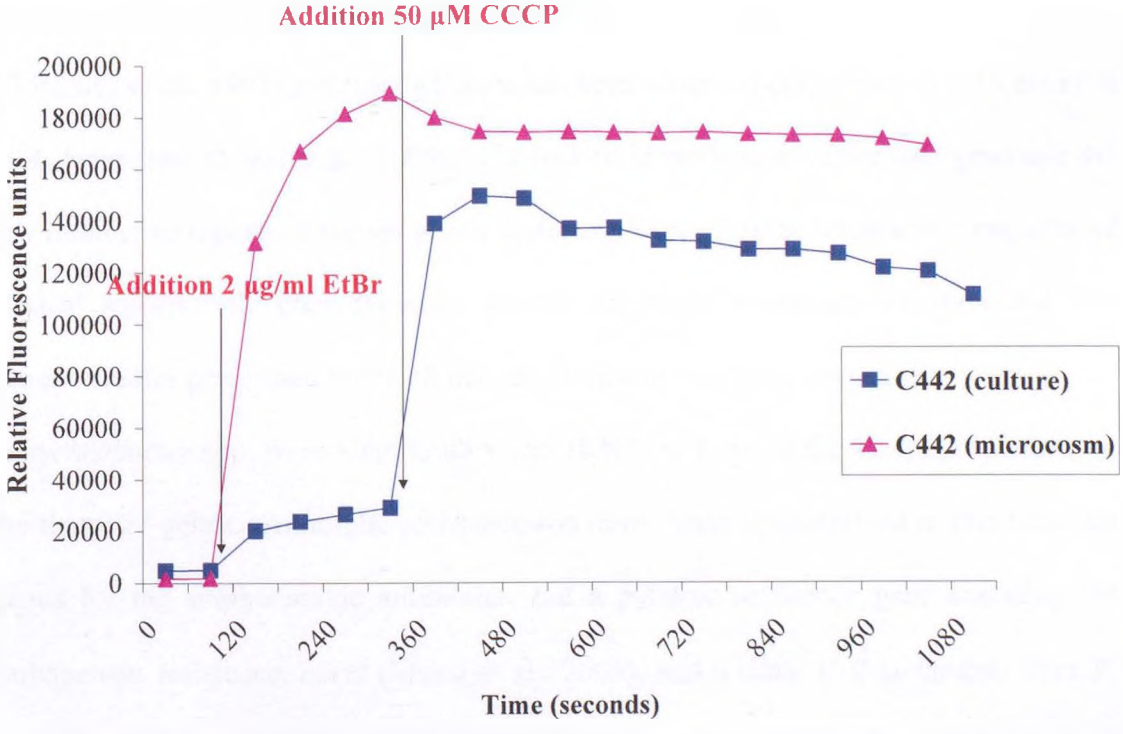
Both the control and soil passaged C442 failed to stain, indicating no production of a polysaccharide/mucoid layer.

7.3.10 Efflux data

As an indication of antibiotic extrusion from the cells to account for the increased MIC phenomena, efflux of ethidium bromide was investigated in C442 maintained in culture (C442 culture) and C442 after its passage through soil microcosms (C442 soil) (Fig.

7.10). Ethidium bromide is highly fluorescent when bound to DNA inside the cell. Low levels of fluorescence were observed with the addition of EtBr to C442 (culture) with a slight increase with time before the addition of CCCP. The protonophore uncouples the membrane potential therefore; no energy is available to the MDR pumps. CCCP caused a sudden increase in C442 (culture) fluorescence due to the uncoupling of the efflux pumps and rapid accumulation of EtBr in the cell, but after 600 seconds a decrease in fluorescence was observed. The fluorescence trace for C442 (soil) differed from that of C442 (culture), the addition of EtBr caused a rapid increase to a peak of 1800000 RFU. This high level of fluorescence was decreased and stabilised with the addition of CCCP.

Fig. 7.10 Comparison of ethidium bromide efflux between C442 from culture and C442 from 6 week soil microcosms.



7.4 DISCUSSION

7.4.1 16S rRNA identification

Acinetobacter was the dominant bacterial genus containing a mosaic of the three known *sul* genes from isolates characterised in this study. *Acinetobacter* have been accumulating resistance to a large number of antibiotic groups including the sulphonamides which has made this group of bacteria a serious problem in ICUs in hospitals (Chastre, 2003; Van Looveren and Goossens, 2004). This genus has also been identified previously in soil environments, and it is therefore proposed that the latter hosts the reservoir for this bacterium (Abbo et al., 2005; Seward, 1999). Soil environments also contain a wide range of bacteria with antibiotic resistance genes which maybe available to this genus, hence *A. baumanni* has the potential for the acquisition and selection of these genes in soils and multiple resistant strains could then be transferred to the clinical environment, for example on the soles of shoes. The *sulI* gene has been identified previously in *Acinetobacter* spp. on Class 1 integrons (Gonzalez et al., 1998) and the *sulII* gene has been observed only once, on a Tn element in *A. baumannii* (Segal et al., 2005). The lack of identification of the *sul3* gene and the low number of reports of the *sul* genes in *Acinetobacter* may be because the majority of clinical reports only phenotypically identify antibiotic resistance and there are few genetic studies performed in the clinical environment into these organisms.

Psychrobacter spp. were identified by 16S rRNA as 4 out of the 12 isolates encoding the three *sul* genes. Antibiotic resistance has never been characterised in this bacterial genus for the sulphonamide antibiotics, but a putative resistance gene encoding for carbapenem resistance, *carO* (Mussi et al., 2005), and a Class C β-lactamase from *P. immobilis* A8 (Feller et al., 1995) have been reported. Integrons have also not been

reported previously in this genus and their presence in these isolates indicates an opportunity for the bacterium to gain multiple antibiotic resistance genes. There are two reports of the *sulII* gene being characterised in another marine psychrotrophic bacteria (*MstK25/27*), in these cases the gene was flanked by a partial *glmM* gene, encoding for phosphoglucosamine mutase and not the *strA/B* genes (Berg, 2001).

One of the 12 isolates containing the *sul* mosaic was identified as a Gram positive *Bacillus* sp.. The *sul* genes have been characterised previously in Gram positive bacteria; the *sulI* and *sul3* genes in *Corynebacterium spp.* (Ploy et al., Unpublished; Tauch et al., 2002) and the *sulII* gene in *Mannheimia* spp. (Kehrenberg and Schwarz, 2002). Sulphonamide resistance, but not the *sul* genes, has been observed in a number of *Bacillus* spp. previously. Le et al., (2005) reported mutations in the chromosomal *folP* gene which confers resistance to sulphonamides in *B. subtilis*. The literature has published reports of *Bacillus* resistance to the majority of major antibiotic groups including the MLS group (Kim et al., 1993), quinolones (Bast et al., 2004) and β-lactamases (Bryskier, 2002) and Barrow et al., (2004) observed natural resistance to trimethoprim in a *B. anthracis* strain. The presence of the three known alternative DHPS enzymes in the bacterial genus of *Bacillus* means mobilisation of these genes has occurred and is significant in that genes once thought only to be the domain of *Enterobacteriacae* are now recorded in a broader spectrum of hosts. Whether these reports are because detection techniques and tools are becoming more powerful and sophisticated or whether there is increasing movement between bacterial phyla, remains to be determined.

7.4.2 Sulphonamide resistance

Of the 12 isolates encoding the *sul* gene mosaic, 11 were collected from SCP selective plates, but this figure was not significant in comparison to the numbers of isolates collected from SCP selective plates or the total number of isolates for this study. The selection of the 11 isolates may have indicated that the encoding of the three *sul* genes conferred a higher resistance to sulphonamides. This was observed not to be the case. Isolates that contained one or no *sul* gene were calculated to have similar SCP MIC values as isolates encoding three *sul* genes. As discussed previously, the presence of a gene does not provide evidence for its transcription or translation and further work should be performed to investigate the transcription rates of these genes by RTPCR and QRTPCR.

From the results, it was observed that half of the *sul* mosaic isolates were selected from year 1, pre-application soils. This could indicate the co-selection of sulphonamide resistance due to the presence of tylosin, in the soil or the pig intestinal environments, or through selection by other agents. Co-selection of sulphonamide and macrolide resistance has been reported previously, in a study characterising the plasmid pRSB101, isolated from activated sludge bacterial communities, which encoded resistance to multiple antibiotics including erythromycin and sulphonamides (Szczepanowski et al., 2004). No reports to date, have specifically detailed co-selection for tylosin and sulphonamides, especially as only a small number of genes have been reported to confer resistance to tylosin (Fouces et al., 1999; Jost et al., 2003; Jost et al., 2004; Oh et al., 1998; Singh et al., 2001).

The calculated SCP MIC values led to the conclusion that sulphonamide resistance was not genera dependant. The presence of the three *sul* genes in one isolate can result

in an SCP MIC in *Acinetobacter* equal to that recorded in *Psychrobacter* or *Bacillus* isolates, despite the differing membrane structures, antibiotic resistance profiles and altered antibiotic effectiveness against Gram negative and Gram positive bacteria.

Additional investigations into the effects of encoding different combinations of the *sul* genes in *Acinetobacter* hosts, revealed variable MIC levels and no correlation was observed between this and the number of *sul* genes carried. These results have been observed previously; Guerra et al., (2003) determined that the presence of *sulI*, *sulII* and *sul3* in a genome did not always confer resistance to an isolate. The MIC figures in this study were lower than those determined by Skold, (2001), who calculated the MIC for *E. coli* encoding a *sulI* gene to be 2 mM (570 μg/ml), increasing up to >4 mM in an *E. coli* expressing the *sulII* or *sul3* gene. The MIC breakpoint for *Acinetobacter* by BSAC is 32 mg/l, which was significantly higher than the MIC values calculated for the isolates in this study containing the three *sul* genes but is also lower than the concentration first used to collect a number of the isolates (BSAC, 2005).

7.4.3 Multiple antibiotic resistance in isolates

Interestingly, isolates identified by their 16S rRNA genes as *Acinetobacter* spp., displayed phenotypic resistance to the highest and lowest numbers of additional antibiotics tested, again displaying the multiple resistance characteristic of this genus in the clinical setting (Abbo et al., 2005; Coelho et al., 2004).

The *Bacillus* strain (C328) encoding the three known *sul* genes, as well as displaying resistance to sulphonamides, also exhibited resistance to tetracycline, nalidixic acid and chloramphenicol. The bacterial genus of *Bacillus* has been reported to encode MDR efflux pumps, but resistance to these three antibiotics is commonly conferred by chromosomal mutations in the *gyrA* gene (quinolones) (Grohs et al., 2004), acquired

resistance genes (such as the *tet* group) (Safferling et al., 2003) or, the *cat/cm* resistance genes for chloramphenicol resistance (Goldfarb et al., 1981; Laredo et al., 1988).

7.4.4 Resistance genes and integron characterisation

The 12 isolates containing the three *sul* genes were further characterised for the presence of mobile elements such as the Class 1 and 2 integrons or other antibiotic resistance genes. In none of the isolates were the locations of any of the sulphonamide resistance genes determined and none of these isolates were identified as encoding Class 1 integrons despite their carriage of the *sulI* gene. The lack of the *intI1* gene, despite the known link between *sulI* gene and Class 1 integrons, may indicate that *sulI* gene had been mobilised on a different genetic element, or the gene was present on an integron with an altered structure. An altered Class 1 integron structure would prevent annealing of the primers used in this study, especially where the *qacEΔ1* gene was absent as observed in a number of reports (Partridge et al., 2001a; Partridge et al., 2001b; Sundstrom et al., 1988). Further characterisation of these isolates using degenerative primers for all classes of integrons may determine whether the *sulI* was on an alternative Class 1 or other integron structure (Nield et al., 2001; Stokes et al., 2001a).

The isolate, C141 (*A. lwoffi*), was characterised to encode an *intI2* gene, but no Class 2 integron structure carrying *orfX*, and it may be that this isolate also carried an alternative structure than that screened for by PCR. Ploy et al., (2000), sequenced a Class 2 integron from an *A. baumanni* strain which encoded no *orfX* gene but a 3' structure similar to that of Class 1 integrons which would not have been amplified by the primer used in this study. Alternatively, integrase genes are not always present as part of an integron as discussed in Chapter 4 (Fonseca et al., 2005; Park et al., 2003).

Class 2 integron structures were sequenced and annotated in the cases of isolates PGS20 (*P. ikaite*) and C15 (*A. lwoffi*), displaying 99 % similarity with each other, possibly indicating HGT and their selection between bacteria located in the Cranfield slurries and indigenous soil bacteria due to the selective pressure. Although the genes identified on the integron (*sat, aadA1*) were not selected for by the antibiotics used in this study, the MDR nature of these integrons and the possibility that they reside on a MDR plasmid or transposon holds the possibility of co-selection via an as yet unidentified resistance gene. This possibility for co-selection was supported further by the identification of an *ermC* gene in PGS20 and C15. The *ermC* gene encodes for an rRNA methyltransferase which can confer inducible or constitutively expressed resistance to macrolides, specifically tylosin (Allen, 1977; Lyon and Skurray, 1987; Weisblum, 1995). As tylosin was an antibiotic used in this field study, the MIC for tylosin was measured and compared to other isolates to investigate whether this gene conferred resistance to tylosin in these isolates. The MIC for both PGS20 and C15 was >128 mg/l, higher than other *Psychrobacter* spp. without the *ermC* gene, but also the same as *Acinetobacter* spp. lacking the gene. The long-term usage and slurry application of tylosin onto the agricultural land of the farm used in this study may support a theory for the long-term selective pressure of the *ermC* gene in this environment.

In an attempt to localise the *sul* genes and characterise the elements on which they were encoded PCR was performed to investigate whether the *strA-strB* genes were present, as the *sulII-strA-strB* are linked genes which commonly occur on plasmids, such as RSF101 (Schölz et al., 1989). As summarised in Section 7.1.2, the *strA* and *strB* genes confer resistance to streptomycin through the inactivation of the antibiotic by

two phosphotransferase enzymes (Recchia and Hall, 1997). The PCR results and sequencing indicated that in C20 the *sulII* gene was flanked on its 3' terminus by *strA* and possibly *strB* (with 99 % similarity to the p9123 *E. coli* plasmid) (Enne et al., 2004). The physical linkage of these clustered and possibly plasmid-borne resistance genes could facilitate the spread of the different resistance genes by co-selection, even in the absence of a direct selective pressure. Further investigations may localise these genes to a multiple resistance plasmid such as those described by Kehrenberg and Schwarz, (2002) in pMVSCS1 from *Mannheimia varigena* which contain the linked genes and a *catAIII* gene but in a different orientation. Parkhill et al., (2001) annotated a plasmid from a *S. enterica* strain which contained MDR genes including the *mer* operon (for mercury resistance), *dhfr*, *cat*, *bla*, *tet(A)-(R)* genes as well as the linked genes in the same orientation as that displayed in this study. Neither of these published plasmids were investigated for their transfer abilities, although p9123 of which the linked genes in this study had 99 % similarity had been observed to transfer into other Gram negative bacteria (Enne et al., 2004).

7.4.5 Conjugal transfer of *sul* genes from the isolates containing the three *sul* genes

Transfer rates for the known three *sul* genes from the 12 isolates were investigated. The results indicted that both the *sulI* and *sulII* genes were transferable in a number of isolates. In the case of the *Psychrobacter* isolates (PGS16, PGS17, PGS20 and C20) only the *sulI* gene was observed to transfer, as was the case with all transfers the rate was lower into the *E. coli* recipient. Conjugal transfer had not previously been investigated from *Psychrobacter* spp. but indicated the bacterial genus may be capable of uptake of free mobile elements and genetic material. The *sulI* gene may transfer

from *Psychrobacter* spp. due to its' presence on an integron encoded on a transposon, in this way the mechanism for transfer maybe different from that of a plasmid and its size, which may hinder transfer from this organism. It is unknown as to why the rates were always lower in the *E. coli* recipients, possibly lower copy numbers or compatibility (Rawlings and Tietze, 2001; Sakai and Komano, 1996).

In the case of the *Acinetobacter* isolates, C15, C35, C36, C44, and C141, but with the exception of isolate C442, *sulI* and *sulII* genes transferred separately into both recipients. Both genes were observed to transfer at the same rate in the case of C442, suggesting a physical link between the *sulI* and *sulII* genes in this isolate, there are no published reports of a link between the *sulI* and *sulII* genes.

No transfer of any of the three *sul* genes was observed in the *Bacillus* isolate, C328. but if the investigation was repeated with Gram positive recipients there may have been transfer due to compatibility and host range (Rawlings and Tietze, 2001).

The *sul3* gene was not observed to transfer from any of the 12 isolates, which may indicate the gene was integrated into the chromosome of the isolates or was encoded on a low rate transferring plasmid or mobile element, or element of specific host range (Sakai and Komano, 1996).

7.4.6 MIC phenomena and efflux experiments

During the SCP MIC determinations for the 12 isolates, it was noted that all but one of the isolates were collected from SCP selective plates that were at a higher concentration than the MIC values, especially in the case of isolate C442. This led to investigations of inducible sulphonamide resistance and whether isolate phenotypes were altered by the passage from soils to agar plates with subsequent subbing and whether the reduced MIC would increase if the isolates were returned to the soil environment. C442, *A.*

baumanni, was investigated due to its clinical significance as an opportunistic pathogen (Abbo et al., 2005).

No effect on growth or MIC was observed with the addition of SCP to the growth media (liquid or solid), therefore concluding that sulphonamide resistance was not inducible *in vitro* and sulphonamide resistance did not confer a fitness cost on C442. On the other hand, the SCP MIC in the case of isolate C442, did increase when returned to soils and SCP spiked microcosms. Its' antibiotic resistance profiles altered and reversion of its SCP MIC after growth *in vitro* and on freeze-thawing was observed. These observations suggest that the SCP MIC was not solely due to the presence of the *sul* genes and a different, yet unidentified, mechanism was influencing the bacterium's phenotype. These results may have a profound effect on how isolates are viewed and treated after removal from their natural environment.

A number of possible mechanisms could be proposed for altered phenotypes due to survival and passage through the soil environment including stress responses, MD efflux pump up-regulation in the soil, and the production of a polysaccharide/mucoid layer as a result of biofilm formation. A stress response was not investigated in this study but a number of published investigations may explain the altered phenotype. Cell envelope stress-sensing two component systems have been observed to effect the uptake and efflux of chemicals, for example vancomycin, due to altered cell wall properties by changes in gene expression and cell surface hydrophilicity in *B. subtilis* (Kovacs et al., 2002; Mascher et al., 2004). A further property may have been responsible for altered phenotype, osmolarity. Overtime in soil, the cell shape and cell envelope have been demonstrated to alter in response to the environment (Deutch and Perera, 1992). On removal and plating onto agar there would be an immediate change in the osmotic

environment of the cell, which may trigger cell changes observed to be instrumented by mechano-sensitive channels, rapidly and non-specifically changing the ion and solute content of the cell (Deutch and Perera, 1992; Morbach and Kramer, 2002). This non-specific mechanism may mean the extrusion of antibiotics, as compounds such as methylamides, sulphonamide analogues and phenols have been reported to be extruded through these channels (Morbach and Kramer, 2002). No studies have been published which investigate antibiotic removal under these conditions but the published data suggests they may be removed via these channels under certain conditions.

MDR efflux pumps can be intrinsic, encoded on the chromosome giving a cell a natural resistance to a number of antibiotics and toxic compounds or acquired, such as antibiotic-specific pumps (for example, Tet (K) and CmlA) encoded on transferable plasmids and transposons (Roberts, 1996; Van Bambeke et al., 2000). MDRs can be strictly controlled by the presence of antibiotics for their regulation, therefore were investigated within C442 by observing resistance levels to a number of groups of antibiotics; the macrolides, sulphonamides and tetracyclines (Roberts, 1996). The results for the macrolide drugs suggested MDR regulation was not the causative mechanism for altered MIC values after soil passage. Although tetracycline resistance was elevated, therefore there may be up-regulation of specific efflux pumps, although no *tet(K)* or *tet(M)* genes were identified in this isolate and no tetracycline was present in the soil to induce up-regulation. Tylosin resistance was observed to decrease with passage through the microcosms, possibly due to reversion of tylosin resistance mutations in the 23SrRNA gene in the *in vitro* culture due to a lack of selection or regulation of an unidentified resistance gene, for example, *ermT* or an unkown gene (Prapasarakul et al., 2003; Whitehead and Cotta, 2001).

Efflux data was used to determine the efflux capabilities of a cell and efflux kinetics of particular molecules, allowing investigations into MDR regulation following isolate passage through the soil environment. To investigate further the efflux capabilities of C442 and to examine up-regulation in the soil environment, an EtBr uptake experiment was performed. Ethidium bromide is highly fluorescent when bound to DNA inside the cell. CCCP is a protonophore, which dissipates the membrane potential used in a number of efflux mechanisms for energy, therefore when CCCP is added to a cell which is actively transporting EtBr out of the cell, the MDR will not function and EtBr will accumulate in the cell, ultimately causing cell death. In the case of C442 (culture) a normal fluorescence curve for EtBr uptake and dissipation was observed with a slight increase with the addition of EtBr due to its binding in the cell which represents an influx verses efflux kinetic equilibrium. With the spiking of the culture with CCCP, a sudden increase in fluorescence was observed due to the uncoupling of the efflux pumps and rapid accumulation of EtBr in the cell. The decrease in fluorescence, after 600 seconds, was possibly due to cell death and lysis. In the case of C442 (soil), the fluorescence data indicates that the efflux mechanisms had been down-regulated, and therefore, CCCP had little or no effect on dissipating the membrane potential and preventing efflux of the EtBr from the cell. EtBr may have saturated available sites in the cell explaining the rapidly reached high and stable peak. Similar results have been observed in *E. faecalis* during investigations into an MDR efflux pump encoded by the *norA* homologue which confers norfloxacin resistance in the absence or presence of the MexAB-OprM MDR in *P. aeruginosa* (Jonas et al., 2001; Xu et al., 2003).

Biofilms and polysaccharide/mucoid layers are produced in bacteria as an environmental protection mechanism, forming barriers which reduce environmental

stresses and prevent diffusion of toxic chemicals such as antibiotics, commonly studied in the clinical but not soil environments (Patel, 2005). The production of a polysaccharide/mucoid layer due to C442's presence in a soils or a soil biofilm seems unlikely after microscopy staining revealed no positive results of their presence. Although it is difficult to assess biofilm formation or polysaccharide formation on plates after the bacterial cells have been removed from the soil environment, additional studies should be attempted in soils. Staining was attempted on soils, but due to the soil matrix, staining was unsuccessful and no fixing took place. Further work in this area could be performed due to the lack of published data in this area.

7.5 FURTHER WORK

As discussed a number of times, an investigation into the transcription and translation rates of the three *sul* genes present in the 12 isolates should be investigated to demonstrate whether the genes are regulated in the presence or absence of the antibiotic and in the soil environment. RTPCR and QRTPCR have been in previous studies to investigate antibiotic resistance gene expression in bacterial cells and expression with relation to resistance levels. The expression of the *ampC*, *bla1* and *bla2* genes were investigated in relation to penicillin resistance in *E. coli* (Perichon and Courvalin, 2004) and *B. anthracis (Chen et al., 2004)*, and *vanA* expression has been recorded in vancomycin resistant *S. aureus* (Tracz et al., 2005). RTPCR is currently the most sensitive technique for mRNA detection and quantification of gene expression. It could be used to assess all three *sul* genes simultaneously using a multiplex PCR approach with different fluorescent dyes with different emission spectra to differentiate the three genes.

Where strains, for example of *Acinetobacter*, are identified as similar by 16S rRNA identification but give different antibiotic resistance profiles it may have been beneficial to further type these strains. This could be done using Box PCR (Rademaker and Bruijn, 1992) or a technique widely used for *Acinetobacter* species of ribotyping, which distinguishes between different ribotypes having possibly originated from one strain and is based on the rRNA operon, *rrnB* (Wu et al., 2004).

A number of the MIC results should be repeated using species specific MIC breakpoints according to BSAC as there maybe some underestimates based on the general breakpoints used, especially in the case of the *Bacillus* strain (BSAC, 2005).

Further characterisation of the 12 *sul* mosaic isolates using degenerate primers for all classes of integrons may determine whether the *sulI* is on an alternative Class 1 or other class of integron structure (Nield et al., 2001; Stokes et al., 2001a). A number of attempts were made in this study to use the primers but they were non-specific and the cloning of each band would have been required for the identification of integrons and their cassette genes, the time restrictions of the project prevented this work.

The sequences for the *sulI*, *sulII* and *sul3* genes of the *Psychrobacter* spp. need to be further characterised with the 5' and 3' flanking regions sequenced if possible for submission into a nucleotide sequence database and possible completion for a publishable paper into antibiotic resistance in the *Psychrobacter* genus. The further characterisation of the *sul* genes and their locations in all the strains would yield interesting results. In the *Bacillus* strain, this additional characterisation would allow an investigation into the *sul* genes transfer into Gram positive bacteria and determine whether lateral transfer has occurred between Gram positive spp. in the soil environment.

Studies into the varying MIC values between species and the non-accumulative effects of the three *sul* genes could be investigated, studying co-selection (especially of efflux genes in the case of C442), promotion, induction and expression of these genes.

The issue of co-selection was raised in relation to a number of antibiotics and bacterial species in this work. Co-selection into tylosin and sulphonamides may have occurred in this study and further investigations into this area would include the full characterisation of a number of plasmids and integrons, with particular attention to genes encoding for tylosin resistance and physical linkage between those and sulphonamide resistance genes. A study into agricultural soils without the application of tylosin pig slurry may act as a control and elucidate whether the co-selection was due to the long-term effects of the application of slurry from tylosin fed pigs.

Further studies could be performed which investigate the formation of biofilms and polysaccharide/mucoid layer production. Nicodenz extraction of bacteria from soils may allow direct staining of soil bacteria without the need for enrichment and growth away from the soil environments allowing phenotypic changes to be investigated.

7.6 Conclusions

This study provides several pieces of evidence for co-selection of sulphonamide resistance by tylosin due to the long-term usage of this antibiotic as a growth promoter in animal husbandry and the application of tylosin-fed pig slurry onto agricultural land, the selection occurring in both the swine intestine and the soil environment.

A number of investigations performed in this study report the first characterisation of MDR in the genus *Psychrobacter*, both genetically and phenotypically. Characterisation of a new MDR containing genus demonstrates the lateral and

horizontal movement of antibiotic resistance genes and provides further evidence of the problem that antibiotic resistance has and is becoming.

Furthermore, this study provides an indication that soils are the environmental reservoir of *Acinetobacter*, in a background rich in the prospective gain of antibiotic resistance genes, providing a pool of resistance bacteria with the potential to cause opportunistic hospital infections.

CHAPTER 8:

DISCUSSION

Chapter 8: DISCUSSION

There has been increasing concern about the release of antibiotics into the environment through the application of polluted animal slurries to soils. Reports have been discussed in the preceding chapters in which antibiotic mobility investigations have provided evidence for the persistence of antibiotics in the environment, especially soils and slurries (Boxall et al., 2002; Tolls, 2001). These reports have led to assessments of the impacts of chemicals in agriculture on the environment and on the microbial communities of the enteric gut, slurry and indigenous soil populations (Buckley and Schmidt, 2003; Schmitt et al., 2004). Assessments have also been performed to investigate the impacts on microbial communities with respect to carriage of resistance genes (Aarestrup et al., 2001; Muller et al., 2002), the distribution of mobile genetic elements (Class 1 and 2 integrons) and their dissemination by HGT (Antunes et al., 2005; Gaze et al., 2005). The assessment of impacts over time and the relationship between presence of antibiotics in soils/slurries and phenotypic effects on the slurry/soil bacterial populations has not been performed in the past. The aim of this study was to relate data from a previous investigation into the chemical fate of antibiotics in soil/slurry to prevalence of antibiotic resistant bacteria in the soil/slurry. The antibiotics, SCP, OTC and tylosin were selected as study compounds due to their differences in mobility and persistence in the environment (Boxall et al., 2002; Kay et al., 2004, 2005b). The effects of long-term applications of antibiotic containing slurries on phenotypic bacterial antibiotic resistance were investigated with respect to tylosin and short-term with respect to SCP and OTC. The issues of genotypic effects specifically on sulphonamide resistance genes and mobile genetic elements, such as Class 1 and 2 integrons, in the soils and slurries was also investigated with a view to

relating shifts in bacterial genotype and phenotype with the persistence of antibiotics in the environment over time.

High RQ values for SCP, OTC and tylosin were determined in an organic slurry sample, but significantly no *intI1* or *intI2* genes were identified in bacterial isolates collected from this slurry or from a control slurry sample which had not been spiked with SCP and OTC, where a low phenotypic resistance was observed to these antibiotics and tylosin at higher concentrations. The lack of Class 1 and 2 integrons from the organic slurries may have been in part due to the low numbers of *Enterobacteriacae* isolated. Class 1 and 2 integrons have been reported to be widely distributed in this bacterial group (White et al., 2001).

In the case of the Cranfield slurry, RQ values decreased with increased concentrations of SCP and OTC, but resistance levels were elevated at higher concentrations of tylosin. Results showed that both CFUs and bacterial resistance were higher in the slurry sample than soils, possibly as result of advantageous growth conditions, higher nutritional content, and temperatures (De la Torre et al., 2000). The general trend of phenotypic resistance displayed in the Cranfield soils was of an initial increase in the soils RQ values and numbers of *sul* genes with slurry application, followed by a decrease over time. The results following the application of slurry to soils, displayed the long-term survival and die off of enteric bacteria and the possible persistence of resistance genes acquired through HGT from the enteric bacteria. The death of sensitive bacteria in the soils due to the presence of a selective pressure (as indicated by HPLC data for OTC and SCP) and increased domination of resistant indigenous bacteria, such as those belonging to the Gammaproteobacteria, was also indicated by the results. Previous investigation have reported discernable effects due to the application of antibiotic

through slurries, Schmitt et al., (2004) observed the development of a 10 % pollution-induced community tolerance with initial exposure to SCP, and Sengeløv et al., (2003) observed a corresponding relationship between tetracycline resistance and soil fertilized by manure with an initial increase after application which decreased over time to normal levels.

The results of this study concluded that there was no overall discernable effect on measurable phenotypic resistance against SCP and OTC due to the application of spiked pig slurry onto soils. The persistence of OTC in the environment and lack of evidence for sustained elevated resistance on the bacterial population in this study indicates that this antibiotic was not bio-available, conflicting with the study of Chander et al., (2005).

In the case of tylosin, resistance remained high throughout both years of the study, although tylosin resistance genes were only identified in a number of isolates. Further work may reveal more tylosin resistance genes in the soil isolates. The constancy of tylosin resistance in the soils could be explained as a result of continual sub-lethal doses of the antibiotic applied to the soils via pig application slurry for over 20 years (tylosin-fed pigs) with the potential to maintain a constant selective pressure, although the HPLC data indicated no tylosin was present in the soil or slurry samples (Kay *et al.*, 2004). This effect of continual sub-lethal doses has been described in a number of studies; Langlois et al., (1978) reported the excretion of multiple resistant microorganisms after pigs received therapeutic antibiotic doses and van den Bogaard et al., (2000) recorded a high prevalence of multiple antibiotic resistant bacteria where antibiotics were used as growth promoters. The higher resistance for tylosin may be a result of a shift in the microbial community in this perturbed soil, also observed in a study by Westergäard et al., (2001) where tylosin treatments of soils caused a shift in

the microbial community even after traces of tylosin or its degradation products could no longer be detected. A comparison between the diversities of microbial communities for untreated control soils and treated soils would be needed before this statement could be qualified.

This study demonstrated a low diversity in the culturable bacterial populations identified in the slurries and soils investigated compared to published literature, although this would need to be confirmed using significantly higher numbers of bacterial isolates (Borneman et al., 1996; Chander et al., 2005; Snell-Castro et al., 2005). The slurry diversities incorporating bacteria originating in the pig gastrointestinal tract, as also identified by Snell-Castro et al., (2005) and the pre-application soils displaying some effect of a community shift towards tylosin resistant Gammaproteobacteria due to long-term usage and application of tylosin, the Gammaproteobacteria being the dominate genus in the soils.

A link in this study between the *intI1* gene and tylosin resistance was proposed. The association may have been established due to the continual selection in the pig gut by tylosin in the feed. No published reports have observed an association between *intI1* and tylosin resistance.

As a result of using more than one antibiotic concentration in examining antibiotic resistance phenotypes, the data exhibited trends with bimodal distributions which could perceivably be used to assess functional resistance mechanisms, the antibiotics effects on bacterial cell physiology and metabolism. This bimodal distribution has not been reported previously in the literature, possibly because most studies investigate only one antibiotic concentration in regard to CFU counts and RQ values (Halling-Sørensen et al., 2005; Langlois et al., 1978). Further work would be needed to provide evidence for

the theory that at lower antibiotic concentrations, in the first phase of the curve, the effect on the constitutive expression, production and rate of general efflux pumps may be being observed and then at higher concentrations, in the second phase of the curve, an induction effect may be being displayed for acquired genes. The fact that the bimodal curve was not observed with increasing concentrations of OTC may hold the key to the functional relevance, if any, of the different response curves.

Of the bacteria isolated in this study, 9.7 % were *intI2* positive and 5.64 % encoded the *intI1* gene, on its own or in a number of different combinations with other screened genes. Previous environmental investigations have reported similar frequencies; Gaze et al., (2005) recorded a prevalence of *intI1* as 7.98 % in isolates from a QAC-polluted environment and Rosser and Young (1999) reported 3.6 % of bacteria isolated in their study carried a Class 1 integron. The *intI1* gene was detected by PCR in 33 of the 583 bacterial isolates cultured in this study, only 11 of which also encoded the *sulI* gene. In this study, the majority of the *intI1* positive isolates did not encode the common Class 1 integron structure with the *sulI* gene in its conserved 3' backbone (Sundström et al., 1988). This suggests environmental selective pressures influence integron structures, as also observed by a study into a QAC-polluted environment, which observed a dominance of Class 1 integrons with *qacE* on its 3' conserved backbone (Gaze et al., 2005) as opposed to *qacEΔ1* and *sulI* (Sundström et al., 1988). Of the *intI1* positive isolates, four were identified as putative Gram positive bacteria, as were 8 of the 52 *intI2* positive isolates. Three *intI1* isolates were identified as *Bacillus* spp. and one isolate was putatively identified as an *Arthrobacter* sp. Class 1 integrons have previously been observed widely distributed within the Gram negative bacteria, particularly the *Enterobacteriacae* (Fluit and Schmitz, 1999), but recent reports have

observed these structures in a number of Gram positive bacteria (Nandi et al., 2004). The integron from the *Arthrobacter* isolate was characterised in more detail, revealing a Class 1 integron with 99 % similarity to an integron structure encoded on a plasmid first identified in a *C. glutamicum* strain, with an *intI1Δ1* gene (Tauch et al., 2002). Significantly, the *intI1* and *sulI* genes on the Class 1 integron and a *sulII* gene detected in the *Arthrobacter* isolate were demonstrated to undergo conjugal transfer to Gram negative recipients, *E. coli* and *P. putida* indicating localisation onto plasmids with broad host ranges and the ability for horizontal gene transfer.

The *intI2* gene was recorded in this study at a higher frequency than the *intI1* gene, 9.7 % of isolates compared to 5.4 % of *intI1* positive isolates. A higher frequency of *intI2* has been observed previously in published investigations; Du et al.,(1998) reported a dominance of Class 2 integrons in *A. baumannii* isolates from Chilean hospitals. A number of isolates were also characterised in which both screened integrase genes were detected. Noted, was the lack of incidence of *intI1* and the *sul3* gene in the same isolate in this investigation but they have been reported together in a study by Antunes et al., (2005), possibly indicating an incompatibility with the mobile element encoding the latter gene.

Twelve isolates were cultured which encoded all three sulphonamide resistance genes, as first reported by Antunes et al., (2005) in *Salmonella* spp. Investigations into the three *sul* gene mosaic isolates revealed no fitness cost was being incurred, and no increase in protection against higher concentrations of sulphonamides was observed compared to carriage of singular or two *sul* genes, in agreement with other studies, therefore questioning the isolates continual maintenance of the three genes (Enne et al., 2004; Sköld, 2001).

The results of the current study displayed a lower distribution of *sul3* compared to *sulI* and *sulII*, as observed previously in other investigations (Antunes et al., 2005; Guerra et al., 2004), possibly attributed to a latter evolution, its distribution on a lower rate transferable element, or latter dissemination to a mobile genetic element (Perreten and Boerlin, 2003).

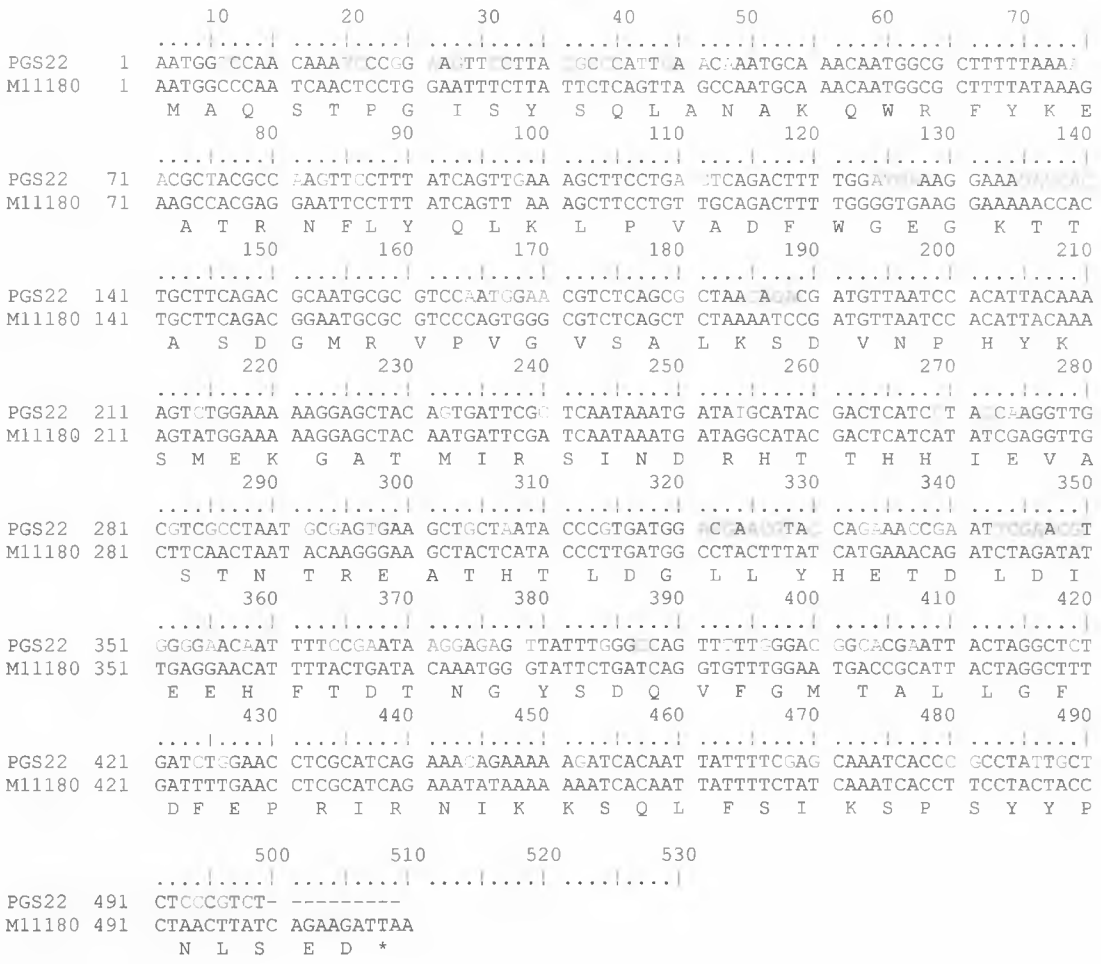
New observations included the first characterisation of Class 1 integrons in the bacterial genera *Carnobacterium*, *Psychrobacter* and *Aerococcus* of which the latter two also displayed the first reports of sulphonamide resistance conferred by *sul* genes and Class 2 integrons. These first identifications provide further vindication of new studies into the diversity of soil and slurry microbial communities and their role as reservoirs of resistance genes where there is little knowledge (Torsvik et al., 1998; Torsvik and Ovreas, 2002).

CHAPTER 9:

APPENDICES

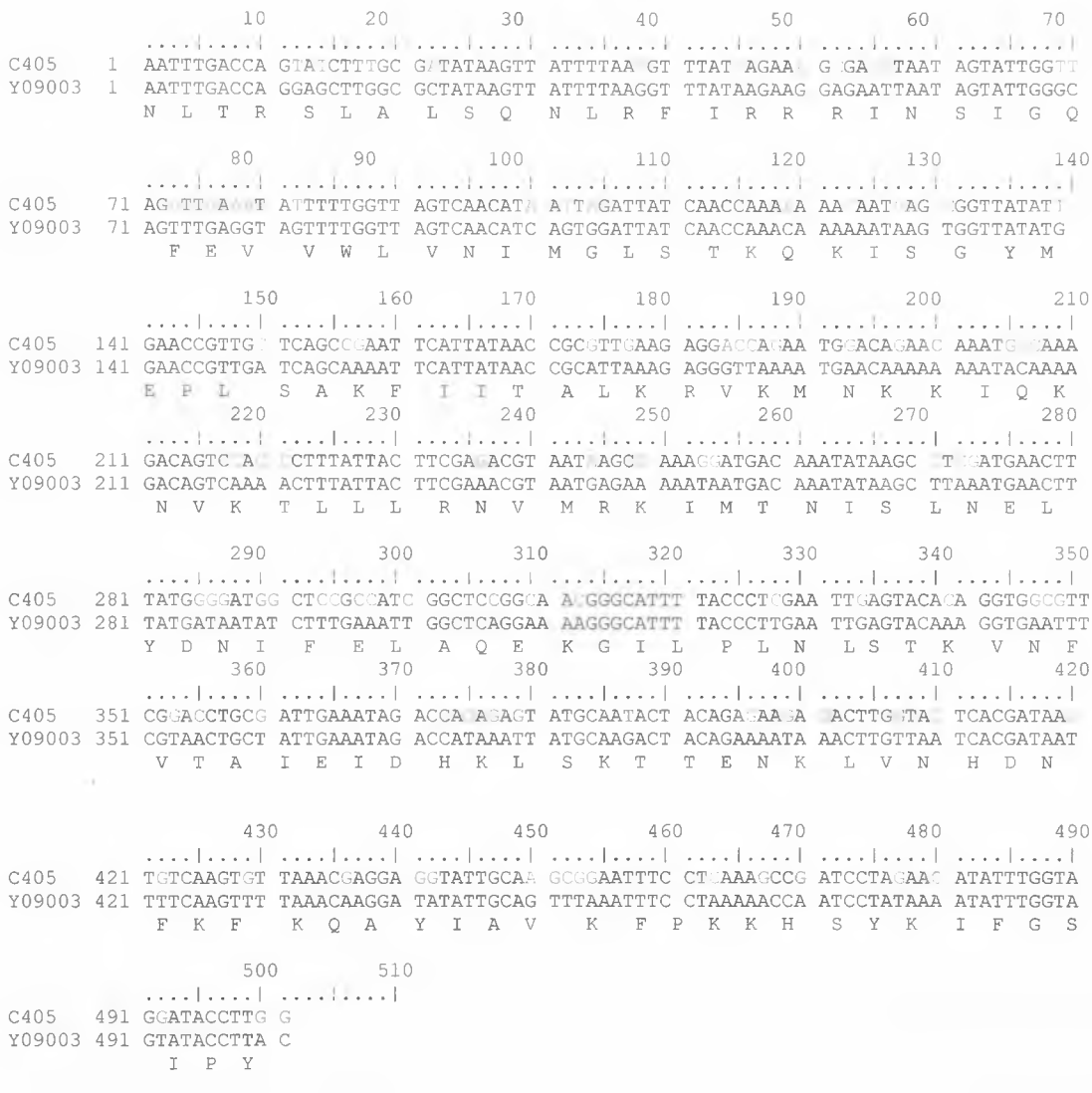
App. A1 Nucleic acid sequence alignments of (a) PGS22 *ermB* PCR fragments with the *ermB* gene on Tn*917* from *Enterococcus faecalis* DS16 (GenBank accession no. M11180), and (b) C405 *ermC* PCR fragment with the *ermC* gene on plasmid pSES21 from *Staphylococcus hyicus* (GenBank accession no. Y09003) with protein translations for M11180 and YO90003.

(a)



PGS22 *ermB* PCR fragment aligns with the *ermB* gene, M11180 with 99 % similarity (black aligned bases) at the nucleic acid level.

(b)



C405 *ermC* PCR fragment aligns with the *ermC* gene, YO9003 with 96 % similarity (black aligned bases) at the nucleic acid level.

App. A2 Sequence alignments of C405, C507 and PGS22 isolates *tet(M)* PCR fragments with the *tet(M)* genes from Tn*1545* from *Streptococcus pneumoniae* and from *Entereococcus faecalis* (GenBank accession nos. X04338 and X92947) (Martin et al., 1986; Perreten et al., 1997) and protein translation for XO4388.

```
                  10         20         30         40         50         60         70
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
C405      1  TGGAGGGCGA GGTCACGAAG ACGGATAATA CGCTTTTAGA ACGTCAGAGA GGAATTACAA TTCAGACGGC
C507      1  TGGAGAGGCA GGTCACGAGG ACGGATAATA CGCTTTTAGA ACGTCAGAGA GGAATTACAA TTCAGACAGG
PGS22     1  TGGGAGGGCA GGTCACGAAG ACGGATAATA CGCTTTTAGA ACGTCAGAGA GGAATTACAA TTCAGACAGG
X04388    1  TGGACAGAGG TACAACGAAA ACGGATAATA CGCTTTTAGA ACGTCAGAGA GGAATTACAA TTCAGACGGC
X92947    1  TGGACAAAGG TACAACGAGG ACGGATAATA CGCTTTTAGA ACGTCAGAGA GGAATTACAA TTCAGACAGG
               D  R  G   T  T  K   T  D  N  T   L  L  E   R  Q  R   G  I  T  I   Q  T  A

                     80         90        100        110        120        130        140
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
C405     71  GATAACCTCT TTTCAGTGGA AAAATACTAA GGTGAACATC ATAGACACGC CAGGACATAT GAATGTTCTC
C507     71  AATAACCTCT TTTCAGTGGG AAAATACGAA GGTGAACATC ATAGACACGC CAGGACATAT GAATGTCCTC
PGS22    71  AATAACCTCT TTTCAGTGGG AAAATACGAA GGTGAACATC ATAGACACGC CAGGACATAT GAATGTTC-C
X04388   71  GATAACCTCT TTTCAGTGGA AAAATACTAA GGTGAACATC ATAGACACGC CAGGACATAT GGATTTTTTA
X92947   71  AATAACCTCT TTTCAGTGGG AAAATACGAA GGTGAACATC ATAGACACGC CAGGACATAT GGATTTCTTA
               I  T  S   F  Q  W  K   N  T  K   V  N  I   I  D  T  P   G  H  M   D  F  L

                     150        160        170        180        190        200
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
C405     141 GTAGAAGTAT ATCGTTCATT ATCACCAGAG AGTGGGGTAA TTCTACTGAT TCTGGCTTTT
C507     141 CTTCAAGTTT CCCGTTCAGT ATCTGTTTTA GATGGGGCCA TTTT------ ----------
PGS22    141 CTTC---TTT CC------GG ---------- ---------- ---------- ----------
X04388   141 GCAGAAGTAT ATCGTTCATT ATCAGTATTA GATGGGGCAA TTCTACTGAT TTCTGCAAAA
X92947   141 GCAGAAGTAT ATCGTTCATT ATCAGTTTTA GATGGGGCAA TTCTACTGAT TTCTGCAAAA
               A  E  V  Y   R  S  L   S  V  L   D  G  A  I   L  L   I   S  A  K
```

C405 *tet(M)* gene PCR fragment displays 98 % similarity to the *tet(M)* gene on Tn*1545* from *S. pneumoniae* (GenBank accession no. X04388) and the PGS22 *tet(M)* gene PCR fragment had 98 % similarity to the *tet(M)* gene from *E. faecalis* (GenBank accession no. X92947) (Martin et al., 1986; Perreten et al., 1997).

App. A3 Sequence alignments of (a) PGS22 *qacE* PCR fragment with the *qacE* gene from *Klebsiella aerogenes*, 99 % similarity (GenBank accession no. X68232) and (b) C506 *qacEΔ1* PCR fragment with the *qacEΔ1* gene within Tn*1696* on plasmid R1033 from *Pseudomonas aeruginosa,* 96 % similarity (GenBank accession no. U12338) (Paulsen et al., 1998; Wohlleben et al., 1989). (a)

```
                  10        20        30        40        50        60        70
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PGS22     1  CCCTCTCCCA TTCCGCCGAT GTCATAATCG GTTATGTCAT CGCATCTTAT TGCCTTTCTC TGGTTCTGAA
X68232    1  --CTTGCCCC TTCCGCCGTT GTCATAATCG GTTATGGCAT CGCATTTTAT TTTCTTTCTC TGGTTCTGAA
               L  A  P   S  A  V   V  I  I  G   Y  G  I   A  F  Y  F  L  S  L   V  L  K

                  80        90       100       110       120       130       140
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PGS22    71  ATCCATCCCT GTCGGTGTTG CTTATGCAGT CTGGTCGGGA CTCGACGTCG TCATAATTAC AGCCATTGCC
X68232   71  ATCCATCCCT GTCGGTGTTG CTTATGCAGT CTGGTCGGGA CTCGGCGTCG TCATAATTAC AGCCATTGCC
               S  I  P   V  G  V  A   Y  A  V   W  S  G   L  G  V  V   I  I  T   A  I  A

                 150       160       170       180       190       200       210
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PGS22   141  TGGTTGCTTC ATGGGCAGGA GCTTGATGCG TGGGGCTTTG TAGGTATGGG GCTCATAATT AGTGCTGCCG
X68232  141  TGGTTGCTTC ATGGGCAAAA GCTTGATGCG TGGGGCTTTG TAGGTATGGG GCTCATAGTT AGTGGTGTAG
               W  L  L  H   G  Q  K   L  D  A   W  G  F  V   G  M  G   L  I  V   S  G  V  V

                 220       230       240       250       260
             ....|....| ....|....| ....|....| ....|....| ....|....|
PGS22   211  TACTTTTACC GTTTGCTCGC CAACGATCTG CCCACGTGG AATCGCTG
X68232  211  TAGTTTTAAA CTTGCTTTCC AAAGCAAGTG CCCACTAAT AAACTCAG
               L  N  L   L  L  F   K  A  S  A   H  *
```

(b)

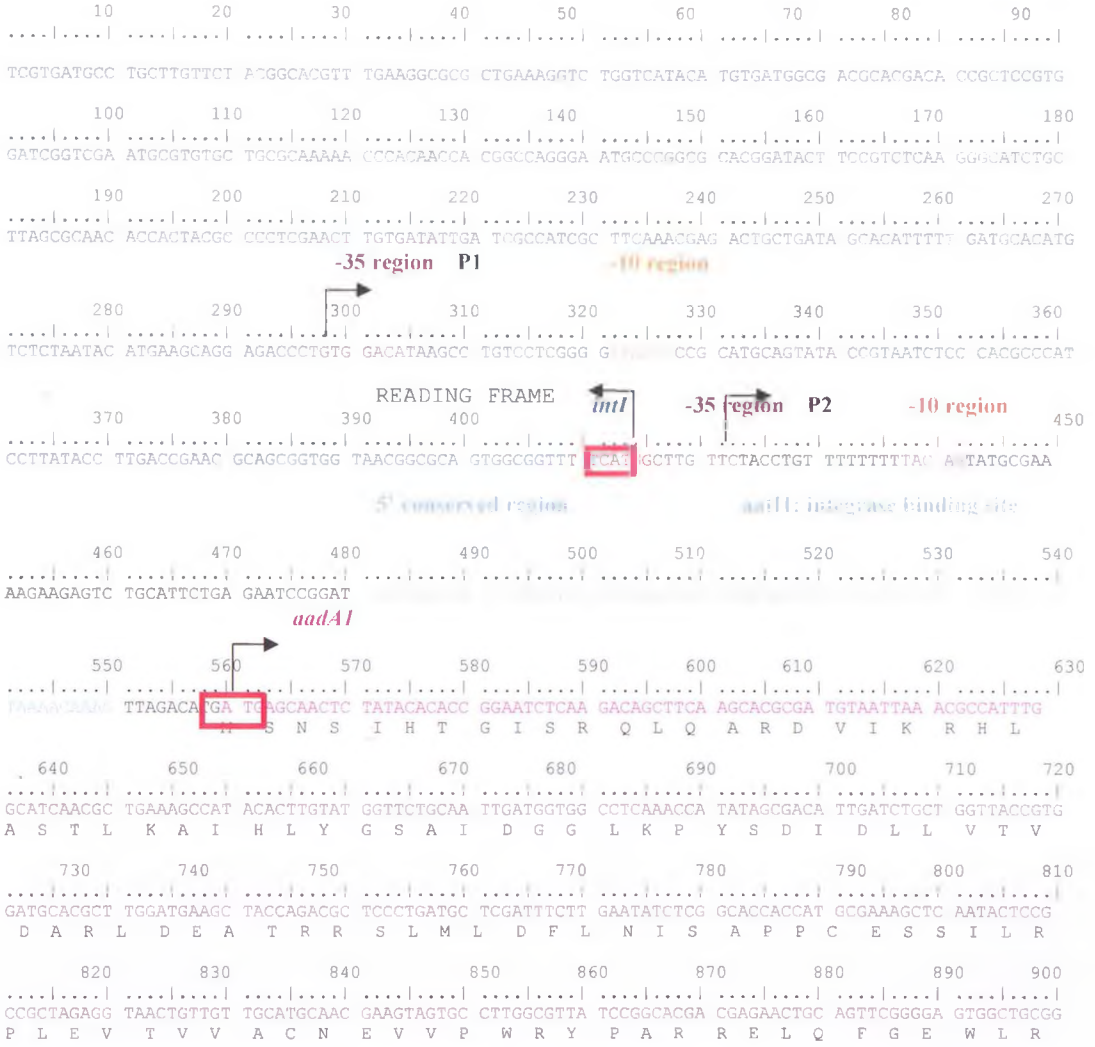
```
                  10        20        30        40        50        60        70
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
C506      1  -ATGAAAGGC TGGCTTTTTC TTGTTATCGC AATAGTTGGC GAAGTAATCG CGACATCCGC ATTAAAATCT
U1233     1  TCATGAAAGG CTGGCTTTTT CTTGTTATCG CAATAGTTGG CGAAGTAATC GCAACATCCG CATTAAAATC
               M  K  G   W  L  F   L  V  I  A   I  V  G   E  V  I   A  T  S  A   L  K  S

                  80        90       100       110       120       130       140
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
C506     71  AGCGAGGGCT TTACTAAGCT TGCCCCTTCC GCCGTTGTCA TAATCGGTTA TGGCATCGCA TTTTATTTTC
U1233    71  TAGCGAGGGC TTTACTAAGC TTGCCCCTTC CGCCGTTGTC ATAATCGGTT ATGGCATCGC ATTTTATTTT
               T  E  G   F  T  K  L   A  P  S   A  V  V   I  I  G  Y   G  I  A   F  Y  F

                 150       160       170       180       190       200       210
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
C506    141  TTTCTCTGGT TCTGAAATCC ATCCCTGTCG GTGTTGCTTA TGCAGTCTGG TCGGGACTCG GCGTCGTCAT
U1233   141  CTTTCTCTGG TTCTGAAATC CATCCCTGTC GGTGTTGCTT ATGCAGTCTG GTCGGGACTC GGCGTCGTCA
            L  S  L  V   L  K  S   I  P  V  G   V  A   Y   A  V  W   S  G  L   G  V  V  I
                  220       230       240       250       260       270       280
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
C506    211  AATTACAGCC ATTGCCTGGT TGCTTCATGG GCAAAAGCTT GATGCGTGGG GCTTTGTAGG TATGGGGCTC
U1233   211  TAATTACAGC CATTGCCTGG TTGCTTCATG GGCAAAAGCT TGATGCGTGG GGCTTTGTAG GTATGGGGCT
               I  T  A   I  A  W   L  L  H  G   Q  K  L   D  A  W   G  F  V  G   M  G  L

                 290       300       310       320       330       340       350
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
C506    281  ATAATTGCTG CCTTTTTGC TCGCCCGATC CCCATCGTGG AAGTCGCTGC GGAGGCCGAC GCCATGGTGA
U1233   281  CATAATTGCT GCCTTTTTGC TCGCCCGATC CCCATCGTGG AAGTCGCTGC GGAGGCCGAC GCCATGGTGA
               I  I  A   A  F  L  L   A  R  S   P  S  W   K  S  L  R   R  P  T   P  W  *
```

App. A4 Annotated C506 Class 1 integron sequence from primer set (VarF/VarR).



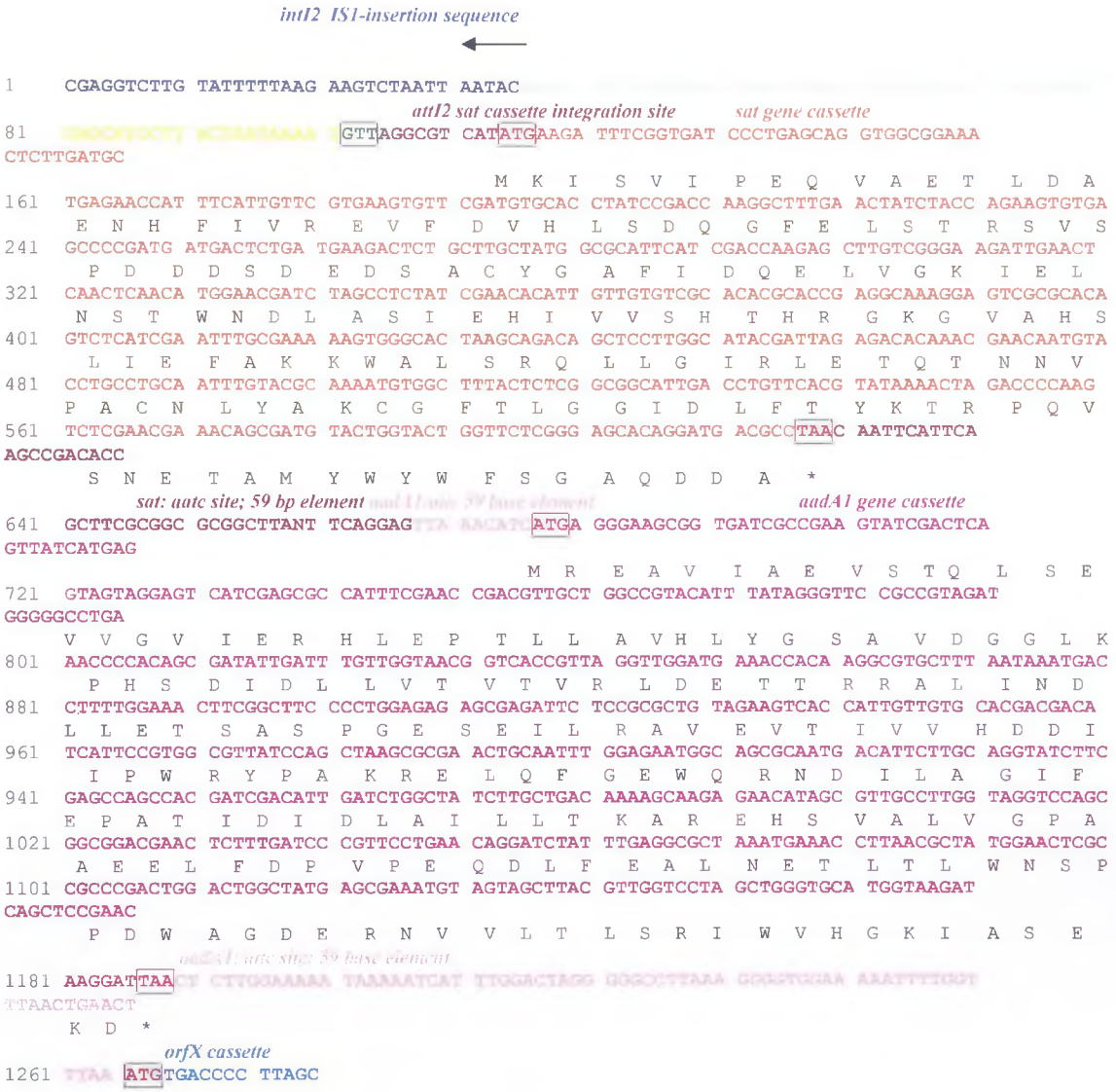
The *intI1* and *aadA1* genes had 99 % similarity with the genes on pHBH01 from *K. pneumoniae* (Genbank accession no. AJ870988) which encodes the genes *intI1*, *vim-1*, *aadA7*, *dhfr1*, *aadA1*, *qacEΔ1* and a truncated *sulI* gene. Only the *intI1* and *aadA1* genes were able to be determined from the sequence due to the quality. The *aadA1* was also confirmed due to the PCR of the gene and sequencing of the fragment.

App. A5 Annotated C361 Class 1 integron sequence with 99 % similarity to Class 1 integron encoded on the plasmid, pTET3 from *Corynebacterium glutamicum* (GenBank accession no. AJ420012) encoding the *intI1Δ1*, *aadA9*, *qacEΔ1* and *sulI* genes.

The C361 Class 1 integron encoded; *intI1Δ1* gene; P1 and P2 promoter sequences; ribosomal binding sequences with -35 and -10 regions; 5' conserved region containing the integrase binding site, aatI1; an *aadA9* gene encoding an adenyltransferase which confers resistance to streptomycin and spectinomycin, its 3' end encodes the 59 bp element with an aadA7-attC site allowing integration of the cassette with the 3' conserved region of the *qacEΔ1* gene; the *qacEΔ1* gene conferring resistance to

quaternary ammonium compounds and a *sul1* gene, conferring resistance to sulphonamides both in the 3' conserved region of the integron (Collis and Hall, 1995).

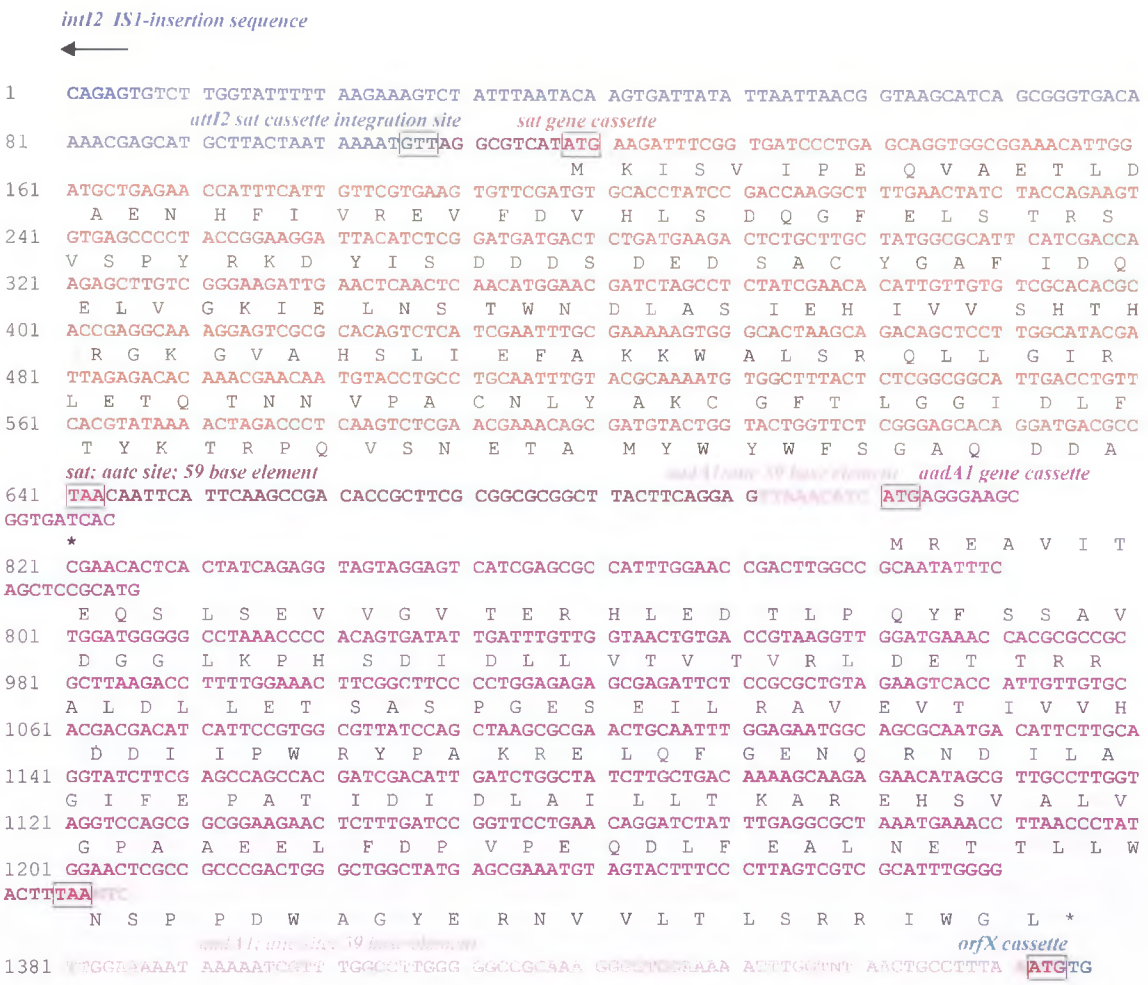
App. A6 Annotated sequence of the fragment from the Class 2 integron from isolate PGS20 (*Psychrobacter ikaite*).



sat gene encodes for streptothricin resistance (streptothricin acetyltransferase); the *aadA1* gene cassette encodes for spectinomycin resistance (aminoglycoside nucleotidyltransferase); and the *orfX* cassette gene encodes for a gene of unknown function. The insertion sequence is of Tn*7* origin. This sequence gave 98% similarity

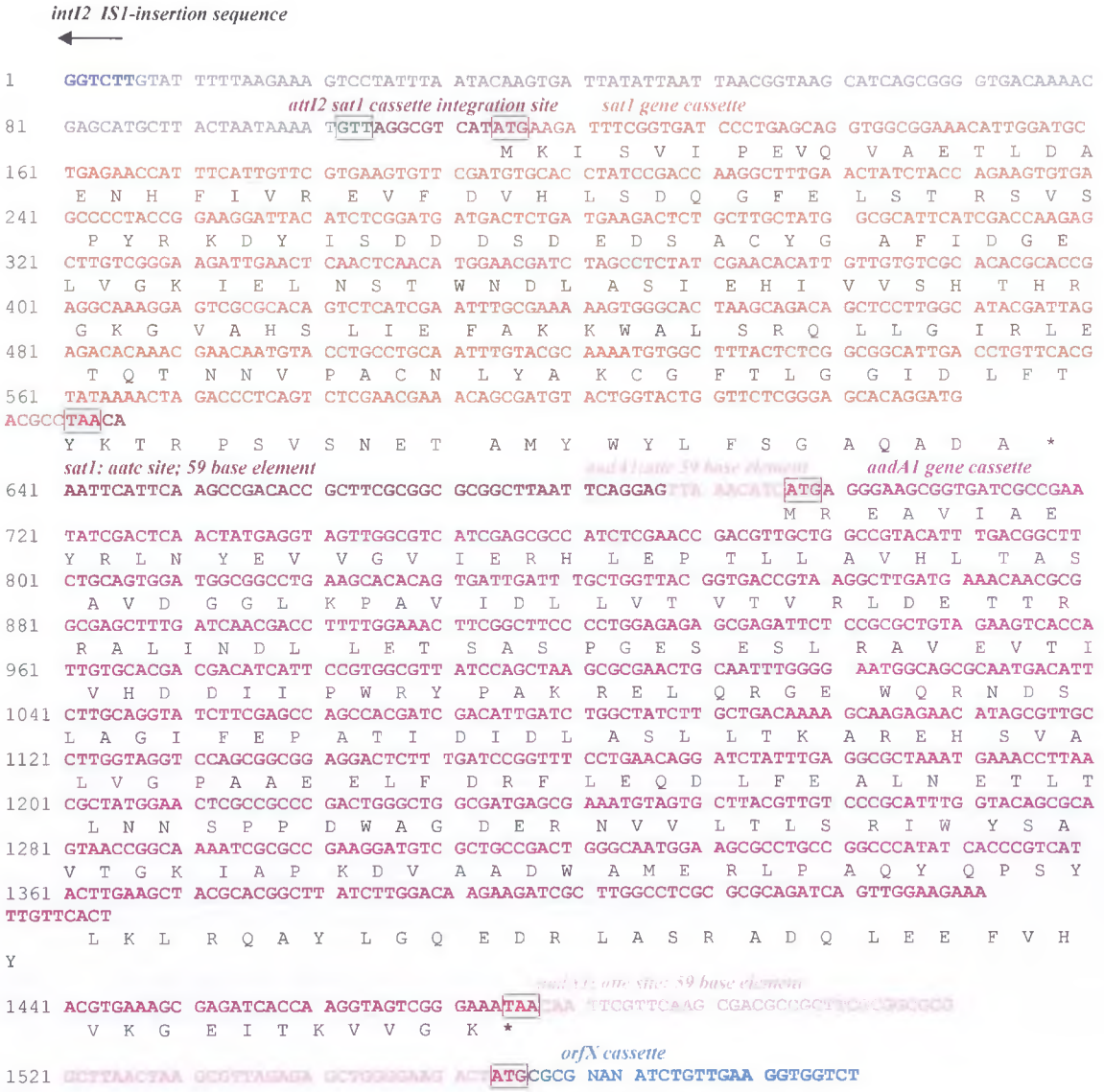
to the pIP1100 sequence from *E. coli* the integron found in this study lacked the *ereA* gene which had been inserted and integrated into the *attc* site between the *sat* and the *aadA1* gene cassettes of pIP110 (Biskri and Mazel, 2003).

App. A7 Annotated sequence of the a fragment from the Class 2 integron from the C15 isolate (*Acinetobacter lwoffi*).



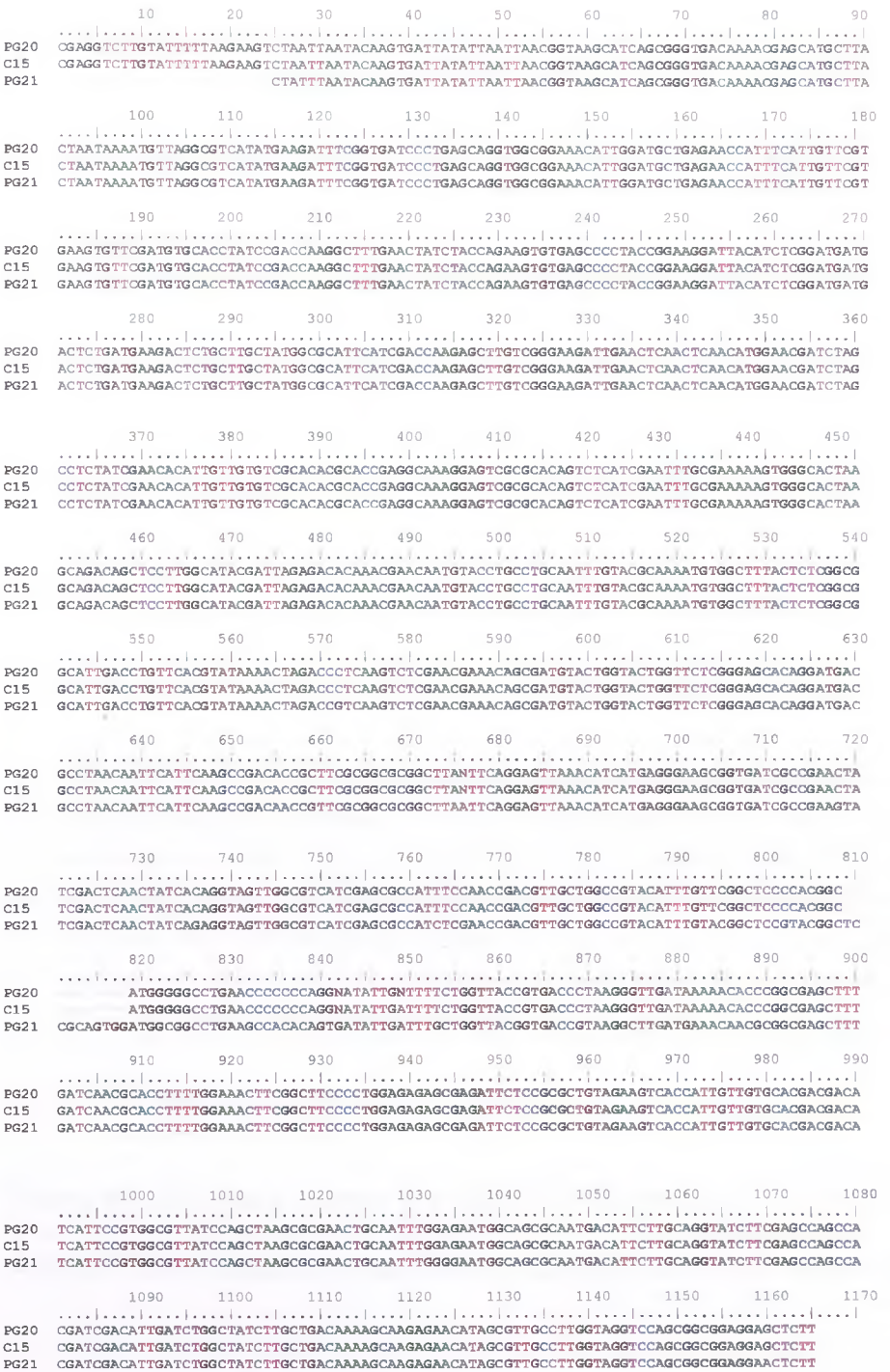
***sat* genes encodes for streptothricin resistance (streptothricin acetyltransferase); the *aadA1* gene cassette encodes for spectinomycin resistance (aminoglycoside nucleotidyltransferase); and the *orfX* cassette gene encodes for a gene of unknown function. The insertion sequence is of Tn*7* origin.**

App. A8 Annotated sequence of a fragment from the Class 2 integron from the PGS21 isolate (*Aerococcus viridians*).

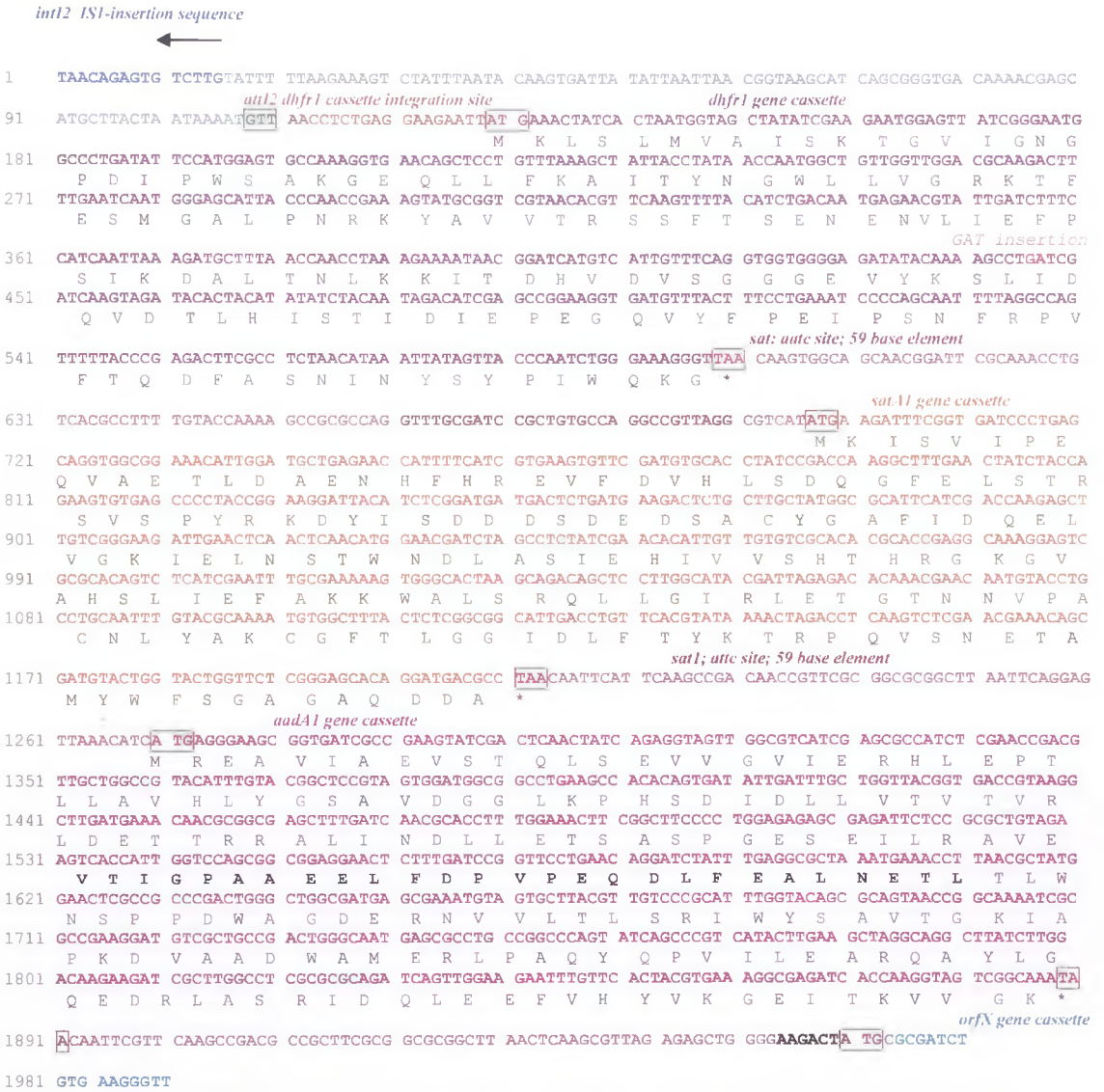


Displays 98 % similarity with the Class 2 integron from *E. coli* ABR88a (GenBank accession no. DQ286458).

App. A9 Sequence alignment of PCR fragments designated to belong to the Type 1 Class 2 integrons demonstrated in this study.

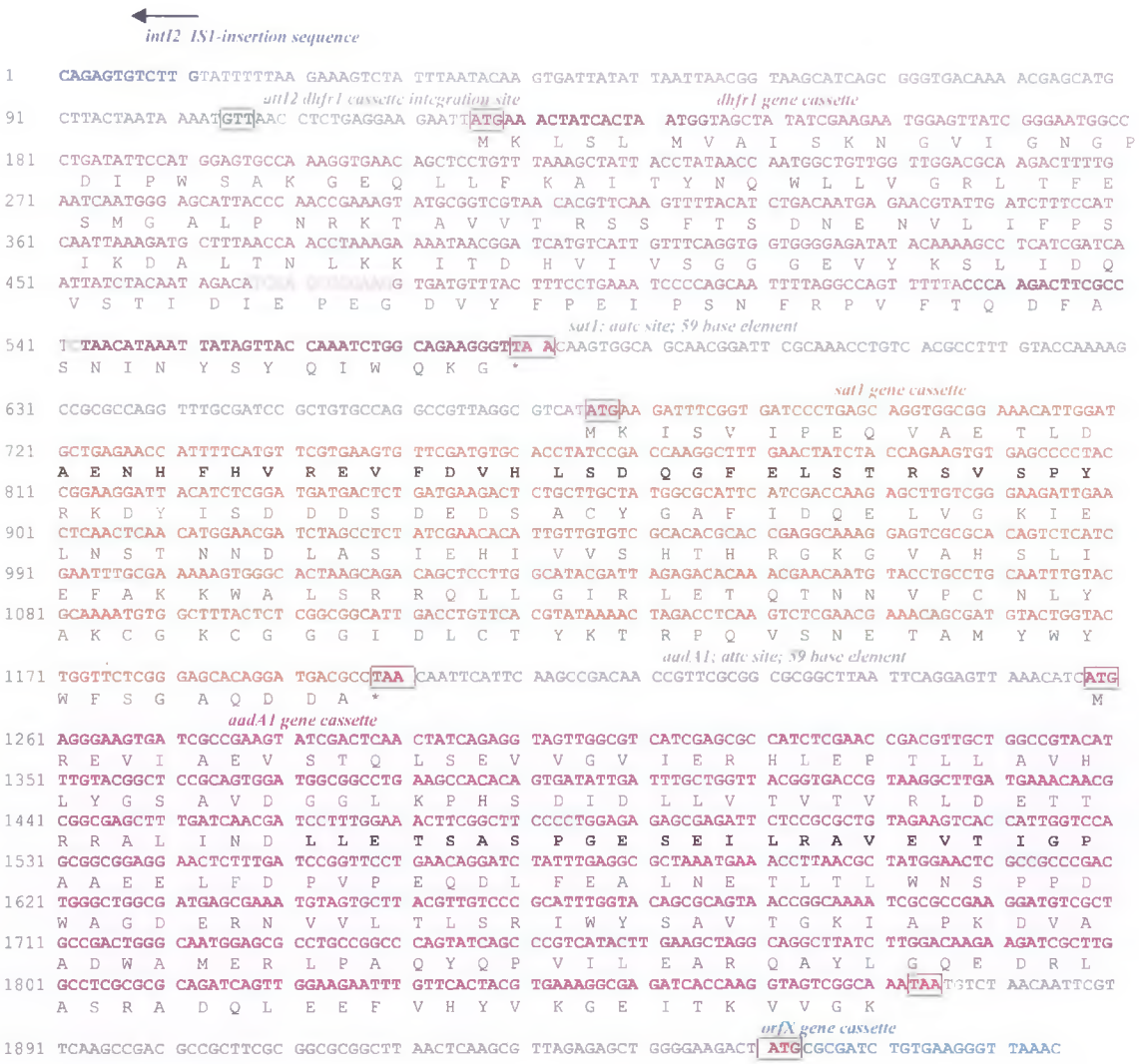


App. A10 Annotated sequence of a fragment from the Class 2 integron from PGS49 isolate (*Acinetobacter sp. AN9*).



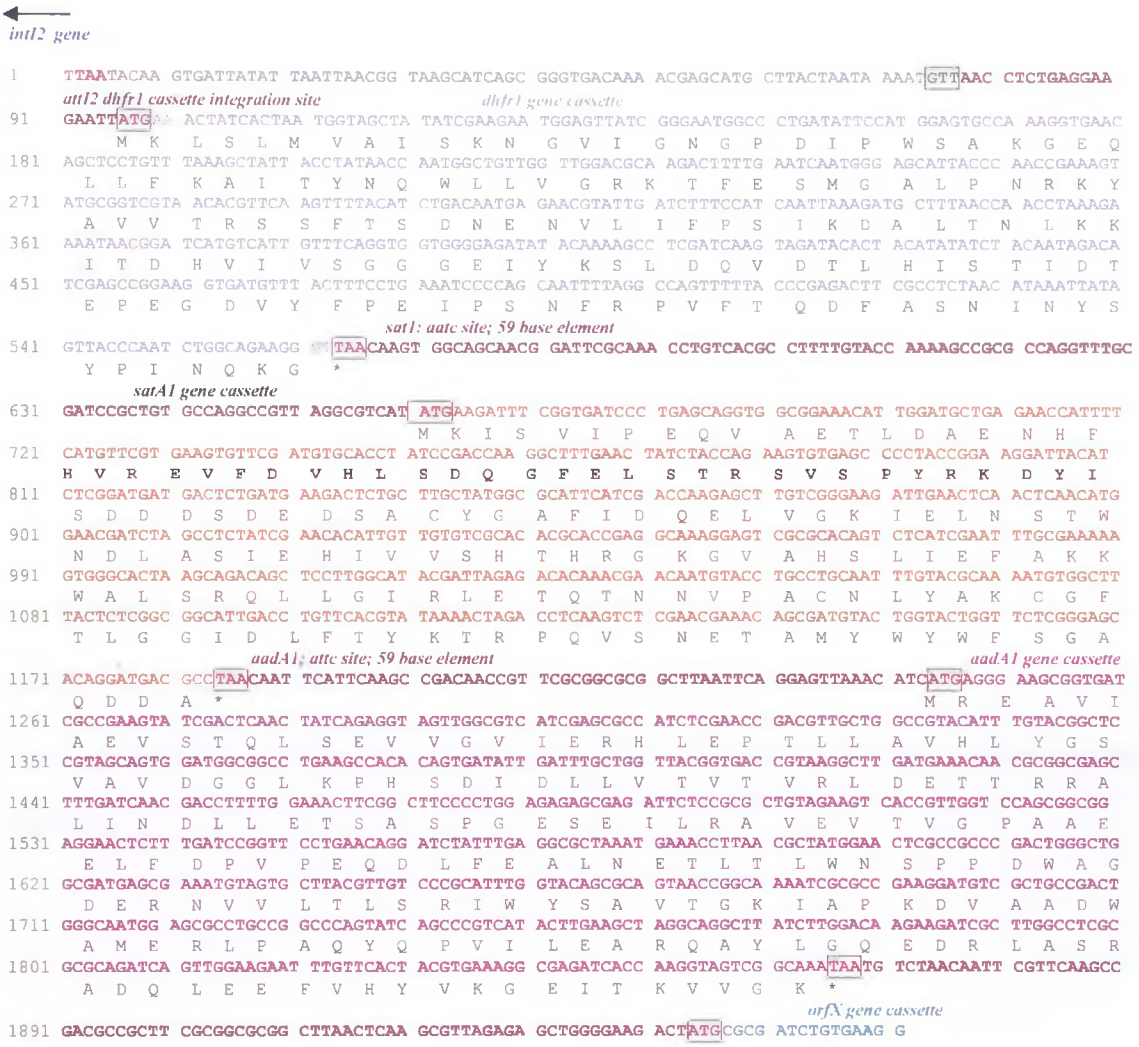
Where the *dhfr1* gene encodes for trimethoprim resistance. Displays 98 % similarity with the Class 2 integron from *Proteus mirabilis* (GenBank accession no. AY736324).

App. A11 Annotated sequence of a PCR fragment of the Class 2 integron from PGS63 isolate (*Psychrobacter* sp. A1-2).



PGS63 fragment displays 97 % similarity to PGS49 Class 2 integron fragment but lacks the GAT insertion in the *dhfr1* gene and displays 96 % similarity with the Class 2 integron from *E. coli* ABR88a (GenBank accession no. AY736324).

App. A12 Annotated sequence of a PCR fragment of the Class 2 integron from isolate PGS65 (*Psychrobacter maritimus*).

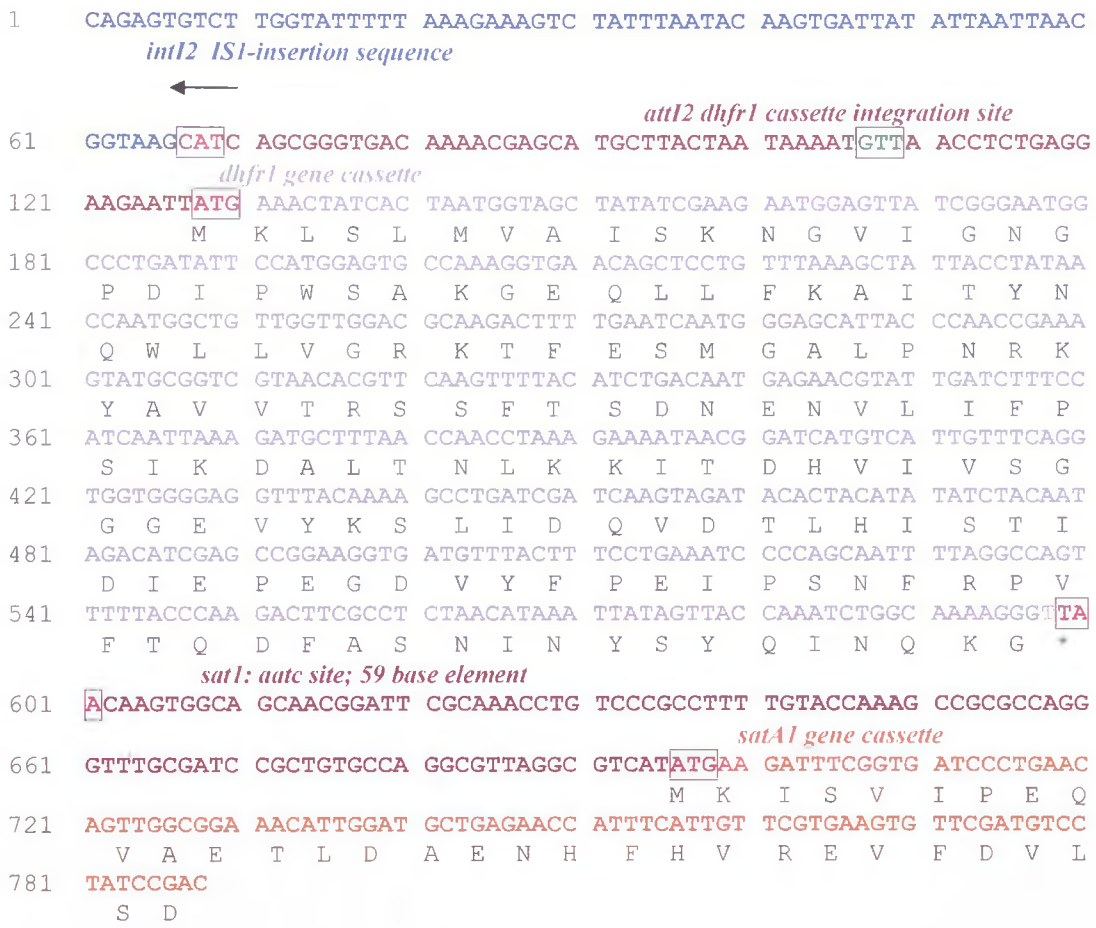


PGS65 fragment has 99 % similarity to PGS63 Class 2 integron fragment and displays 96 % similarity with the Class 2 integron from *E. coli* ABR88a (GneBank accession no. AY736324).

App. A13 Annotated sequence of a PCR fragment from the Class 2 integron of isolate C519 (*Bacillus pumilus*).

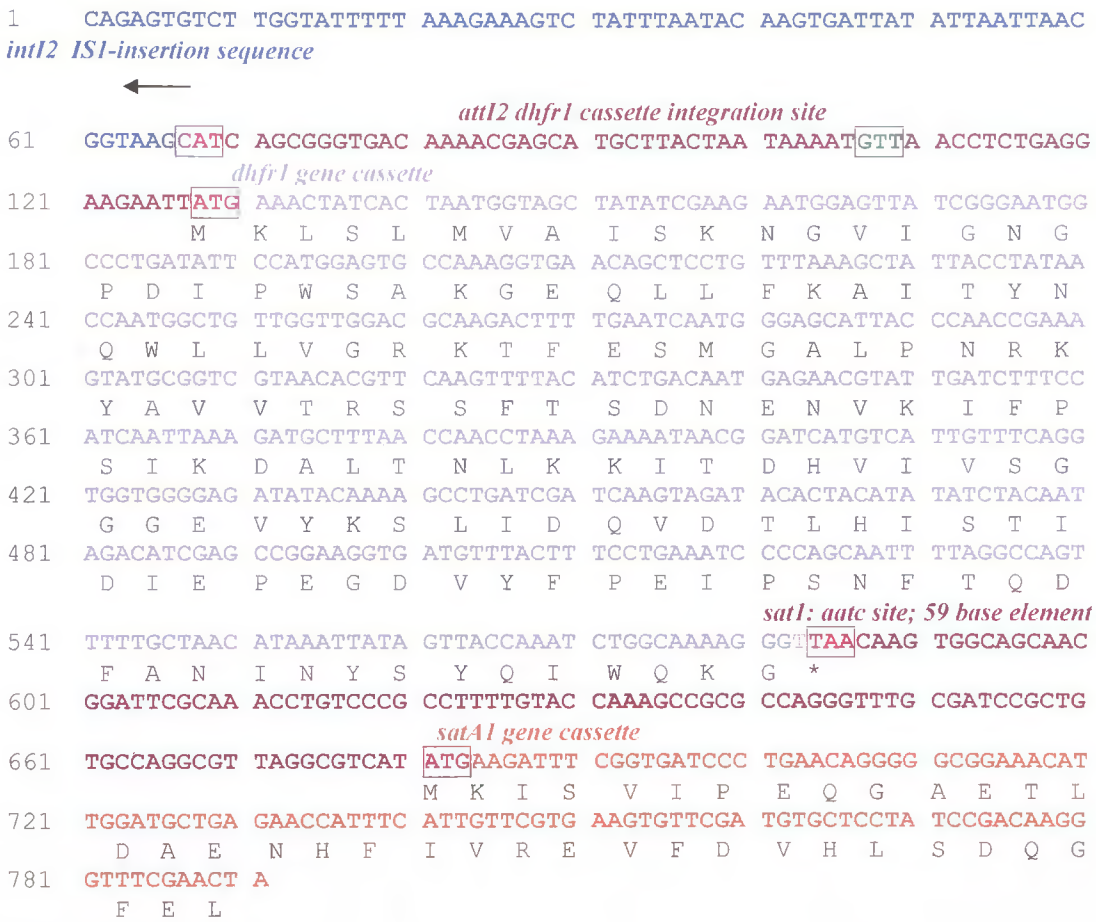
Fragment sequence gave 96 % similarity to the Tn*7* of *P. aeruginosa* (GenBank accession no. DQ176869). This fragment size was identical to those of PGS49, PGS63 and PGS65 but the first gene of the Class 2 integron fragment was sequenced as *dfrA1*, which also encodes for trimethoprim resistance but the gene nomenclature has differed.

App. A14 Annotated sequence of a PCR fragment of the Class 2 integron from isolate C410 (*Psychrobacter ikaite*).



The Class 2 integron fragment for C410 contains a *dhfr1* and a *sat1* gene. The rest of the integron is inferred due to its identical size and starting sequence to PGS49. The fragment displays 98 % similarity with the Class 2 integron from *Morganella morganii* (GenBank accession no. AJ938161).

App. A15 Annotated sequence of a PCR fragment of the Class 2 integron from isolate C402 (*Pseudomonas jessenii*).

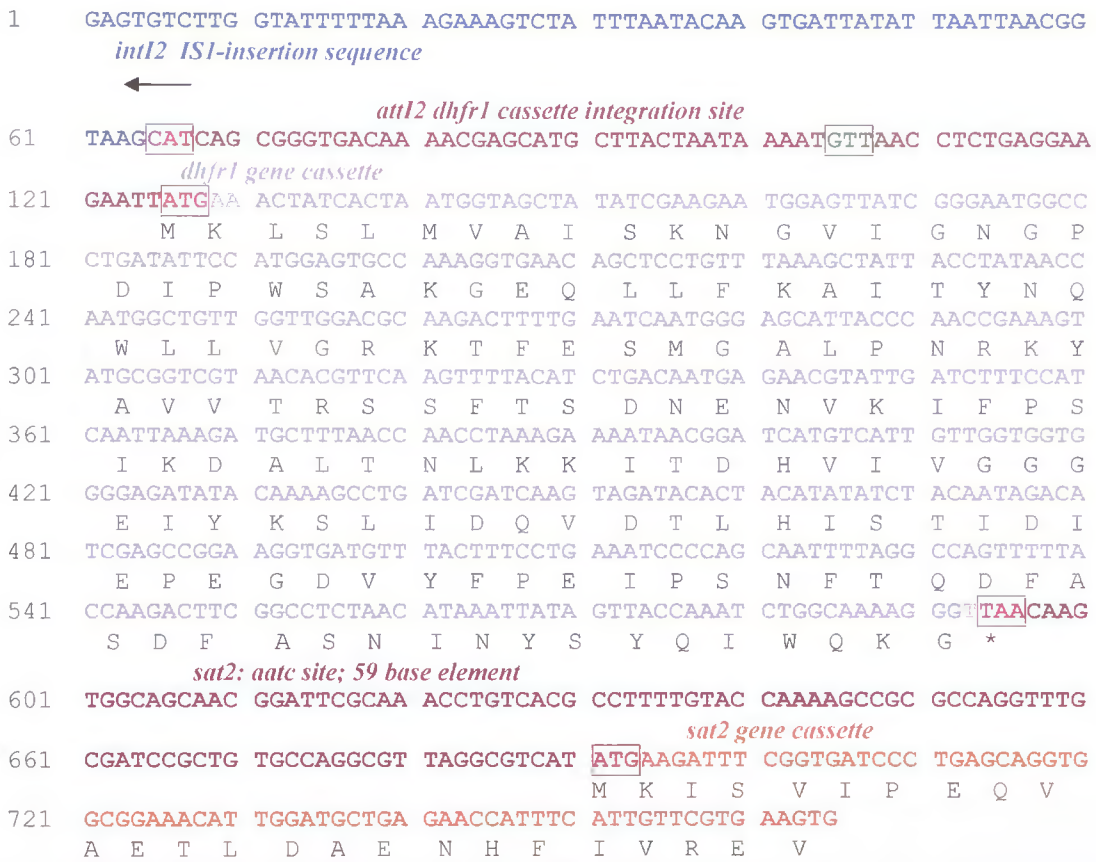


The Class 2 integron fragment for C402 contains a *dhfr1* and a *sat1* gene, as C410. The rest of the integron is inferred due to its identical size and starting sequence to PGS49. Displays 99 % similarity with the Class 2 integron from *Morganella morganii* (GenBank accession no. AJ938161).

App. A16 Annotated sequence of a fragment from the Class 2 integron from the C472 isolate (*Shigella sonnei*).

Two primer sequence runs form the fragment, containing the *dhfr1* and *sat1* gene, as C410. The rest is inferred due to its identical size and starting sequence to PGS49. Displays 99 % similarity with the Class 2 integron from a chromosomally inserted insertion sequence IS911 from *Shigella sonnei* (GenBank accession no. AY639870).

App. A17 Annotated sequence of a PCR fragment of the Class 2 integron from isolate C317 (Uncultured bacterium EV818 EBSCPSA-617).

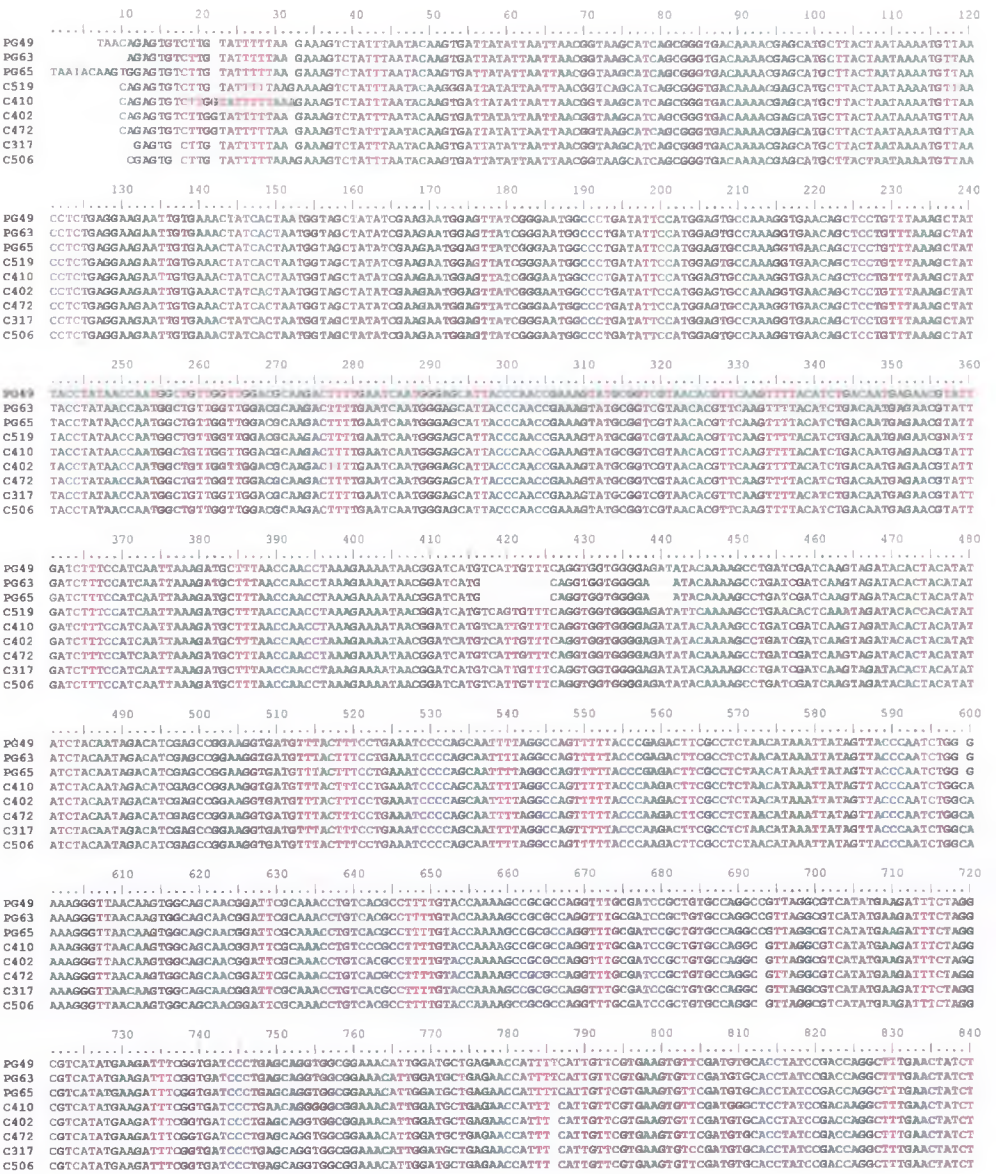


The Class 2 integron fragment for C317 encodes a *dhfr1* and a *sat2* gene. The rest of the integron is inferred due to its identical size and starting sequence to PGS49 and displays 99 % similarity with the Class 2 integron from a Tn*7* from *Acinetobacter baumanni* isolate AB300 (GenBank accession no. DQ176451).

App. A18 Annotated sequence of a PCR fragment from the Class 2 integron of isolate C506 (*Shigella flexneri*).

The Class 2 integron fragment for C506 a *dfr1* and a *sat1* gene. The rest of the integron is inferred due to its identical size and starting sequence to PGS49. Displays 99 % similarity with the Class 2 integron from *Shigella flexneri* (GenBank accession no. AB234886).

App. A19 Sequence alignment from Type 2 Class 2 integron fragments investigated in this study.



App. A20 Annotated sequence of PCR fragment from isolate C20 which contained the *sulII* and *strA* genes.

sulII gene (start site not sequenced)

```
1    TCTCCGATGG AGGCCGGTAT CTGGCGCCAC ACGCAGCCAT TGCGCAGGCG CGTAAGCTGA
       S  D  G   G  R  Y   L  A  P  D   A  A  I   A  Q  A   R  K  L  M
61   TGGCCGAGGG GGCAGATGTG ATCGACCTCG GTCCGGCATC CAGCAATCCC GACGCCGCGC
       A  E  G   A  D  V   I  D  L  G   P  A  S   S  N  P   D  A  A  P
121  CTGTTTCGTC CGACACAGAA ATCGCGCGTA TCGCGCCGGT GCTGGACGCG CTCAAGGCAG
       V  S  S   D  T  E   I  A  R  I   A  P  V   L  D  A   L  K  A  D
181  ATGGCATTCC CGTCTCGCTC GACAGTTATC AACCCGCGAC GCAAGCCTAT GCCTTGTCGC
       G  I  P   V  S  L   D  S  Y  Q   P  A  T   G  A  Y   A  L  S  R
241  GTGGTGTGGC CTATCTCAAT GATATTCGCG GTTTTCCAGA CGCTGCGTTC TATCCGCAAT
       G  V  A   Y  L  N   D  I  R  G   F  P  D   A  A  F   Y  P  Q  L
301  TGGCGAAATC ATCTGCCAAA CTCGTCGTTA TGCATTCGGT GCAAGACGGG CAGGCAGATC
       A  K  S   S  A  K   L  V  V  M   H  S  V   Q  D  G   Q  A  D  R
361  GGCGCGAGGC ACCCGCTGGC GACATCATGG ATCACATTGC GGCGTTCTTT GACGCGCGCA
       R  E  A   P  A  G   D  I  M  D   H  I  A   A  F  F   D  A  R  I
421  TCGCGGCGCT GACGGGTGCC GGTATCAAAC GCAACCGCCT TGTCCTTGAT CCCGGCATGG
       A  A  L   T  G  A   G  I  K  R   N  R  L   V  L  A   P  G  M  G
481  GGTTTTTTCT GGGGGCTGCT CCCGAAACCT CGCTCTCGGT GCTGGCGCGG TTCGATGAAT
       F  F  L   G  A  A   P  E  T  S   L  S  V   L  A  R   F  D  E  L
541  TGCGGCTGCG CTTCGATTTG CCGGTGCTTC TGTCTGTTTC GCGCAAATCC TTTCTGCGCG
       R  L  R   F  D  L   P  V  L  L   S  V  S   R  K  S   F  L  R  A
601  CGCTCACAGG CCGTGGTCCG GGGGATGTCG GGGCCGCGAC ACTCGCTGCA AAGCTTGCCG
       L  T  G   R  G  P   G  D  V  G   A  A  T   L  A  A   E  L  A  A
661  CCGCCGCAGG TGGAGCTGAA TTCATCCGCA CACACGAGCC GCGCCCCTTG CGCGACGGGC
       A  G  G   G  A  E   F  I  R  T   H  D  P   R  P  L   R  D  G  L
721  TGGCGGTATT GGCGGCGCTG TAATTCGTGA ATTGCAAATT CGCTATATTT CTCGATATTC
       A  V  L   A  A  L
781  GCGCTTCATC AGAAAACTGA AGGAACCTCC ATTGAATCGA ACTAATATTT TTTTTGGTGA

841  ATCGCATTCT GACTGGTTGC CTGTCAGAGG CGGAGAATCT GGTGATTTTG TTTTTCGACG
                    strA gene
901  TGGTGACGGG CATGCCTTCG CGAAAATCGC ACCTGCTTCC CGCCGCGGTG AGCTCGCTGG
                M  L  R   K  I  A   P  A  S   R  R  G  E   L  A  G
961  AGAGCGTGAC CGCCTCATTT GGCTCAAA
       E  R  D   R  L  I  W   L  K
```

Where the *sulII* gene encodes for sulphonamide (an alternative DHPS) and *strA* gene.

CHAPTER 10:

BIBLIOGRAPHY

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