

**The identification and characterisation of novel antimicrobial resistance
genes from human and animal metagenomes**

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This thesis is submitted for the degree of PhD

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Declaration

I, Liam Joseph Reynolds, confirm that the work detailed in this thesis is my own except where stated otherwise.

Saliva samples were collected from volunteers by Mr. Supatthep Tansirichaiya (Eastman Dental Institute, UCL, United Kingdom). Calf faeces was collected by various members of staff at the Animal Plant and Health Agency.

The creation of the human saliva and calf faecal 16S Illumina libraries was conducted in conjunction with Ms. Catryn Williams (Eastman Dental Institute, UCL, United Kingdom).

Illumina MiSeq sequencing of these 16S libraries was carried out by Dr. Tony Brooks (Institute of Child Health, UCL, United Kingdom).

End sequencing of a subset of the human saliva metagenomic library was conducted by myself and Mrs. Supanan Sucharit.

A subset of the human saliva metagenomic library was screened by Mr. Gianmarco Cristarella under my supervision as part of his master's research project. Analysis of the CTAB-resistant clone identified during this screen was completed in conjunction with Mr. Gianmarco Cristarella and Mr. Supatthep Tansirichaiya.

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Abstract

Antimicrobial resistance genes are harboured by bacteria in the human oral cavity and ruminant faeces and they are shed in particularly high abundances in calf faeces.

Furthermore, bacteriocin (antimicrobial peptide) producing bacteria have been isolated from these environments. In recent times bacteriocins have received much attention as potential alternatives to antibiotics.

Human saliva and calf faeces harbour 'yet-to-be cultured bacteria' that can only be studied by analysing their DNA. To this end, two metagenomic libraries were created from human saliva and calf faeces metagenomic DNA with the aim of identifying novel antimicrobial resistance and bacteriocin genes. Screening these libraries for tetracycline resistance identified two tetracycline resistant clones.

Clone PS9 was also tigecycline resistant and contained a 7,765 bp insert that encoded two half-ABC transporter genes; subcloning of these genes showed that they were responsible for the observed resistance phenotype. As the ABC transporter conferred resistance only to tetracyclines and its putative amino acid sequence showed <80 % identity to known tetracycline resistance proteins, it was named TetAB(60).

Clone TT31 contained a 14,226 bp insert. 7, 216 bp of the insert had 97 % nucleotide identity to Tn916 and contained part of *tet(M)* and a full length *tet(L)* gene. This gene organisation has not been described in Tn916-like elements and it may represent a novel Tn916-like element.

The human saliva library was also screened for antiseptic resistance revealing a CTAB resistant clone. Random transposon mutagenesis of the 19.1 Kb insert and subcloning of a UDP-glucose 4-epimerase revealed it to be solely required for the observed resistance.

This study identified novel tetracycline, tigecycline and CTAB resistance genes from the human saliva metagenome, demonstrating the importance of this environment as a source of resistance genes that may compromise the effectiveness of these antibiotics and antimicrobials. Additionally, this work highlights the relevance of house-keeping genes to the development of antimicrobial resistance.

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Abbreviations

ABC	ATP-Binding Cassette Protein
aa-tRNA	Aminoacyl-transfer Ribonucleic Acid
AEP	Acquired Enamel Pellicle
AmpR	Ampicillin Resistance
ARG	Antimicrobial Resistance Gene
ATP	Adenosine Triphosphate
BAC	Bacterial Artificial Chromosome
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
°C	Degree Celsius
cDNA	Complementary DNA
CHX	Chlorhexidine
CFU	Colony Forming Unit
CmR	Chloramphenicol Resistance
CPC	Cetylpyridinium Chloride
CTAB	Cetyltrimethylammonium bromide
cIAI	Complicated Intra-abdominal Infections
cUTI	complicated urinary tract infections
CSP	Competence Stimulating Peptide
CTn	Conjugative transposon
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
ECDC	European Centre for Disease Control
EmrR	Erythromycin Resistance
EPS	Extracellular Polymeric Substances
ED ₅₀	Median Effective Dose
EF (EF-G and EF-Tu)	Elongation Factor
EDTA	Ethylenediaminetetraacetic acid
ESBL	Extended Spectrum β -lactamase

EtOH	Ethanol
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
FACS	Fluorescence Associated Cell Sorting
g	Gravitational Force
GCF	Gingival Crevicular Fluid
GI	Gastrointestinal
GlcNAc	N-acetylglucosamine
GRAS	Generally Regarded As Safe
GFP	Green Fluorescence Protein
GTP	Guanosine Triphosphate
h	Hour
HGT	Horizontal Gene Transfer
IM	Inner Membrane
IPTG	Isopropyl- β -D-thiogalactopyranoside
IS	Insertion Sequence
Kb	Kilobase
Kg	Kilogram
LAB	Lactic Acid Bacteria
LB	Luria Bertani
LPS	Lipopolysaccharide
MATE	Multidrug and Toxin Compound Extrusion
μ l	Microlitre
μ m	Micrometer
M	Molar
MDR	Multidrug resistance
MBL	Metallo- β -lactamase
MFS	Major Facilitator Super family
MIC	Minimum Inhibitory Concentration
min	Minute
mg	Milligram

MGE	Mobile Genetic Element
ml	Millilitre
MLS	Macrolide, Lincosamide and Streptogramin
μ F	microfarad
μ M	Micromolar
mM	Millimolar
mRNA	Messenger RNA
MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
MurNAc	N-acetylmuramic acid
mV	Millivolts
NADPH	Nicotinamide adenine dinucleotide phosphate
NBD	Nucleotide Binding Domain
NCBI	National Centre for Biotechnology Information
ng	Nanogram
nm	Nanometer
NMR	Nuclear Magnetic Resonance
NaOAc	Sodium Acetate
Ω	Ohm
OM	Outer Membrane
OMP	Outer Membrane Porin
OD	Optical Density
ORF	Open Reading Frame
OriT	Origin of Transfer
OTU	Operational Taxonomic Unit
PBP	Penicillin Binding Proteins
PCR	Polymerase Chain Reaction
PIGEX	Product Induced Gene Expression
PTMs	Post Translational Modifications
QAC	Quaternary ammonium compound
qPCR	Quantitative Polymerase Chain Reaction
RBS	Ribosome Binding Site

rDNA	Ribosomal Deoxyribonucleic Acid
RNA	Ribonucleic Acid
RNAP	RNA Polymerase
RND	Resistance Nodule Division
ROS	Reactive Oxygen Species
rRNA	Ribosomal Ribonucleic Acid
RPM	Revolutions Per Minute
RPP	Ribosomal Protection Proteins
RT-PCR	Real Time Polymerase Chain Reaction
S (16S)	Svedberg unit
s	Second
SBL	Serine β -lactamase
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SIGEX	Substrate Induced Gene Expression
SMR	Small Multidrug Resistance
TMD	Transmembrane Domain
Tn	Transposon
tRNA	Transfer Ribonucleic Acid
VRE	Vancomycin Resistant <i>Enterococcus</i>
U	1 Unit of enzyme required to digest 1 μ g of DNA in 1 h at 37°C
X-gal	5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside

Chapter 1

Introduction

1.1 Antibiotics: Discovery

1.1.1 Pre-Antibiotic Era

The time before antibiotics were discovered and brought into large scale use is often referred to as the 'pre-antibiotic era'. By the beginning of the First World War it was widely accepted that microorganisms caused infection but even with this knowledge treatment options were limited. Wound infections were treated with saline, antiseptics such as iodine and in some cases corrosive compounds including H₂O₂ (Runcie, 2015). As a result of these treatment limitations infections were associated with high mortality rates. In fact, a retrospective epidemiological study of the Third Ottoman Army (March 1915 – February 1916) revealed that typhus and dysentery were the cause of 48 % of all recorded deaths during this time (Erdem *et al.*, 2011).

High mortality rates due to infection were not isolated to the frontline; civilian infection cases had equally high mortality rates, and in 1890 the death rate due to infection is believed to have been 797 per 100,000 people (Walsh and Wright, 2005). In fact, it has been estimated that one third of all deaths were a result of just three infections: pneumonia, tuberculosis (TB) and infections that caused diarrhoea (Department of Commerce and Labor, 1906).

1.1.2 Discovery and Introduction of Antibiotics

Some antimicrobials were used (with mixed success) during the 'pre-antibiotic era'. For instance, Emmerich and Low used a *Pseudomonas aeruginosa* extract called pyocyanase as an antimicrobial therapy. However, due to instabilities in the extract and its toxic nature, development of this treatment ceased (Hays *et al.*, 1945). Later, in 1909 Paul Ehrlich identified an arsenic-containing molecule with activity against *Treponema pallidum*, the causative agent of syphilis. The drug was marketed as Salvarsan and was successfully used in treating this infection for over 30 years (Ehrlich, 1910).

Following the discovery of Emmerich's and Ehrlich's compounds came the famous discovery of penicillin by Alexander Fleming who in 1928 identified contaminating moulds producing zones of clearance in *Staphylococcus aureus* growing on agar plates (Fleming, 1929).

Penicillin was the first in a series of antibiotic discoveries during a time referred to as the 'golden age' of antibiotic discovery (Lewis, 2013). It was followed by prontosil, the first sulfadruug identified in 1932, which was actually marketed before penicillin, while the 1940s saw the discovery of five classes of antibiotics, the chloramphenicols, tetracyclines, aminoglycosides, macrolides and fidaxomicin, Table 1.1.

Table 1.1 Timeline of Antibiotic Discovery.

Antibiotic Class	Year of Discovery	Year Introduced	Mechanism of Action
Sulfadruugs; prontosil	1932	1936	Dihydropteroate synthetase inhibition
β -lactams; penicillin	1928	1938	Cell wall synthesis inhibition
Aminoglycosides	1943	1946	Binds 30S ribosomal subunit
Chloramphenicols	1946	1948	Binds 50S ribosomal subunit
Macrolides	1948	1951	Binds 50S ribosomal subunit
Tetracyclines	1944	1948	Binds 30S ribosomal subunit
Rifamycins	1957	1958	Binds RNA polymerase β -subunit
Glycopeptides	1953	1958	Cell wall synthesis inhibition
Quinolones	1961	1968	Inhibitor of DNA Synthesis
Oxazolidinones	1955	2000	Binds 50S ribosomal subunit

Amended from Lewis *et al.* (Lewis, 2013).

The introduction of antibiotics marked a dramatic change in medicine and paved a path for the development of modern medical practices. Not only did the use of antibiotics allow for the treatment of infectious diseases but also for the development of more invasive life-saving surgeries and cancer treatments when used prophylactically.

1.1.2.1 Tetracyclines

1.1.2.1.1 Discovery and Structure

In 1948 the antibiotic aureomycin, isolated from *Streptomyces aureofaciens*, was first described in the literature and in the same year received Food and Drug Administration (FDA) approval (Duggar, 2011). A semi-synthetic derivative with increased solubility and antimicrobial activity called tetracycline was created from this, Figure 1.1(b) (Conover *et al.*, 1953).

The structure of tetracycline was determined following its introduction to clinical use and was found to have a characteristic naphthacene core which is composed of four linearly arranged 6-membered rings, Figure 1.1 (a) (Stephens *et al.*, 1954). The elucidation of the structure of tetracyclines allowed for further development of semisynthetic derivatives of this core structure resulting in the synthesis of the second-generation doxycycline (lacks the 6-hydroxy group) and through modification of this, minocycline (contains a 7-dimethyl group) (Stephens *et al.*, 1963; Martell and Boothe, 1967). More recently the third-generation tigecycline containing a 7-dimethylamine and a 9-dimethylglycylamido side chain was developed and brought to market, Figure 1.1 (c) (Zhanel *et al.*, 2004).

Figure 1.1

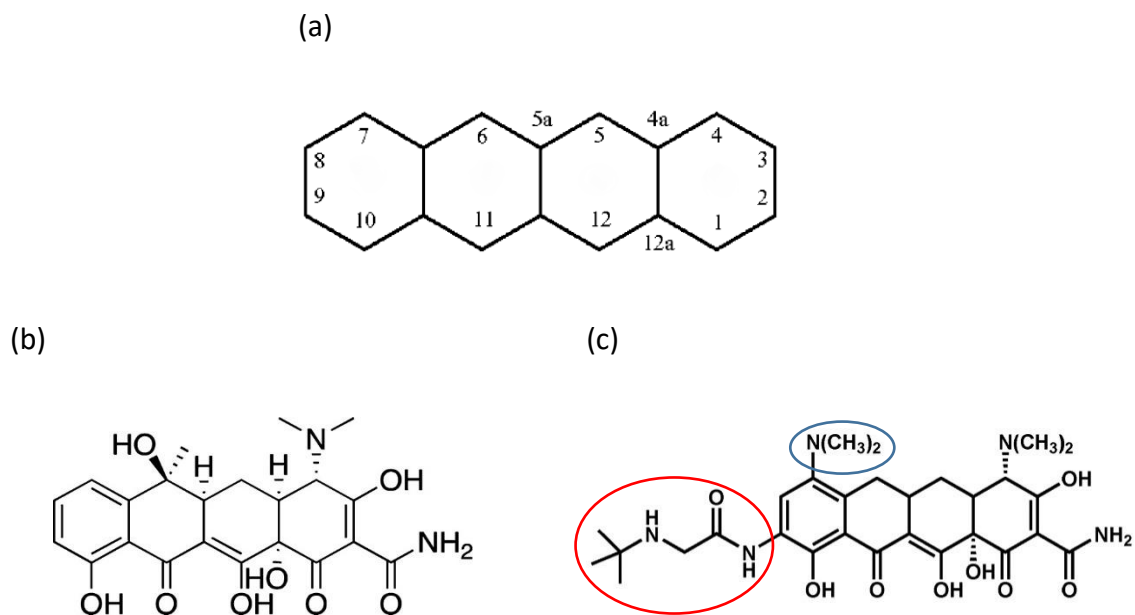


Figure 1.1 Structure of Tetracyclines. (a) A depiction of the naphthalene core of the tetracycline class with the carbon atoms numbered (1, 1a, 2, 2a...). (<http://patentimages.storage.googleapis.com>) (b) Shows the structure of tetracycline, figure reproduced from Jameson *et al.* (Jameson *et al.*, 2012). (c) Illustrates the structure of tigecycline, the 7-dimethylamine and the 9-dimethylglycylamido side chains are circled in blue and red respectively. This figure was reproduced from Olson *et al.* (Olson *et al.*, 2006).

1.1.2.1.2 Mode of Action

Tetracycline is a broad-spectrum bacteriostatic antibiotic (as it stalls the growth of the bacterial cell rather than killing it) with activity against Gram-positive and Gram-negative pathogens. It elicits its bacteriostatic effect by interfering with the cell's ability to synthesise proteins (Chopra and Roberts, 2001).

Tetracycline is an ionophore as it can coordinate dicationic metals including Ca^{2+} and Mg^{2+} via ketone and hydroxyl structures at carbon 11 and 12, and by the ketone, carboxamide and hydroxyl groups at positions, 1, 2 and 3 respectively, Figure 1.1 (a-c), Figure 1.2 and Figure 1.2 (a-b) (Chopra *et al.*, 1992; Nelson, 1998).

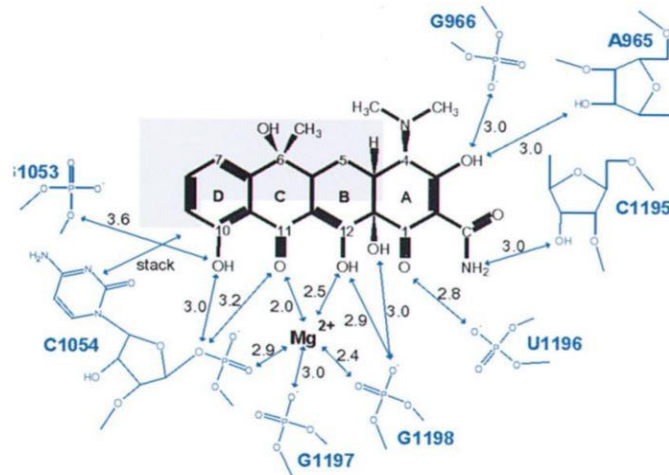
Tetracycline, as a dicationic metal complex, can enter Gram-negative cells through the OmpF and OmpC outer membrane porins (OMPs) (Mortimer and Piddock, 1993; Thanassi *et al.*, 1995). In fact, mutants exhibiting a loss of or reduced *ompF* expression showed reduced tetracycline susceptibility as they accumulated less of the antibiotic (Cohen *et al.*, 1988; Thanassi *et al.*, 1995). Once in the periplasm it is believed that tetracycline is neutralised before it diffuses through the lipid bilayer of the inner membrane (IM) (Sigler *et al.*, 2000). Similarly, the neutral form of tetracycline is able to traverse the Gram-positive peptidoglycan cell wall and diffuse across its cell membrane to enter the cytoplasm (Nikaido and Thanassi, 1993).

Once in the cytoplasm tetracycline chelates Mg^{2+} and in this form, targets the 30S ribosomal subunit. Crystal structures of the 30S ribosomal subunit of *Thermus thermophilus* in complex with tetracycline were obtained independently by Brodersen *et al.* (2000) and Pioletti *et al.* (2001). These structures revealed six binding sites for tetracycline on the 30S subunit. The so-called primary or Tet-1 binding site is positioned above the tRNA binding site. When the tetracycline- Mg^{2+} complex enters the site, it interacts with the phosphate backbone of 16S rRNA forming salt bridges. This blocks entry of charged tRNA molecules in the A site of the ribosome, stalling peptide chain extension. Four of the remaining tetracycline binding sites are located on the 16S rRNA with the final site found in a hydrophobic pocket on the ribosome peptide. Tetracycline binding at these sites are believed to act in synergy with

binding at the primary site to halt protein translation (Brodersen *et al.*, 2000; Pioletti *et al.*, 2001). The recently published x-ray crystallography structure of tigecycline binding the 30S ribosomal subunit of *Thermus thermophilus* revealed a similar pattern of binding by these two antibiotics, although the bulky 9-dimethylglycylamido side chain was observed to interact with the A-site and 16S rRNA likely creating further steric interference for the incoming tRNA molecules and preventing conformational changes required for the extension of the peptide chain, Figure 1.2(b) (Schedlbauer *et al.*, 2015). These observations are in agreement with *in vitro* translation and competition assays that showed tigecycline to be able to bind the ribosome with 100-fold greater affinity and inhibit translation with 20-fold greater efficiency than tetracycline (Olson *et al.*, 2006). It has been posited that the selective inhibition of prokaryotic protein synthesis by tetracyclines is a result of eukaryotic ribosomes being protected in the nucleus. In this cell organelle there is less Mg^{2+} available for chelation by tetracycline and so the antibiotic's affinity for the eukaryotic ribosome is reduced compared with the prokaryotic ribosome (Chukwudi *et al.*, 2016).

Figure 1.2

(a)



(b)

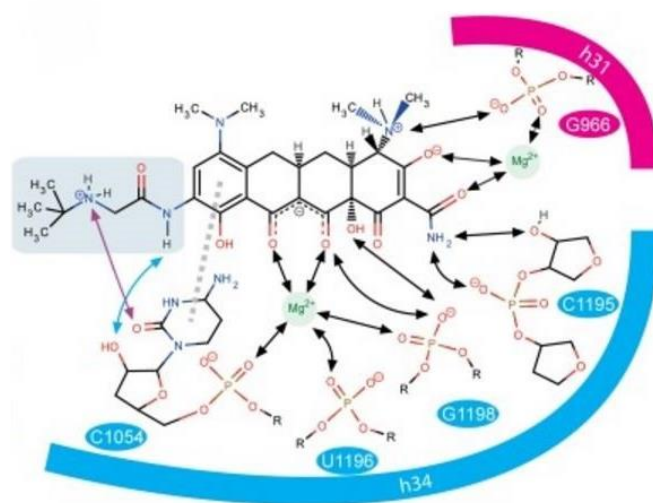


Figure 1.2 Ribosomal Binding Sites of Tetracycline and Tigecycline. (a) The interactions between Mg²⁺ chelating tetracycline (black) and the 16S rRNA backbone in the 30S ribosome (blue), the shaded area is the region of the naphthacene core that can be modified without reducing the antibiotic's activity, reproduced from Hlavka and Booth (Hlavka and Booth, 1985). (b) tigecycline molecule (black) chelating two Mg²⁺ ions and binding the 16S rRNA molecule (blue and pink). The shaded region indicates the 9-dimethylglycylamido that also interacts with the 16S rRNA molecule, reproduced from Schedlbauer *et al.* (Schedlbauer *et al.*, 2015).

1.1.2.1.3 Clinical Uses of Tetracyclines

Although the rise of antimicrobial resistance has resulted in reduced reliance on this group of antibiotics they are still used to treat some human infections including those caused by Chlamydia, acne and some eye infections such as trachoma (Hu *et al.*, 2010; Dukers-Muijers *et al.*, 2015; Zaenglein *et al.*, 2016).

Tigecycline is administered intravenously to treat skin and abdominal infections as well as some cases of community acquired pneumonia (Rubinstein and Vaughan, 2005; Shen *et al.*, 2015; Van Berkel *et al.*, 2016). Additionally, the novel tetracycline derivatives omadacycline and eravacycline are currently in phase 3 trials and show promise as novel therapeutics as discussed in 1.1.2.1.5.

1.1.2.1.4 Agricultural Uses of Tetracyclines

Tetracyclines were among the first classes of antibiotics to be identified as having growth promoting properties in feed animals, as fermentation waste containing traces of aureomycin were shown to increase the growth of pigs (Jukes *et al.*, 1950). Though antibiotics are now banned as growth promoters in the EU [European Parliament and Council Regulation (EC) No. 1831/2003] they are still used in this capacity in the USA. In the UK tetracyclines are also the most purchased antibiotic for agricultural use representing 40 % of total sales in 2014 (Veterinary Medicines Directorate, 2015).

Tetracyclines are also used therapeutically in livestock. Oxytetracycline for example is one of the antibiotics of choice for treating bovine pneumonia and is also used in the treatment of swine dysentery and enteritis when *Escherichia coli* or *Clostridium perfringens* is the causative agent (Dunlop *et al.*, 1998; Constable *et al.*, 2009).

1.1.2.1.5 Novel Tetracycline Derivatives

Omadacycline is a tetracycline derivative and the first of the class aminomethylcyclines. The antibiotic has activity against *S. aureus* and *Haemophilus influenzae* as well as *Streptococcus* spp. and *Legionella* spp. It has also been demonstrated to overcome the activity of efflux and ribosomal protection protein (RPP) mechanisms of resistance as a result of a dimethylamine and a 2,2-(dimethylpropyl) aminomethyl modification on the seventh and ninth carbon respectively, Figure 1.3 (Macone, 2014; Draper *et al.*, 2014). It had minimum inhibitory concentrations (MIC) of 0.125 µg/ml and 0.25 µg/ml against *S. aureus* strains expressing *tet(M)* (encodes an RPP) or *tet(K)* (an MFS transporter) respectively, which represents a greater than 128-fold difference compared with tetracycline (Draper *et al.*, 2014). Paratek Pharmaceuticals, Inc. are currently conducting phase 3 trials for the intravenous and oral use of omadacycline in treating skin infections and phase 3 trials for its use in treating community-acquired bacterial pneumonia are also planned (Paratek, 2016; Sun *et al.*, 2016).

Figure 1.3

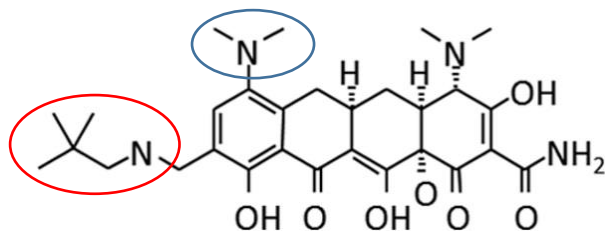


Figure 1.3 Omadacycline Structure. A diagram of the structure of omadacycline with the 7-dimethylamine and 2,2-(dimethylpropyl) aminomethyl side chain highlighted by a blue and red circle respectively, reproduced from Draper *et al.* (Draper *et al.*, 2014).

Eravacycline is a fully synthetic tetracycline also currently being evaluated and is of the fluorocycline family, Figure 1.4. It exhibits a broad spectrum of activity being able to inhibit methicillin-susceptible and methicillin-resistant *S. aureus* (MIC₉₀ of 0.13-0.25 µg/ml), *E. coli* (MIC₉₀ of ≤0.5 µg/ml), *K. pneumoniae* (MIC₉₀ of 2 µg/ml) and anaerobic pathogens including *Clostridium difficile* (MIC₉₀ of 0.13 µg/ml). It has also been demonstrated to be more efficacious (MIC₉₀ values were ≥2-fold lower) than tigecycline against a range of pathogens including *E. coli*, *S. aureus* and various *Bacteroides* spp. (Sutcliffe *et al.*, 2013). Tetrphase Pharmaceuticals, INC are planning phase 3 clinical trials for the intravenous use of eravacycline in the treatment of complicated intra-abdominal infections (cIAI) and complicated urinary tract infections (cUTI) (Tetrphase, 2016; Solomkin *et al.*, 2016).

Figure 1.4

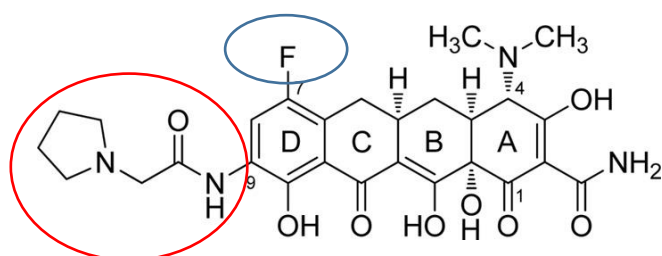


Figure 1.4 Eravacycline Structure. The eravacycline structure with the fluoride and 2-(pyrrolidin-1-yl)ethanamide side chains highlighted in blue and red circles respectively (Sutcliffe *et al.*, 2013).

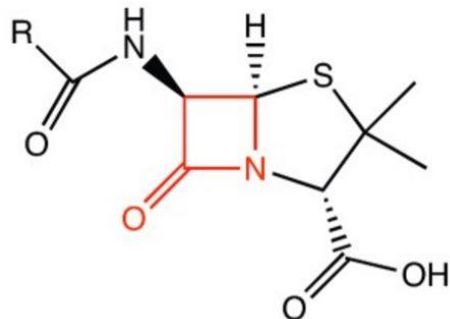
1.1.2.2 β -lactams

1.1.2.2.1 Structure and Diversity

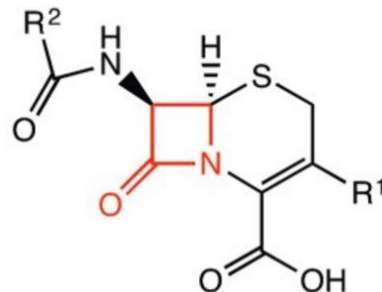
Penicillins contain a 5-membered thiazolidine ring fused to a 4-membered β -lactam ring to which a variable acyl side chain is attached, Figure 1.5 (a): It is alterations in this side chain that produce different penicillin antibiotics. Following the discovery of penicillin, more classes of β -lactams were discovered. The cephalosporins and cephamycins were discovered in the 1950s and 1970s respectively, and both contained the typical β -lactam ring fused to a 6-membered dihydrothiazine ring, Figure 1.5(b) (Newton and Abraham, 1955). The first carbapenem was isolated from *Streptomyces cattleya*, and like the penicillins contains a β -lactam ring fused to a 5-membered ring, although this ring contains no sulfur atom and is unsaturated, Figure 1.5(c) (Kahan *et al.*, 1979). The monobactams are the most recently identified β -lactams to be used clinically. They are typified by the presence of a β -lactam ring that is not fused to another ring structure, Figure 1.5(d) (Sykes *et al.*, 1981).

Figure 1.5

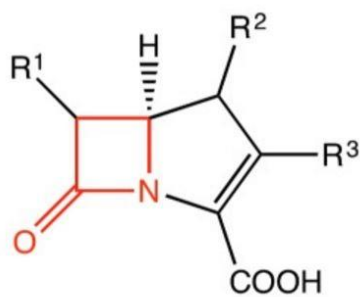
(a)



(b)



(c)



(d)

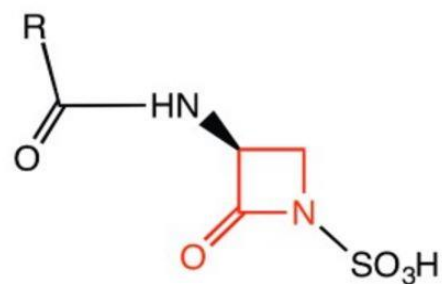


Figure 1.5 Structure of β -lactam Antibiotics. The molecular structure of (a) the penicillins, (b) the cephalosporins/cephamycins, (c) the carbapenems and (d) the monobactams. The 'R' in each structure represents the variable side chains of each antibiotic. Figures reproduced from Lee *et al.* (Lee *et al.*, 2016).

Modifications to the side groups present on the core structure of the β -lactam antibiotics have resulted in the development of over 50 approved semi-synthetic derivatives with extended antimicrobial activity and with activity in the presence of resistance mechanisms to earlier generation β -lactams (Wright *et al.*, 2014).

1.1.2.2 Mode of Action

β -lactam antibiotics are broad-spectrum, target cell wall synthesis and are bactericidal at high concentrations. The first suggestions that penicillin targeted the cell wall came from microscopic studies that showed altered cell division and cell surface morphology (Duguid, 1946). It was subsequently observed that a build-up of cell wall precursors (uridine peptides) occurred in *S. aureus* cells exposed to penicillin (Park and Strominger, 1957).

The cell wall of Gram-positive and Gram-negative bacteria contains peptidoglycan composed of polymers of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) with peptide side chains cross-linked by pentaglycine bridges. The formation of this pentaglycine bridge is mediated by a transpeptidase enzyme that recognises the D-alanine-D-alanine residue of the amino acid side chain, Figure 1.6 (a) (Typas *et al.*, 2011). Penicillin was shown to inhibit the transpeptidase reaction between *E. coli* GlcNAc and MurNAc pentapeptide *in vitro* (Izaki *et al.*, 1968). As the CO-N configuration of the penicillin β -lactam amide bond is highly similar to that found in the D-alanine-D-alanine structure, it can act as substrate for the transpeptidase enzyme (Tipper and Strominger, 1965). It is now known that the β -lactam is bound by the D-alanine-D-alanine transpeptidase, also referred to as a penicillin binding protein (PBP), and an ester bond is formed between the carbonyl group of the β -lactam ring and the active site serine of the enzyme Figure 1.6 (b). An ester bond is also formed between the D-alanine-D-alanine transpeptidase and the D-alanine-D-alanine carbonyl, however the ester bond between the β -lactam and the enzyme is hydrolysed far more slowly and so effectively prevents the D-alanine-D-alanine

transpeptidase from taking part in further reactions, resulting in a loss of cell wall cross-linking and ultimately bacterial cell lysis (Zapun *et al.*, 2008).

Figure 1.6

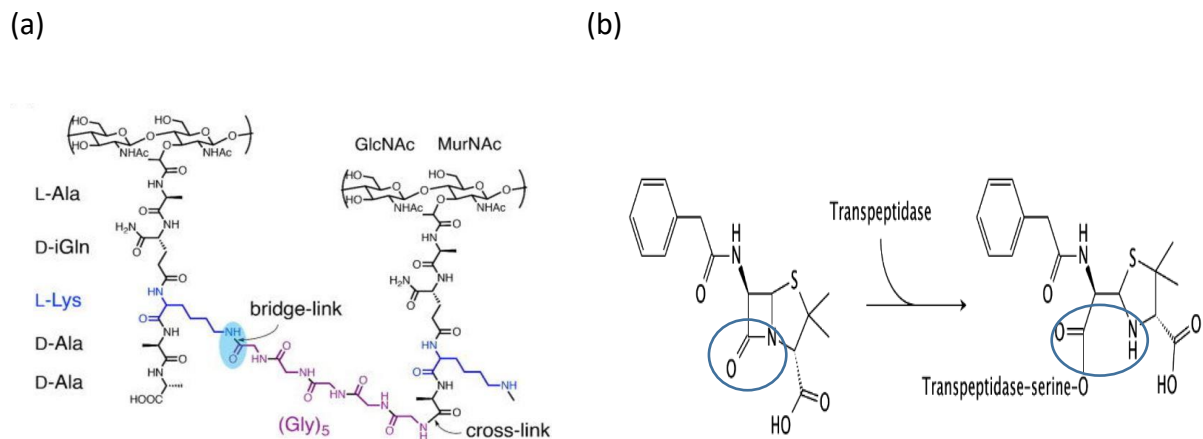


Figure 1.6 Illustration of Peptidoglycan Transpeptidation. (a) A diagram of the structure of two peptidoglycan molecules of *S. aureus* including the GlcNAc, MurNAc and peptide side chain. The pentaglycine bridge crosslinking the molecules between the L-lysine and D-alanine residues is highlighted in blue and purple. Figure reproduced from Zhou *et al.* (Zhou and Cegelski, 2012). (b) A diagram illustrating the formation of an ester bond between the carbonyl group (highlighted by a blue ring) of a β-lactam (benzylpenicillin) and the serine residue of the D,D-transpeptidase active site, reproduced from <http://watcut.uwaterloo.ca/webnotes/Pharmacology/microbesBacterialCellWall.html>.

1.1.2.2.3 Clinical Uses of β-lactams

Although resistance has arisen to penicillin G in most pathogens it is still a first-line drug for the treatment of syphilis caused by *Treponema pallidum*, and still shows use for the treatment of penicillin-susceptible *Streptococcus* spp. infections (Holman and Hook, 2013).

According to the European Centre for Disease Control (ECDC), penicillins are the most frequently used class of antibiotic in European countries representing 22-66 % of prescriptions (European Centre for Disease Prevention and Control (ECDC), 2012).

The cephalosporins are characterised into generations (from first to fifth) based on when they were developed, and are typified by a general increase in activity against Gram-negative organisms with decreasing activity against Gram-positive bacteria. The third-generation ceftriaxone is used to treat *Neisseria gonorrhoeae* infections, and as it can cross the blood-brain barrier it is used to treat meningitis infections (Llarrull *et al.*, 2010; Molyneux *et al.*, 2011). The cephalosporins and carbapenems are used in treating pneumonia and cIAls (Wilson, 1988; Flanders *et al.*, 2006; Breilh *et al.*, 2013).

Aztreonam is currently the only monobactam available for clinical use and is used in the treatment of *P. aeruginosa* infections including lung infections in cystic fibrosis patients (Assael, 2011).

1.1.2.2.4 Agricultural Uses of β -lactams

In the UK β -lactams represented 21 % of total sales of antibiotics for use in agriculture in 2014 (Veterinary Medicines Directorate, 2015). Ceftiofur and cefquinome are third and fourth-generation cephalosporins respectively that along with amoxicillins are used in the treatment of bovine uterine and mastitis infections, as well as bovine and porcine respiratory disorders (De Briyne *et al.*, 2014; Veterinary Medicines Directorate, 2015).

1.1.2.2.5 Novel β -lactam Therapies

Theravance Inc. currently have a glycopeptide-cephalosporin conjugate formula (TD-1792) in phase 3 clinical trials for complicated skin and soft tissues infections (cSSTIs) caused by Gram-positive bacteria (Theravance Biopharma: Programs infectious diseases, 2016). Early studies showed that one of their vancomycin-cephalosporin conjugates was more effective in treating MRSA (methicillin resistant *S. aureus*) than vancomycin in a mouse thigh model, with median effective doses (ED₅₀) of 0.5 mg/kg and 9 mg/kg respectively (Fatheree *et al.*, 2011).

1.2 Antimicrobial Activity of Metals and Cationic Antiseptics

1.2.1 Metals

1.2.1.1 History of Metals as Antimicrobials

The antimicrobial activity of metals was being utilised long before the discovery of microorganisms; for example, silver and copper were used by ancient Greeks to make vessels for storing water as they kept the water fresh. The first description of copper and silver being used in medicine dates back to between 2600 and 2200 BC when it was used to sterilise wounds (Dollwet, 1985; Alexander, 2009).

Later in the 19th and early 20th century silver found multiple uses in medicines, as silver nitrate was used in eye drops to treat gonorrhoeal ophthalmia and showed usefulness in

cleaning burn wounds. Silver arsphenamine was also used to treat neurosyphilis by injection into the spine (Hobman and Crossman, 2015). At this time inorganic and organic copper formulations were used in the treatment of syphilis, cholera, impetigo and other maladies (Dollwet *et al.*, 1985).

1.2.1.2 Mode of Action of Copper and Silver Antimicrobial Activities

The antimicrobial activity of copper is multifaceted. The redox potential of Cu^{2+} ions in the cell act as oxidising agents to deplete cellular thiol-containing biomolecules including cysteine and glutathione. The resulting Cu^+ ions then mediate the synthesis of H_2O_2 from the generated H^+ creating reactive oxygen species (ROS) that can oxidise protein and lipids in the cell, Figure 1.7 (Prudent and Girault, 2009).

Figure 1.7

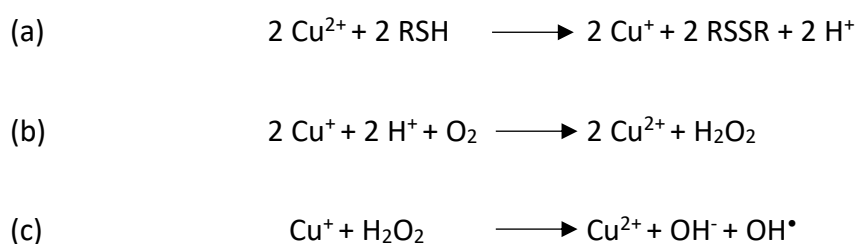


Figure 1.7 Redox Activity of Copper. (a) The oxidation of thiol groups by Cu^{2+} , where R represents the molecule with a thiol group. (b) The reduction of O_2 to H_2O_2 mediated by Cu^+ . (c) The Cu^+ mediated reduction of H_2O_2 to form hydroxide radicals.

Silver ions have also been demonstrated to interfere with thiol-containing biomolecules, and Liao *et al.* (1997) found that exogenous thiol-containing compounds including cysteine were able to protect *P. aeruginosa* from the activity of silver nitrate (Liao *et al.*, 1997). Furthermore, silver ions can induce ROS; Park *et al.* (2009) demonstrated that incubation with 1 mg/ml silver nitrate induced a 1.2- and 1.7-fold greater log inactivation of *S. aureus* and *E. coli* respectively, under aerobic conditions compared with anaerobic conditions (Park *et al.*, 2009).

Copper ions may also directly damage protein function by competing with coordinated iron in enzymes resulting in a loss of function. For example, Macomber and Imlay (2009) showed that the addition of Cu^+ resulted in a loss of iron from the purified fumarase A enzyme *in vitro* (Macomber and Imlay, 2009).

Additionally, accumulation of copper and copper-mediated membrane damage has been reported in *E. coli* and *Bacillus subtilis* while the metal has been shown to disrupt *Enterococcus* spp. DNA in another study (Warnes *et al.* 2010; Santo *et al.* 2011). Similarly, silver ions have been demonstrated (biochemically and microscopically) to be able to destabilise the OM of *E. coli* as well as induce DNA damage in *E. coli* and *S. aureus* cells (Feng *et al.*, 2000; Dibrov *et al.*, 2002). Furthermore, silver can disrupt ATP generation in *E. coli* as Lok *et al.* (2006) demonstrated that following treatment with AgNO_3 , the cell membrane potential fell with a concomitant drop in cellular ATP concentration (Lok *et al.*, 2006).

1.2.1.3 Uses of Silver and Copper

Silver and copper are present in cosmetics, dental amalgams used to fill tooth cavities (caries) and because of their antimicrobial activity they are also made use of in medicine (Ferracane, 2001; Borkow and Gabbay, 2009; Mijndonckx *et al.*, 2013). Copper surfaces induce rapid killing of both Gram-positive and Gram-negative bacteria and as such they may be of use in the control of spread of pathogens. A number of clinical trials have demonstrated the use of copper alloy surfaces in controlling bioburden and healthcare-associated infections in hospital environments (Casey *et al.*, 2010; Salgado *et al.*, 2013). Silver is used as an antimicrobial coating on instruments such as catheters and intubation apparatus, as well as in wound dressings, to prevent infection (Fernandez *et al.*, 2012). In agriculture, copper sulfate is approved by the EU for use as a growth promoter in feed animals and is also used to control diarrhoea in piglets and calves, following weaning (Verstegen and Williams, 2002). Copper sulfate is also used as an antimicrobial to protect growing crops and seeds (Russell, 2005).

1.2.2 Cationic Antiseptics

1.2.2.1 Chlorhexidine

The antimicrobial activity of chlorhexidine (CHX) was first reported by Davies *et al.* in the 1950s (Davies *et al.*, 1954). CHX is a bisbiguanide 1,1'-hexamethylenebis[5-p-chlorophenyl]biguanide], consisting of a rigid hexamethylene chain connecting two cationic

chlorophenylbiguanide groups, Figure 1.8. It exhibits bactericidal activity against Gram-positive and Gram-negative bacteria and yeasts as well as antibiofilm activity (Jones, 1997; Hope and Wilson, 2004).

At physiological pH the positively charged biguanide groups interact with the negatively charged surface components of the cell surface including lipopolysaccharides (LPS) and phospholipids. The rigid nature of the hexamethylene chain prevents its insertion in the cell membrane and instead it acts as an inflexible bridge between the negatively charged cell surface components resulting in reduced membrane fluidity, and potassium and phosphate ion leakage; this effect is bacteriostatic (Gilbert and Moore, 2005).

At high enough concentrations membrane integrity is compromised and CHX can enter the cell and form complexes with cytoplasmic proteins and DNA resulting in their precipitation. These complexes have been observed by scanning electron microscopy in both Gram-positive and Gram-negative cells and their formation results in cell death (Jones, 1997; Cheung *et al.*, 2012).

Figure 1.8

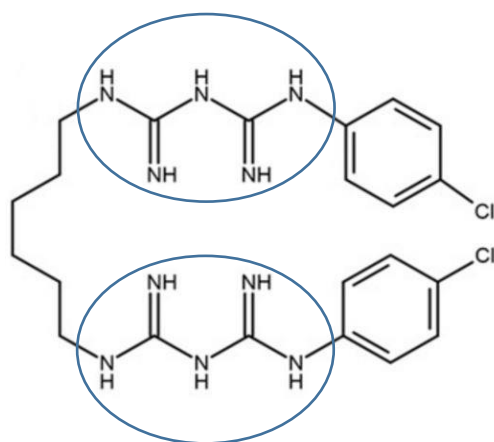


Figure 1.8 Chlorhexidine Structure. The structure of chlorhexidine with the two positively charged biguanide groups circled in blue; amended from Carrijo-Carvalho *et al.* (Carrijo-Carvalho *et al.*, 2016).

1.2.2.2 Quaternary Ammonium Compounds

Quaternary ammonium compounds (QACs) are amphiphilic cationic detergents with a broad range of targets inhibiting Gram-positive, Gram-negative bacteria and yeasts (Jimenez and Chiang, 2006).. The cationic nature of these compounds is derived from the presence of a nitrogen atom surrounded by hydrophobic carbon chains of various lengths. The structure of two QACs, cetyltrimethylammonium bromide (CTAB) and cetylpyridinium chloride (CPC) are shown in Figure 1.9 (a) and (b), respectively.

Like CHX, the cationic nature of QACs allows them to interact with negatively charged components of the cell surface. However, unlike CHX the hydrophobic carbon chains of QACs can insert into the cell membrane with their charged head group facing out from the cell, resulting in a decrease in membrane hydrophobicity (Gilbert and Moore, 2005). At

bacteriostatic concentrations QACs disrupt the membrane structure inducing leakage of K^+ and H^+ (Ferreira *et al.*, 2011; Inacio *et al.*, 2016). As concentrations of QACs increase, membrane structure and membrane protein functionality are disrupted affecting cellular respiration and ion transport across the membrane, which has been demonstrated by decreased cellular ATP concentrations in *E. coli* following treatment with QACs (Inacio *et al.*, 2016). At concentrations used in product formulations QACs form micelles that can dissolve cell membranes which has been demonstrated for *S. aureus* and *E. coli* cells (Ioannou *et al.*, 2007 Inacio *et al.*, 2016).

Different QACs have slightly different mechanisms of actions as determined by their differing binding affinities and bactericidal activity. Indeed, Ahlström *et al.* (1990) showed that CTAB (C_{16} carbon chain length) more effectively bound to *S. enterica* serovar Typhimurium than QACs with C_{14} and C_{12} chains (Ahlström *et al.*, 1999). In a later study, Ioannou *et al.* (2007) demonstrated that a QAC containing didecyldimethyl chains exhibited greater killing of *S. aureus* than QACs with alkyldimethyl chains (Ioannou *et al.*, 2007).

Figure 1.9

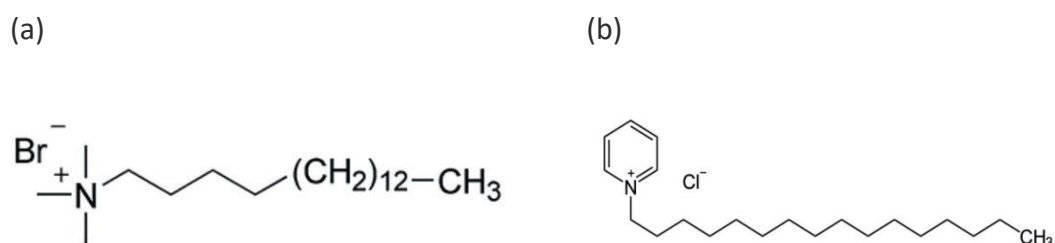


Figure 1.9 Structure of Cetyltrimethylammonium Bromide (CTAB) and Cetylpyridinium Chloride (CPC). (a)

CTAB, reproduced from Bhardwaj *et al.* (Bhardwaj *et al.*, 2014) and (b) CPC, reproduced from Zarei *et al.* (Zarei *et al.*, 2013).

1.2.2.3 Uses of Cationic Antiseptics

Due to their antimicrobial activity CHX, CTAB and CPC are included in antimicrobial hand washes and as preservatives in many cosmetic products (Buffet-Bataillon *et al.*, 2012; Silvestri and McEnery-Stonelake, 2013). Chlorhexidine is also used as an oral rinse in the treatment of gingivitis and periodontitis and is also found in over-the-counter mouthwashes, typically at concentrations between 0.1% and 0.2% (Eberhard *et al.*, 2008; Najafi *et al.*, 2012). CPC also exhibits efficacy in mouthwashes (Rao *et al.*, 2011). Additionally, QACs and chlorhexidine are also used as topical antiseptics for the sterilisation of surgical sites as well as wounds (McDonnell and Russell, 1999; Darouiche *et al.*, 2010).

1.3 Antimicrobial Resistance

1.3.1 The Increasing Threat of Antibiotic Resistance

Soon after Fleming discovered penicillin, he observed resistance to it as the producing mould was unable to inhibit some Gram-negative organisms including *E. coli* and *K. pneumoniae* isolates (Abraham and Chain, 1988). Following this first observation of resistance, the clinical introduction of every antibiotic has been followed, or even preceded, by the development of resistance to it in human pathogens, Table 1.2 (Lewis, 2013).

Table 1.2 Timeline of Clinical Antibiotic Resistance Development.

Antibiotic Class	Year Introduced as a Therapeutic	Year Clinical Resistance First Observed
Sulfadruugs; prontosil	1936	1942
B-lactams; penicillin	1938	1940
Aminoglycosides	1946	1946
Chloramphenicols	1948	1950
Macrolides	1951	1955
Tetracyclines	1948	1953
Rifamycins	1958	1962
Glycopeptides	1958	1988
Quinolones	1968	1968
Oxazolidinones	2000	2001

Amended from Lewis *et al.* (Lewis, 2013).

Today infections caused by antimicrobial-resistant pathogens continue to rise and it has been estimated that 25,000 deaths and a loss of €1.5 billion in lost productivity result from infections caused by these antimicrobial resistant pathogens in Europe alone, each year (European Centre for Disease Prevention and Control (ECDC), 2009).

In Europe, multidrug resistance (MDR) *Enterobacteriaceae* are a growing concern and the European Antimicrobial Resistance Surveillance Network (EARS-Net) has reported that between 2012 and 2015 *E. coli* and *K. pneumoniae* isolates with combined resistance to fluorquinolones, aminoglycosides and third-generation cephalosporins increased by 0.4 % (to 5.3 %) and 0.8 % (to 18.6 %) respectively. Additionally, although still at low levels carbapenem resistant *K. pneumoniae* isolates rose to 8.2 % with most of isolates exhibiting combined resistance to the three previously mentioned antibiotics. In such cases only

colistin treatment remains (European Centre for Disease Prevention and Control (ECDC), 2012). This is particularly worrying given the identification and dissemination of the plasmid-borne *mcr-1* (colistin resistance) gene which has been found in *E. coli* strains susceptible only to tigecycline and doxycycline (Yao *et al.*, 2016).

Such is the threat surrounding antibiotic-resistant bacteria that the UK government commissioned a major review on the problem. Lord Jim O'Neill's (2014) subsequent report estimated that by 2050 infections caused by antimicrobial resistant pathogens will cause 10 million deaths per year (O'Neill, 2014). The report also highlighted the increased risk associated with procedures such as chemotherapy and invasive surgeries when no viable antimicrobials are available. These procedures require prophylactic antibiotic use to prevent infection that may result due to the suppression of the patients' immune system and / or the massive microbial load introduced into the body. The report estimates that globally between now and 2050, a loss of \$210 trillion globally will result from the rise of antibiotic resistance (O'Neill, 2014).

However, de Kraker *et al.* (2016) have more recently cast doubt on the report. In their essay, they highlight the inaccuracies associated with using non-population based EARS-Net data, questionably validated infection mortality rates and unsubstantiated increases in antibiotic-resistant pathogens to estimate future mortality rates (De Kraker *et al.*, 2016).

Nonetheless, the rise of antibiotic resistance has resulted from the overuse and misuse of these therapeutic agents such as not finishing a course of antibiotics which results in sublethal concentrations of the antibiotic in the patient. This will result in bacteria that have obtained resistance genes or mutations surviving the treatment and spreading while the more susceptible strains die. Additionally, global antibiotic use is increasing, Van Boeckel *et*

al. (2014) determined that between 2000 and 2010 antibiotic consumption increased by 36 % from 54 billion to 74 billion standard units (where a standard unit is equal to a single dose) (Van Boeckel *et al.*, 2014). Worryingly, the use of antibiotics in agriculture greatly outweighs their use in human therapy. In the USA, where antibiotics are used for growth promotion, it has been estimated that over 70 % of antibiotics are used in feed animals, and the Food and Drug Administration (FDA) estimated that over 15 million Kg of antibiotics were sold for use in food-producing and non-food-producing animals (FDA, 2015).

1.3.2 Intrinsic Resistance

Intrinsic resistance is typically conserved in members of a species and is independent of antibiotic selection in its native host. Intrinsic resistance generally relies on the absence of a target or reduced permeability and/or increased efflux of the antimicrobial.

In Gram-negative bacteria, the outer membrane contains LPS the saturated fatty acid chains of which insert into the OM decreasing its fluidity, and thus reducing the ability of antibiotics to cross it. The presence of porins in the OM are responsible for the uptake of nutrients required for cell growth, but also act to restrict the movement of antibiotics based on size limits, charge and hydrophobicity incompatibility (Nikaido *et al.*, 1983; Decad and Nikaido, 1976). Further to this, loss of expression of porins can result in decreased susceptibility to antibiotics including carbapenems, as has been demonstrated for clinical *P. aeruginosa oprD* mutants (Sakyo *et al.*, 2006).

Intrinsic resistance may also be provided by the production of efflux mechanisms that have functions other than antibiotic efflux. For example, *P. aeruginosa* encodes a tripartite resistance nodule division (RND) efflux pump; deletion of which has been demonstrated to result in a loss of resistance to β -lactams, chloramphenicol and nalidixic acid (Morita *et al.*, 2001). This transporter plays other roles in the cell including inter-cell communication and virulence (Hirakata *et al.*, 2002; Evans *et al.*, 1998).

More recently, the screening of transposon mutant libraries for hyper-susceptible mutants has revealed that genes involved in cell metabolism are also involved in intrinsic resistance in bacterial species, which highlights the role that the metabolic state of the cell can have on its response to antibiotics. For example, insertions in the histidine and arginine synthesis genes, *hisF* and *argH*, in *Acinetobacter baylyi* resulted in an 8-fold increase in susceptibility to ampicillin, while insertion in riboflavin synthesis genes in *S. aureus* resulted in increased susceptibility to daptomycin (Gomez and Neyfakh, 2006; Blake and O'Neill, 2013).

1.3.3 Acquired Resistance

Acquired antimicrobial resistance refers to the development of resistance in a bacterial cell that was previously susceptible. Acquired resistance can occur following the acquisition of DNA containing resistance genes, through horizontal gene transfer (HGT), or from mutations that alter the cells susceptibility to an antibiotic.

1.3.3.1 Mutation Contributes to Antimicrobial Resistance

Decreased susceptibility to antibiotics can result from the acquisition of mutations in the antibiotic's target or from mutations that alter the regulation of genes involved in resistance. In some species mutations are the sole cause of antimicrobial resistance. For example, isoniazid and rifampin resistance in *Mycobacteria tuberculosis* has resulted solely from the acquisition of mutations (Ahmed *et al.*, 2016).

In clinical *E. coli* isolates, quinolone resistance occurs through the acquisition of mutations in *gyrA* that encodes one of the gyrase monomers that is the target of this group of antibiotics. These mutations are typically found in the 'quinolone resistance determinant region' of *gyrA* that corresponds to amino acids between 67 and 101 (Yoshida *et al.*, 1990). Single mutations generally result in resistance to nalidixic acid while multiple mutations are typically required for fluoroquinolone resistance (Cambau *et al.*, 1993; Ruiz *et al.*, 2002).

AmpC is an inducible β -lactamase (an enzyme that hydrolyses the β -lactam ring; that will be discussed in more detail later in this chapter) produced by *Enterobacteriaceae* and *P. aeruginosa*. Transcription of *ampC* is induced by the presence of β -lactams including carbapenems due to an increase in 1,6-anhydromuropeptide peptidoglycan precursors as cell wall synthesis is inhibited (Jacobs *et al.*, 1994). AmpD negatively regulates *ampC* transcription by processing these precursors (Lee *et al.*, 2009). Mutations in the *ampD* gene can result in a loss of β -lactam controlled *ampC* transcription. Clinical *P. aeruginosa* strains with AmpC hyperproduction had between 8- and >256-fold greater ceftazidime resistance compared with *P. aeruginosa* PAO1 (Juan *et al.*, 2005).

1.3.3.2 Horizontal Gene Transfer and Antimicrobial Resistance

HGT was first demonstrated in 1947 by Tatum and Lederberg when they observed their mutant *E. coli* strains had been complemented without the acquisition of further mutations (Tatum and Lederberg, 1947). The extent to which HGT has influenced bacterial genomes has been highlighted by the increasing amount of genome sequences available which allows for the identification of genes present or absent in different species. As nucleotide composition and codon usage differs between transferred and host DNA, these differences can also be used to determine the contribution of HGT to the genome (Langille *et al.*, 2010). Using such approaches, HGT-obtained genes have been shown to account for up to 40 % of the coding sequences in bacteria. For example, in *E. coli* HGT contributes between 10 and 40 % of coding sequences while in *P. aeruginosa* and *B. subtilis* HGT accounts for 20 % and 14.47 % of coding sequences respectively (Lawrence and Ochman, 1998; Garcia-Vallve *et al.*, 2000; Pohl *et al.*, 2014).

1.3.3.2.1 Mechanisms of Horizontal Gene Transfer

1.3.3.2.1.1 Conjugation

Conjugation involves the transfer of DNA, a mobile genetic element (MGE), from a donor to a recipient through cell-to-cell contact via pilus or adhesion interactions, Figure 1.10 (Furuya and Lowy, 2006). Conjugative transposons (DNA that can excise and insert into different chromosomal location; CTns) or conjugative plasmids (circular DNA molecules that can

replicate independently of the host genome) that encode the conjugation machinery as well as accessory genes (including antimicrobial resistance genes; ARGs) are transferred in this way, Figure 1.10 (van Hoek *et al.*, 2011).

Figure 1.10

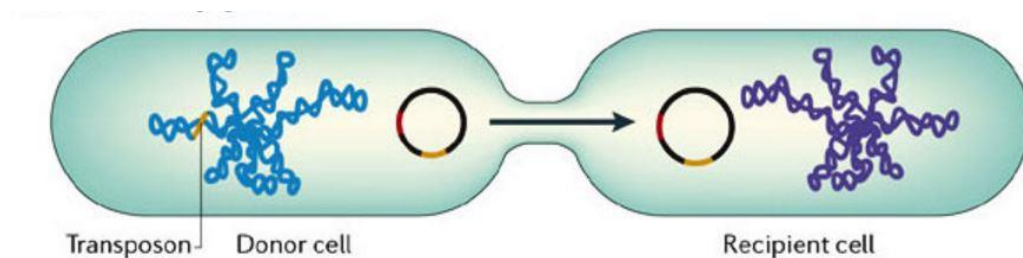


Figure 1.10 Illustration of the HGT Mechanism, Conjugation. Reproduced from Furuya *et al.* (Furuya and Lowy, 2006).

Transposition and conjugation of a CTn first involves its excision from the donor cells chromosome forming a double-stranded DNA (dsDNA) circular intermediate. Following the formation of the circular intermediate, the mechanism of transfer for conjugative plasmids and CTNs are similar (Grohmann *et al.*, 2003; Johnson and Grossman, 2015). A relaxase, encoded by the conjugative MGE, creates a single strand (ss) nick at the origin of transfer (*oriT*) on the circular structure and binds to the 5' end of the ssDNA. The helicase activity of the relaxase protein then unwinds the ssDNA and it is transferred to a type IV secretion system (T4SS) via an ATPase-type coupling protein. The T4SS interacts with the recipient cell and transports the ssDNA conjugative MGE from the donor to the recipient cell. Once in the cytoplasm of the recipient cell, the second strand of the ssDNA MGE is synthesised resulting

in the complete conjugative plasmid or a circular intermediate CTn that can insert into the host chromosome (Grohmann *et al.*, 2003; Johnson and Grossman, 2015).

Conjugation is likely to have the greatest impact on the dissemination of ARGs as conjugative MGEs have a broader host range than bacteriophages (viruses that infect bacteria cells), and the transferred DNA is less prone to degradation than DNA free in the environment (Furuya and Lowy, 2006). Tn916-like CTns are discussed in more detail in chapter 6.

1.3.3.2.1.1.1 Mobilisation of Non-Conjugative Elements

Conjugative MGEs may also mediate the transfer of non-conjugative MGEs to recipient cells. This can be mediated in *trans* as some non-conjugative MGEs can use the conjugation machinery encoded by a conjugative MGE to transfer to a new host. For example, the pWBG749 family of *S. aureus* conjugative plasmids can mobilise non-conjugative plasmids that carry its *oriT* (O'Brien *et al.*, 2015). Alternatively, the insertion of a non-conjugative MGE into or adjacent to a conjugative MGE may result in the formation of a co-integrate whereby both MGEs can be transferred to a recipient host as one. For example, Tn5253 is a composite conjugative CTn as it contains two distinct elements, Tn5251 and $\Omega_{cat}(pC194)$ (Iannelli *et al.*, 2014).

Integrations are non-conjugative elements composed of an integrase gene, *intI*, an *attI* recombination site and a promoter (Gillings, 2014). IntI mediates the capture of gene cassettes via site-specific recombination allowing these MGEs to capture gene cassettes

including those encoding resistance genes; this can result in multidrug resistance encoding elements (Krauland *et al.*, 2009). Further to this, integrons may be associated with conjugative MGEs, thus expanding their dissemination (Mazel, 2006).

Insertion sequence (IS) elements are small transposable elements (700-2,500 bp) composed of a transposase-encoding gene, flanked by two inverted repeats that are substrates of the transposase (Chandler and Mahillon, 2002). When two identical IS elements flank a genomic region, they can mediate its transposition which can result in the deletion, inversion or duplication of genes (Wei *et al.*, 2003; Kothapalli *et al.*, 2005; Iguchi *et al.*, 2006). For example, *bla*_{SHV}, encoding a β -lactamase is associated with IS26 on composite transposons and conjugative transposons in *K. pneumoniae* and *Enterobacter cloacae* (Chen *et al.*, 2015).

1.3.3.2.1.2 Transformation

Transformation is a process by which bacterial cells take in DNA from their environment, including ARGs, Figure 1.11. In 1951, Hotchkiss first demonstrated the acquisition of ARGs in this way when penicillin and streptomycin-sensitive *Streptococcus pneumoniae* strains were shown to become resistant following incubation with DNA from other resistant strains (Hotchkiss, 1951).

Figure 1.11

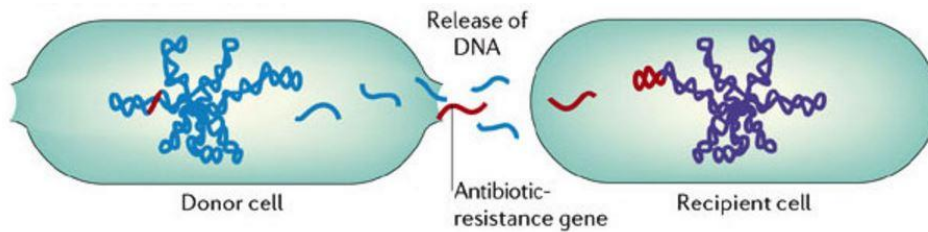


Figure 1.11 Illustration of the HGT Mechanism, Transformation. Reproduced from Furuya *et al.* (Furuya and Lowy, 2006).

In order for cells to be able to take up DNA they must be competent and although some cells are inherently so, for example *Neisseria spp.*, in other cells it must be induced (Spurling, 1966). Induction can result from the production of peptides that stimulate competence when cells reach a certain density or in response to stress. For example, *S. pneumoniae* secretes competence stimulating protein (CSP) into its environment which at a certain threshold concentration activates the *com* genes required for inducing competence. The *com* genes in *S. pneumoniae* can also be induced by stress, including antibiotic-induced stress. Prudhomme *et al.* (2006) used a luciferase transcription fusion assay to demonstrate that sublethal concentrations of fluorquinolones and aminoglycosides induced *com* gene expression and the uptake of extrachromosomal DNA (eDNA) (Prudhomme *et al.*, 2006).

In a recent analysis of the Haihe River basin in China, Mao *et al.* (2014) developed a method to isolate eDNA separately from bacterial chromosomal DNA and found that the former was more abundant in sediment samples. Additionally, this eDNA contained higher abundances

of ARGs including tetracycline resistance genes, indicating the potential importance of eDNA and transformation in the dissemination of antimicrobial resistance (Mao *et al.*, 2014).

1.3.3.2.1.3 Transduction

Transduction is the process by which DNA is transmitted from one cell to another by a virus; in the case of bacterial DNA transmission, the transfer is mediated by bacteriophages, Figure 1.12.

Figure 1.12

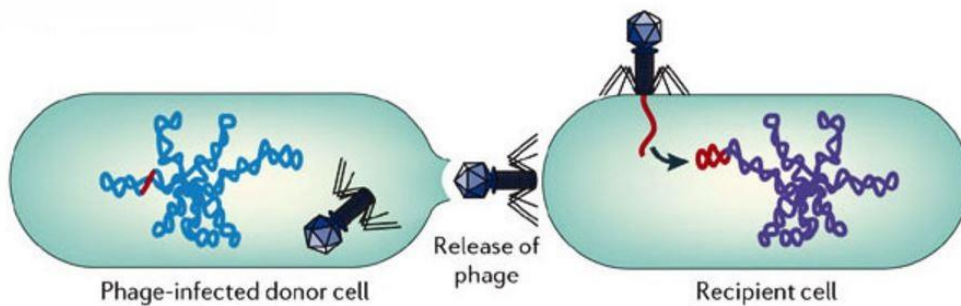


Figure 1.12 Illustration of the HGT Mechanism, Transduction. Reproduced from Furuya *et al.* (Furuya and Lowy, 2006).

Transduction can be described as being either generalised, whereby only non-bacteriophage DNA is transferred, or specialised, when bacteriophage DNA bound by bacterial DNA is transferred.

Bacteriophages are the most abundant biological entity on the planet with estimates putting their numbers at between 10^{30} and 10^{32} , and as these virus particles are vehicles for the transport of genetic material between cells, they represent an important avenue for the spread of ARGs. Studies using quantitative PCR (qPCR) have demonstrated the carriage of ARGs in bacteriophages from numerous environments including wastewater samples and human and feed animal faeces (Colomer-Lluch *et al.* 2011; Quiros *et al.* 2014; Marti *et al.* 2014). Indeed, a study conducted by Shousha *et al.* (2015) found a statistically significant relationship between the presence of kanamycin-resistant *E. coli* and kanamycin resistance gene-carrying bacteriophages in chicken meat samples indicating their importance in the dissemination of resistance genes (Shousha *et al.*, 2015).

1.3.4 Dissemination of Antimicrobial Resistance

Two recent studies focusing on the uncultivable resistome (the collection of all antibiotic resistance genes in an environment) of different environments demonstrated that clustering of resistance genes occurred based on environment. For example, Gibson *et al.* (2015) found that the resistome of soil and the human gut clustered separately and were dictated greatly by the presence of tetracycline and β -lactam resistance genes in these environments (Gibson *et al.*, 2015). Similarly, Munck *et al.* (2015) found little overlap between the resistome of a waste water treatment plant and ARGs found in the National Centre for Biotechnology Information (NCBI) database (which primarily contains sequence information from pathogenic bacteria), with less than 10 % of their identified ARGs exhibiting greater

than 95 % nucleotide identity to ARGs in the database (Munck *et al.*, 2015). These results suggest that there is limited dissemination between the environments tested.

Although the studies discussed above suggest otherwise, there is also evidence that dissemination of ARGs from the environment to clinical pathogens has occurred. Forsberg *et al.* (2012) identified and annotated 110 functional ARGs from a Proteobacteria-enriched soil sample. Of these ARGs, 16 had 100 % nucleotide identity with ARGs from known pathogens, with some also exhibiting synteny (the genomic location of a gene) suggesting that recent HGT events had occurred (Forsberg *et al.*, 2012).

Environmental bacteria may represent the origin of a number of ARGs. For example, many *Kluyvera* spp. encode chromosomal ESBLs (extended spectrum β -lactamases) and transfer of the *bla*_{CTX-M-2} gene from *Kluyvera ascorbata* to a recipient *E. coli* host has been demonstrated *in vitro* (Lartigue *et al.*, 2006). Similarly, the quinolone resistance gene, *qnrA*, and the *bla*_{OXA-48} may have environmental origins having both been identified in *Shewanella algae* (Poirel *et al.*, 2005; Poirel *et al.*, 2012).

There is also evidence that human processes further contribute the abundance of ARGs in the environment through the release of active antibiotics, ARGs and antimicrobial resistant bacteria. For example, it has been estimated that following oral administration of oxytetracycline to sheep, 21 % of the dose is excreted in its active form, while young bulls will excrete up to 75 % of the active chlortetracycline they receive as treatment. As manure from farm animals is used as a fertilizer, the presence of excreted antibiotics will select for the maintenance of ARGs in the faecal microbiota that will then contaminate the soil and adjacent water systems (Boxall *et al.*, 2004). In a study on the effect of manure spreading on the abundance of ARGs in soil, Hong *et al.* (2013) used a qPCR to show that tetracycline

ARGs were at least 6-fold more abundant following spreading and that they remained at elevated levels for at least 16 months (Hong *et al.*, 2013). In another study, *Enterococcus* spp. in ground water and surface water down-gradient of a swine feeding operation were found to be more abundant and have MIC₉₀ values between 1.5- and 64-fold greater than those isolated up-gradient (Sapkota *et al.*, 2007).

Humans can be exposed to these ARGs and resistant bacteria through the food chain via contaminated crops and meats, or through bathing in or drinking contaminated water. Once ingested these microorganisms can transfer their ARGs to pathogens within the human gastrointestinal (GI) tract, or if pathogenic themselves, can cause infections that are recalcitrant to treatment (Chang *et al.*, 2015).

1.3.5 Selection of Antimicrobial Resistance

Natural antimicrobials have existed for billions of years, giving their producer a selective benefit by inhibiting neighbouring sensitive cells that would otherwise compete with them for resources (Aminov, 2009). Just as antibiotic production is an ancient attribute of microorganisms, so is antimicrobial-resistance, as microorganisms that could survive in the presence of antimicrobials would be selected for over their sensitive counterparts (Perron *et al.*, 2015).

Although ARGs preceded the introduction of antibiotic use in the clinic and agriculture, it is now accepted that their increased use and misuse has contributed to the dissemination of ARGs and the rise of antimicrobial resistance in bacteria (Austin *et al.*, 1999; Fleming-Dutra

et al., 2016). For example, in a Serbian tertiary care unit an increased administration of ceftriaxone (measured as defined daily dose/1,000 patient days) over a 9 year period was associated with an increase in the isolation of ceftriaxone resistant *E. coli* from patients (Velickovic-Radovanovic *et al.*, 2015).

The relationship between antimicrobial use and antimicrobial resistance is particularly exemplified by their use and subsequent withdrawal from use on European farms. Following a ban on the use of avoparcin (a glycopeptide) as a growth promoter in Denmark (1996) and the Netherlands (1997) there was a reduction in the prevalence and abundance of vancomycin-resistant *Enterococcus* (VRE) from pig faeces (van den Bogaard *et al.*, 2000). More recently a voluntary ban on the use of cephalosporins in pig production was introduced in Denmark. This reduction in the use of cephalosporins has resulted in a decrease in the isolation of ESBL-producing *E. coli* from caecal samples at slaughter, Figure 1.13 (Agersø and Aarestrup, 2013).

Figure 1.13

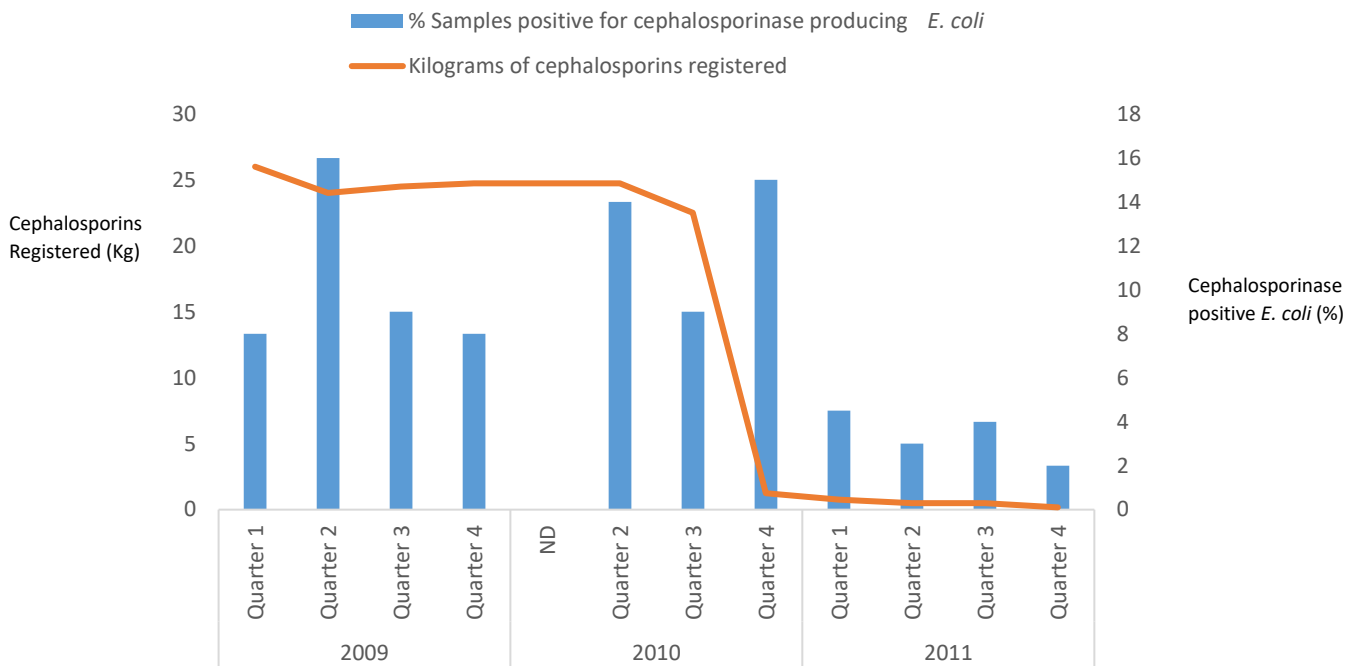


Figure 1.13 Cephalosporin use on Danish Pig Farms and Pig Caecal ESBL *E. coli* Isolates. The above graph shows the relationship between the amount of cephalosporins registered for use in pig production (orange line) and the percentage of pig caecal samples at slaughter positive for ESBL *E. coli* (blue bars). This graph was reproduced from data taken from Agersø *et al.* (Agersø and Aarestrup, 2013).

Similarly, reductions in the isolation of antimicrobial resistant isolates following changes in the use of antimicrobials has been noted in human medicine. For example, Chung *et al.* (2007) found that *Haemophilus* spp. with increased ampicillin resistance (3-fold mean increase) were found in throat swabs in children who were prescribed the antibiotic compared with the control group who had not received treatment. This change was transient however, as after 12 weeks the mean MIC for *Haemophilus* spp. isolates returned to control group levels (Chung *et al.*, 2007). In an earlier study, Seppälä *et al.* (1997) identified a statistically significant association between the reduction of use of erythromycin

and a reduction in the frequency of erythromycin resistant group A *Streptococci* being present in patient throat and pus swabs (Seppälä *et al.*, 1997).

1.3.6 Resistance Can Persist in the Absence of Antibiotic Selection

The previously described examples indicate that following the withdrawal of antimicrobial use there is a decrease in the prevalence of antimicrobial resistant isolates. This is a result of the fitness costs associated with the maintenance of horizontally acquired ARGs or mutations in antimicrobial targets that are vital for cell survival such as cell wall synthesis, translation components and DNA replication.

Some ARGs however may have a neutral or positive fitness effect on the cell without antibiotic pressure, or compensatory mutations may arise that compensate for reduced fitness which could result in their maintenance in the absence of antimicrobials (Nagaev *et al.*, 2001). In growth competition assays conducted by Baker *et al.* (2013), fluoroquinolone resistant *Salmonella enterica* serovar Typhi strains with clinically relevant double mutations in *gyrA* and/or *parC* were shown to have greater fitness than their parent strains (Baker *et al.*, 2013). In another study, carriage of *bla*_{CTX-M-14} (β -lactam resistance) on a pCT plasmid conferred no fitness cost to their *E. coli* or *Salmonella* spp. host compared with the empty pCT plasmid (Cottell *et al.*, 2012).

1.3.7 Co-selection of Antibiotic Resistance Genes

Co-selection of ARGs occurs when one antimicrobial can select for ARGs conferring resistance to another unrelated antimicrobial. Thus, withdrawal of use of one antimicrobial may not result in a decrease in frequency of ARGs if its replacement can co-select for it.

Co-selection can occur due to the genetic linkage of ARGs on an MGE. For example, tetracycline-resistance genes have been identified on a number of CTNs together with macrolide, lincosamide and streptogramin ARGs (Del Grosso *et al.* 2004; Cochetti *et al.* 2008). As a result, the use of tetracyclines is associated with cross-resistance to these antibiotic classes and vice versa (Chaffanel *et al.*, 2015).

Additionally, plasmids and CTNs harbouring metal- and antiseptic-resistance genes as well as ARGs have been identified indicating that the ARGs can be selected for in the presence of these antimicrobials (Davis *et al.*, 2005; Soge *et al.*, 2008; Ciric *et al.*, 2011). Cross resistance between metals, antiseptics and antibiotics may also result from the expression of MDR efflux pumps (Blanco *et al.*, 2016). As discussed above, metals and antiseptics are used in various consumer products and in this context they are creating a selection pressure for the maintenance of ARGs (Wales and Davies, 2015).

1.3.8 Specific Mechanisms of Resistance

1.3.8.1 Tetracycline Resistance

Since their discovery tetracyclines have seen wide clinical, veterinary and agricultural use because of their broad spectrum of activity and their activity as growth promoters; this has resulted in the dissemination of resistance to these antibiotics. Tetracycline-specific resistance genes were originally designated *tet* followed by a letter. However, due to the high number of tetracycline-specific resistance genes identified to date they are now designated *tet* followed by a number, and if the mechanism is encoded by two genes they are denoted 'AB'. A new *tet* gene is designated as such if it encodes a protein that has less than 80 % amino acid similarity to another tetracycline resistance protein (Levy *et al.*, 1999).

Tetracycline resistance is typically mediated by ribosomal protection, efflux of the antibiotic or enzymatic activity, Table 1.3. *tet(U)* encodes a tetracycline-resistance protein with a currently unknown mechanism, although its ability to confer resistance is in question (Ridenhour *et al.*, 1996; Caryl *et al.*, 2012). In addition to these protein-mediated tetracycline resistance phenotypes, mutations in the rRNA of the 30S ribosome subunit can also confer resistance to tetracycline, Table 1.3 (Nguyen *et al.*, 2014).

Table 1.3 Genes and Mutations Conferring Tetracycline Resistance.

Efflux		RPP	Enzymatic	Other Mechanisms	
MFS	Non-MFS	-	-	Unknown	rRNA Mutations
<i>tet(A)</i>	<i>tet(30)</i>	<i>tetAB(46)</i>	<i>tet(M)</i>	<i>tet(X)</i>	<i>tet(U)</i> G1058C
<i>tet(B)</i>	<i>tet(31)</i>	<i>tet(35)</i>	<i>tet(O)</i>	<i>tet(34)</i>	A926T
<i>tet(C)</i>					
<i>tet(D)</i>	<i>tet(33)</i>		<i>tet(Q)</i>	<i>tet(37)</i>	G927T
<i>tet(D)</i>					
<i>tet(E)</i>	<i>tet(38)</i>		<i>tet(S)</i>	<i>tet(47)</i>	A928C
<i>tet(G)</i>	<i>tet(39)</i>		<i>tet(T)</i>	<i>tet(48)</i>	ΔG942
<i>tet(H)</i>					
<i>tet(J)</i>	<i>tet(40)</i>		<i>tet(W)</i>	<i>tet(49)</i>	
<i>tet(K)</i>	<i>tet(41)</i>		<i>tet(32)</i>	<i>tet(50)</i>	
<i>tet(L)</i>					
<i>tetA(P)</i>	<i>tet(42)</i>		<i>tet(36)</i>	<i>tet(51)</i>	
<i>tet(V)</i>	<i>tet(43)</i>		<i>tet(44)</i>	<i>tet(52)</i>	
<i>tet(Y)</i>	<i>tet(45)</i>		<i>tet</i>	<i>tet(53)</i>	
<i>tet(Z)</i>					
	<i>tet(57)</i>		<i>tetB(P)^b</i>	<i>tet(54)</i>	
	<i>tet(58)</i>		<i>otr(A)</i>	<i>tet(55)</i>	
	<i>tet(59)</i>			<i>tet(56)</i>	
	<i>tcr</i>				
	<i>otr(B)</i>				
	<i>otr(C)</i>				

Table 1.3 This table is modified version of the tables found in Nguyen *et al.* and

<https://faculty.washington.edu/marilynr/tetweb1.pdf> [Accessed December 1, 2016] (Nguyen *et al.*, 2014).

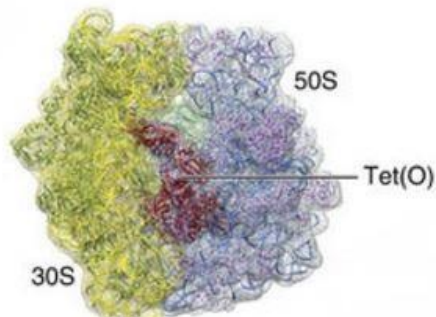
1.3.8.1.1 Ribosomal Protection

Ribosomal protection proteins (RPPs) are paralogs of the elongation factors (EFs), EF-G and EF-Tu. These three proteins have highest amino acid identity in their conserved N-terminal GTPase domains which are vital for their functionality (Sanchez-Pescador *et al.*, 1988; Margus *et al.*, 2007). Both EFs are involved in protein translation; EF-G functions in the translocation of mRNA through the ribosome, and EF-Tu introduces charged aminoacyl-tRNA (aa-tRNA) molecules to the ribosomal A site (Margus *et al.*, 2007).

Of the RPPs identified, Tet(M) and Tet(O) are the most common and the most studied (Chopra and Roberts, 2001). RPPs bind to the 70S ribosome within the 30S and 50S subunits resulting in the dislodgement of tetracycline from the ribosome, which allows the entrance of charged aa-tRNA complexes into the ribosome, Figure 1.14 (a) (Connell *et al.*, 2003). The primary binding site for Tet(O) is located on the 16S rRNA molecule at helix 34A while a model created by Li *et al.* (2013) showed that Tet(O) also binds to the 16S RNA between nucleotides 966 and 1196, forming a tunnel through which tetracycline dissociates following conformational changes in the nucleotide structure, Figure 1.14(b) (Li *et al.*, 2013; Arenz *et al.*, 2015).

Figure 1.14

(a)



(b)

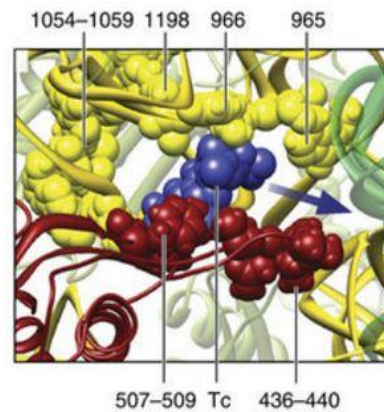


Figure 1.14 Tet(O) Ribosomal Binding Sites. (a) A depiction of the binding site of Tet(O) (red) within the 30S (yellow) and 50S (blue) subunits of the ribosome. (b) Shows the interaction of Tet(O) (red) with the 16S rRNA nucleotides (yellow) that form the channel through which tetracycline (blue) dissociates through following ribosomal protection protein (RPP) binding. The direction of dissociation of the antibiotic is depicted by a blue arrow. These figures were reproduced from Li *et al.* (Li *et al.*, 2013).

The GTPase activity of the RPP is not required for displacement of tetracycline from the ribosome as Tet(O) has been shown to dislodge the antibiotic when complexed with non-hydrolysable GTP analogues (Trieber *et al.*, 1998). However, it is required for the release of the RPP, allowing for the entry of charged EF-Tu-aa-tRNA complexes into the ribosome and recycling the RPP (Connell *et al.*, 2003). Conformational changes within the 16S nucleotide, at position C1054 for example, remain following dissociation of the RPP preventing rebinding of tetracycline following hydrolysis of GTP (Arenz *et al.*, 2015).

Tigecycline is able to prevent rescue of the ribosome by RPPs as Jenner *et al.* (2013) used X-ray crystallography to demonstrate that the 9-dimethylglycylamido side chain of the antibiotic prevents docking of Tet(M) to the ribosome (Jenner *et al.*, 2013).

1.3.8.1.2 Tetracycline Efflux

Tetracycline efflux was first identified in *E. coli* (McMurry *et al.*, 1980). The transporter responsible is a major facilitator superfamily (MFS) efflux protein and at least 32 further have been discovered since (McMurry *et al.* 1980; ARDB-Antibiotic Resistance Genes Database). MFS transporters are secondary transporters as they utilise the energy of solutes (such as a proton) moving along their electrochemical gradients to fuel substrate transport (Quistgaard *et al.*, 2016). Tetracycline resistance MFS transporters are antiporters, as the proton and the antibiotic move across the membrane in opposite directions (Yamaguchi *et al.*, 1990, Quistgaard *et al.*, 2016). MFS transporters contain a transmembrane domain (TMD) that is made up of either 12 or 14 transmembrane helices with the N- and C-domains contributing an equal number of helices (Hassan *et al.*, 2006).

Structural and biochemical analysis have shown the MFS transporter substrate binding site to be located between the N- and C-termini; residues in helices 1, 4, 7 and 10 have been identified as important for substrate co-ordination (Hassan *et al.*, 2006). The substrate recognised by tetracycline exporters is the tetracycline-Mg²⁺ complex (Yamaguchi *et al.*, 1990). Following substrate binding, conformational changes cause the MFS to close access

to the cytoplasm and open to the surface of the periplasm/extracellular space in a rocker-switch type movement (Hirai and Subramaniam, 2004; Yin *et al.*, 2006).

Protons may induce substrate recognition by the MFS and thus drive conformational change as LacY, a lactose permease, is unable to bind its galactoside substrate without protonation at a glutamine residue in helix 10 (Kaback, 2015).

Tetracycline efflux mediated by ATP binding cassette (ABC) transporters have also been identified. For example, TetAB(46) was identified from an oral *Streptococcus australis* strain and was found to confer tetracycline resistance to the cell (Warburton *et al.*, 2013). These transporters hydrolyse ATP to power transport of substrates across membranes and their structure and function are discussed in more detail in chapter 5.

1.3.8.1.3 Enzymes and Tetracycline Resistance

TetX is an NADPH-dependent monooxygenase that was first identified on the transposable elements Tn4351 and Tn4400 from *Bacteroides fragilis* (Speer *et al.*, 1991). As *B. fragilis* is anaerobic the enzyme does not function in this host, however it functions in aerobically grown *E. coli* and has since been identified in clinical isolates able to grow aerobically, including *K. pneumoniae* and *Pseudomonas* spp. (Speer *et al.*, 1991; Leski *et al.*, 2013). The enzyme inactivates even the third-generation tigecycline as it hydroxylates the molecule at carbon 11a, reducing its ability to chelate Mg^{2+} and bind the ribosome, Figure 1.15 (Moore *et al.*, 2005; Volkens *et al.*, 2011).

Figure 1.15

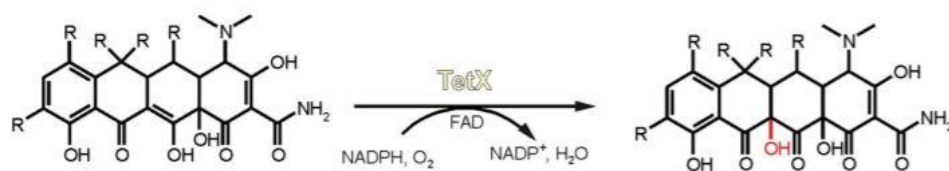


Figure 1.15 Hydroxylation of Tetracycline by Tet(X). The hydroxylation reaction catalysed by Tet(X) inactivates the tetracycline. The hydroxylated 11a carbon is indicated in red. This figure was reproduced from Nguyen *et al.* (Nguyen *et al.*, 2014).

A gene encoding an enzyme that also inactivated tetracycline in an NADPH and oxygen-dependent manner, *tet(37)*, was identified from the human oral cavity, however the enzyme had limited amino acid sequence identity to Tet(X) (Diaz-Torres *et al.*, 2003).

Tet(34) was identified in *Vibrio* sp. no. 6 as it conferred resistance to oxytetracycline in an Mg^{2+} -dependent manner. As the enzyme had 80 % amino acid identity to xanthine-guanine phosphoribosyltransferase, which is involved in guanine nucleotide synthesis, the authors hypothesised that *Tet(34)* conferred resistance by producing excess GTP which accelerated binding of the charged EF-Tu-aa-tRNA to the ribosome (Nonaka and Suzuki, 2002).

1.3.8.1.4 Further Mechanisms of Resistance

tet(U) was identified on the plasmid pKQ10 in *Enterococcus faecium* and was determined by Ridenhour *et al.* (1996) to confer low-level resistance in this host and *E. coli*. The mechanism

of resistance of Tet(U) is unclear as it only has limited amino acid identity (21 %) to the C-terminal of Tet(M) (Ridenhour *et al.*, 1996). More recently, Caryl *et al.* (2012) have demonstrated that *tet(U)* does not confer resistance in *E. coli* and stated that it is not a resistance gene (Caryl *et al.*, 2012).

Mutations in the 16S rRNA of the ribosome also result in resistance to tetracyclines. For example, a G1058C substitution was found to be associated with resistance to tetracycline in *Propionibacterium acnes* and overexpression of this 16S rRNA operon in *E. coli* resulted in an 8-fold increase in resistance to tetracycline and tigecycline (Ross *et al.*, 1998; Bauer *et al.*, 2004).

1.3.8.2 β -lactam Resistance

As with the tetracycline family of antibiotics, the broad use of β -lactams has resulted in the spread of resistance to them in the clinic, community and veterinary practices. Resistance to β -lactams can arise from impermeability of the cell membrane which has been described in *Acinetobacter* spp. or from efflux of the antibiotic from the cell. For example, the MDR transporter MexAB-OprM contributes to carbapenem resistance in *P. aeruginosa* (Sato and Nakae, 1991; Choudhury *et al.*, 2015). However, it is the expression of β -lactamases and PBPs (penicillin binding proteins) that have proven to be the most problematic in preventing infection treatment (Qin *et al.*, 2008; Carrel *et al.*, 2015).

1.3.8.2.1 β -lactamases

1.3.8.2.1.1 Classification of β -lactamases

β -lactamases are enzymes that are able to hydrolyse the amide bond of the β -lactam ring resulting in inactivation of the antibiotic. These β -lactamases can be organised by the Ambler classification system that groups these enzymes based on their amino acid sequence homology (Hall and Barlow, 2005). Groups A, C and D contain the serine β -lactamases (SBLs); they have a serine residue in their active site and are structurally similar around their active sites to each other and to transpeptidases, suggesting a common ancestry between them (Wilke *et al.*, 2005; Fisher *et al.*, 2005). The metallo- β -lactamases (MBLs) require coordination of one or two zinc ions at their active site to function; they are members of the zinc-dependent metallohydrolases and have arisen from a distinct evolutionary path to the SBLs (Daiyasu *et al.*, 2001; Bebrone, 2007).

β -lactamases from each group have members with extended spectrum (ESBLs) and activity against third and fourth-generation cephalosporins, monobactams. In fact, all but the class C β -lactamases have members with carbapenemase activity, Table 1.4 (Al-Bayssari *et al.*, 2015).

Table 1.4 Classes of β -lactamase Enzymes.

Ambler Class	Active Site	Enzyme Type	Host Organisms	Substrates
A	Serine	TEM, SHV	<i>Enterobacteriaceae</i>	Penicillins, cephalothin, third-generation cephalosporins
		ESBL (TEM, SHV, CTX-M)		
		Carbapenemase (KPC, GES, SME)		
B	Zinc	Carbapenemase (VIM, IMP, NDM)	<i>Enterobacteriaceae</i>	All β -lactams
C	Serine	AmpC	<i>Enterobacter</i> spp. and <i>Citrobacter</i> spp.	Cephamycins, third-generation cephalosporins
		CMY, DHA, MOX, FOX, ACC	<i>Enterobacteriaceae</i>	
D	Serine	OXA	<i>Enterobacteriaceae</i>	Penicillins, oxacillin, cephalothin.
		ESBL (OXA-10, -11, -14, -16)	<i>Enterobacteriaceae</i>	third-generation cephalosporins, aztreonam
		Carbapenemase (OXA-58, 143, -162, -163)	<i>Enterobacteriaceae</i>	β -lactams

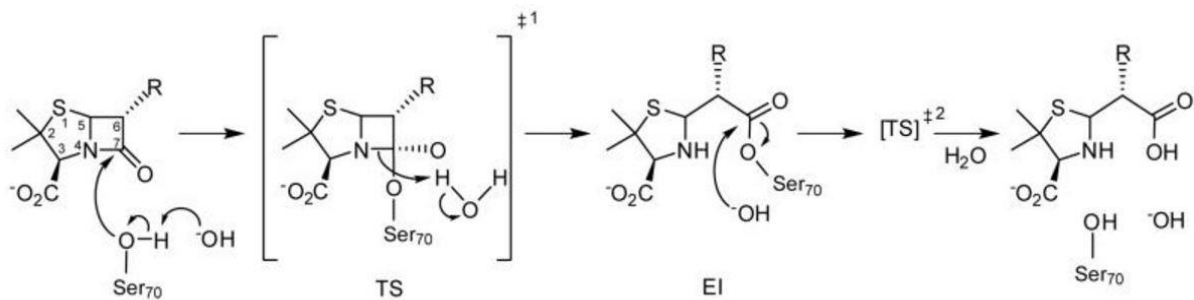
*This table is modified version of the tables found in Kanj *et al.* (Al-Bayssari *et al.*, 2015; Kanj and Kanafani, 2016).

1.3.8.2.1.2 β -lactamase Mechanism of Hydrolysis

The mechanism of β -lactam hydrolysis by SBLs and MBLs differ, although both involve the nucleophilic attack of the carbonyl group of the β -lactam ring. SBLs attack the amide bond of the β -lactam ring via the serine residue of their active site. A covalent bond forms between the hydroxyl oxygen of the serine residue and the β -lactam ring carbon, creating a high-energy transition molecule. The amide bond is then broken as the β -lactam nitrogen is simultaneously protonated resulting in the formation of an acyl-enzyme intermediate. Attack of this intermediate by a catalytic water molecule activates the serine residue covalently bound to the β -lactam carbon creating another transition molecule. The serine oxygen- β -lactam carbon bond is then hydrolysed and the enzyme is released, Figure 1.16(a) (Drawz and Bonomo, 2010).

Figure 1.16

(a)



(b)

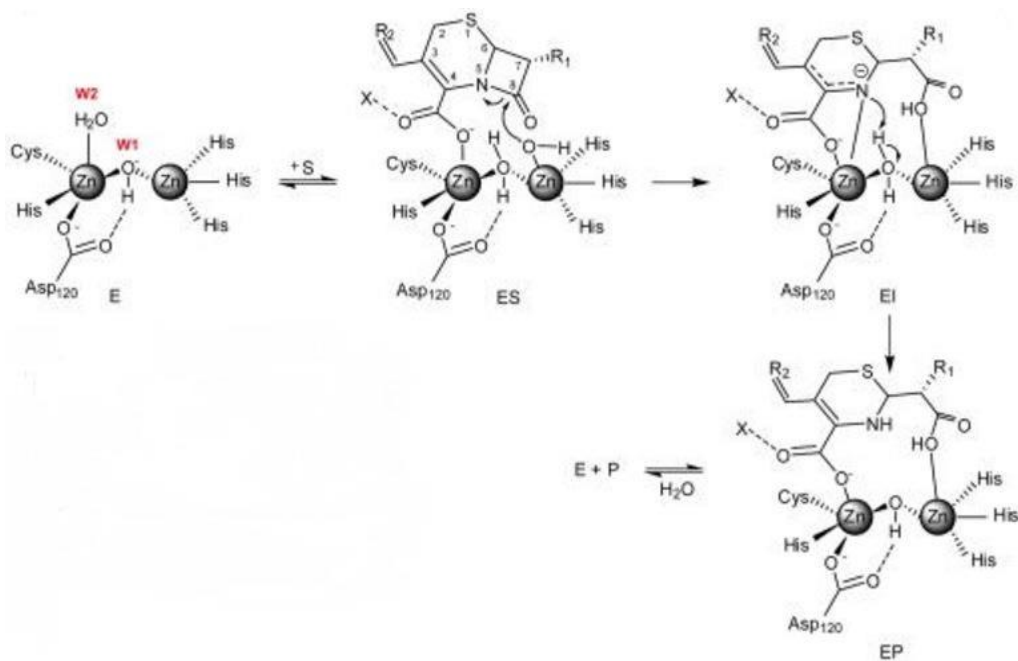


Figure 1.16 β -lactam Hydrolysis by β -lactamases. (a) A depiction of the serine β -lactamase-mediated hydrolysis of the β -lactam ring amide bond. Ser₇₀ represents the active serine residue, curved arrows indicate the movement of electrons, TS stands for transition state. (b) A depiction of the metallo- β -lactamase mediated hydrolysis of the β -lactam ring amide bond of a cephalosporin type β -lactam. Zn1 is on the left, Zn2 is on the right. Curved arrows represent the movement of electrons. Figures reproduced from Meini *et al.* (Meini *et al.*, 2015).

For di-zinc MBLs, Zn1 interacts with the carbonyl carbon while Zn2 interacts with the carboxyl group of the 5- or 6-membered ring, Figure 1.16(b). Instead of an activated serine residue, a hydroxide ion acts as a nucleophile, attacking the carbonyl carbon. The formation of an anionic intermediate containing a negatively charged β -lactam nitrogen atom results from this with the nitrogen atom being subsequently protonated (Palzkill, 2013; Meini *et al.*, 2015). Meini *et al.* (2015) have proposed that the proton donor for this reaction is likely a water molecule that bridges the two Zn ions in the MBL (Meini *et al.*, 2015). Hydrolysis of the bond between Zn1 and the β -lactam carbonyl carbon regenerates the enzyme, Figure 1.16(b).

1.3.8.2.2 Penicillin Binding Protein Affinity

β -lactam resistance can result from the expression of PBPs that have reduced affinity for the antibiotic. Thus, cell wall crosslinking can still occur even in the presence of the antibiotic that can't bind the enzyme. For example, MRSA encode 4 endogenous PBPs as well as a fifth low-affinity PBP, PBP 2a. PBP 2a is encoded by the *mecA* gene and is present on an MGE called the staphylococcal cassette chromosome *mec* (SCC*mec*) (Garcia-Castellanos *et al.*, 2004). Expression of PBP 2a confers resistance to nearly all β -lactams except the fifth-generation cephalosporins, ceftaroline and ceftobiprole (Chambers 1997; Lovering *et al.* 2012; Otero *et al.* 2013).

X-ray crystallography revealed the active site serine of PBP 2a to be too poorly positioned to interact with the amide bond of the β -lactam ring, explaining why acylation of penicillin G by PBP 2a is between 1 and 3 orders of magnitude slower than by the endogenous PBPs

(Graves-Woodward and Pratt, 1998; Lu *et al.*, 1999; Lim and Strynadka 2002).

Conformational changes within the active site allow the active site serine to catalyse cell wall cross linking. Fuda *et al.* (2005) demonstrated that binding of synthetic peptidoglycan fragments at a second 'allosteric' binding site on PBP 2a induced these conformational changes and resulted in an increase in acylation of the PBP (Fuda *et al.*, 2005).

More recently X-ray crystallographic analysis of PBP 2a bound to ceftaroline have shown that a ceftaroline molecule is bound at both the 'allosteric' site and the active site which explains this antibiotic's ability to inactivate this PBP (Otero *et al.*, 2013).

PBPs with low affinity for β -lactams have been implicated in β -lactam resistance in a number of other bacterial species. For example, intrinsic resistance to β -lactams in *Enterococcus* spp. is associated with the expression of the low affinity PBP 5 (Fontana *et al.*, 1983). Loss of expression of PBP 5 can result in hyper-susceptibility, while over expression of PBP 5 with mutations that further decrease its affinity for β -lactams further increases resistance to ampicillin (Fontana *et al.*, 1985; Fontana *et al.*, 1994).

PBPs with lower affinities for β -lactams have also been identified in resistant strains of *S. pneumoniae*, *Neisseria* spp, and *Streptomyces* spp. among others (Bowler *et al.*, 1994; Hakenbeck *et al.*, 2012; Ogawara, 2015).

1.3.8.3 Metal Resistance

Metal resistance, including silver and copper resistance, has been described in a number of bacterial species and is most frequently associated with metal efflux. For example, Gupta *et al.* (1999) described a plasmid from a *Salmonella* spp. isolate from a burns ward as harbouring *silCBA* that encoded a tripartite RND type transporter; such transporters function as cationic antiporters (Gupta *et al.*, 1999). In a later study, Randall *et al.* (2015) showed that these genes conferred silver resistance; with an MIC greater than 256 µg/ml in *E. coli* (Randall *et al.*, 2015). Deletion of any of these genes resulted in a reduction in the MIC to between 4-8 µg/ml. Also, found on this plasmid was a gene, *silE*, that encoded a periplasmic silver binding protein. The purified SilE protein was shown to specifically bind silver and deletion of *silE* from the *E. coli* host resulted in increased susceptibility to silver (Gupta *et al.*, 1999; Randall *et al.*, 2015).

In vitro studies have shown that successive culturing of *E. coli* in sub-inhibitory concentrations of silver can select for mutants with decreased silver susceptibility. This is due to down regulation of outer membrane porins, OmpF and OmpC and loss of repression control of *cusCFBA*, where CusA is a cytoplasmic RND transporter, CusB is a membrane fusion protein, CusC is an outer membrane factor and CusF is a metal chaperone that delivers the metal to the transporter (Li *et al.*, 1997; Lok *et al.*, 2008). However, no such mutations have been observed outside of laboratory conditions (Randall *et al.*, 2015).

Expression of *cusCFBA* is also involved the copper resistance in *E. coli*, Figure 1.17 (Franke *et al.*, 2003). Increased transcription of *cusA* has been demonstrated in *Shewanella oneidensis*

following copper stress, and has also been shown to play a role in copper resistance in *Acidithiobacillus ferrooxidans* (Toes *et al.*, 2008; Navarro *et al.*, 2009).

P-type ATPases such as that encoded by *copA* are also involved in copper efflux from the cell as they hydrolyse ATP to transporter Cu^+ into the periplasm, Figure 1.17 (Rensing *et al.*, 2000). However, once in the periplasm the Cu^+ is still toxic to the cell. Expression of the multicopper oxidase allows the cell to oxidise Cu^+ to the less toxic Cu^{2+} ion, Figure 1.17. CueO is a multicopper oxidase involved in copper resistance. Crystal structures of *E. coli* CueO identified four copper atoms within its structure that mediate the oxidation Cu^+ through four individual electron transfer events (Roberts *et al.*, 2002; Djoko *et al.*, 2010; Singh *et al.*, 2011).

Binding and sequestering of free copper in the cell may also contribute to the copper resistance of some cells. CopT is a copper binding protein required for resistance in *Sulfolobus solfataricus* and CopG and Cot are examples of periplasmic copper binding proteins expressed by *Vibrio cholera* although their full functionality has yet-to-be determined (Villafane *et al.*, 2009; Marrero *et al.*, 2012).

Figure 1.17

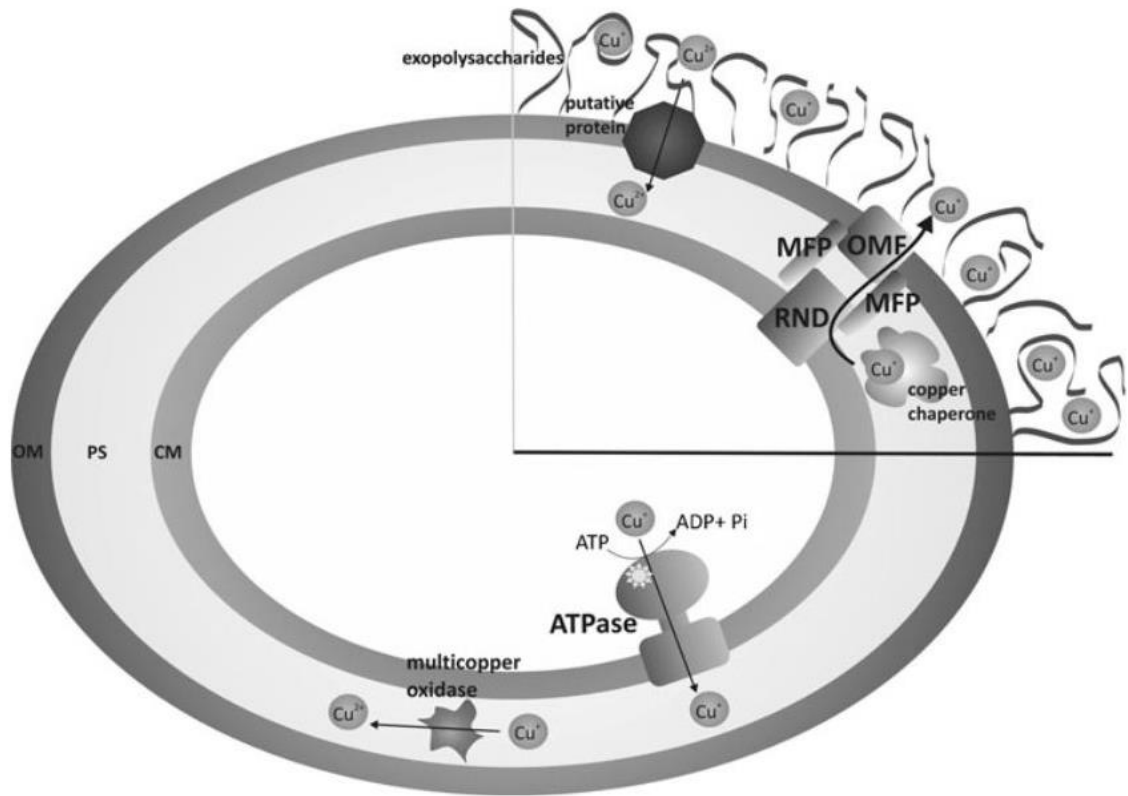


Figure 1.17 Mechanisms of Copper Resistance. A depiction of the cell envelope of a Gram-negative cell: CM is the cytoplasmic membrane, PS is the periplasmic space and the OM is the outer membrane. In the top right corner of the cell is an illustration of the CusCBA transporter that transports Cu^+ from the cell following delivery of the ion by the CusF chaperone. Towards the bottom middle of the cell is a diagram of a P-type ATPase such as CopA that hydrolysis ATP to transport Cu^+ from the cytoplasm into the periplasmic space. At the very bottom of the cell is a representation of the oxidation of Cu^+ to the less toxic Cu^{2+} by multicopper oxidases such as CueO following its transport into the periplasm. This figure was reproduced from Bondarczuk *et al.* (Bondarczuk and Piotrowska-Seget, 2013).

1.3.8.4 Reduced Susceptibility to Cationic Antiseptics

Resistance is typically described as an increase in the MIC of a compound. However, as cationic antiseptics are typically used in concentrations far greater than their MIC, increases in MICs for these compounds do not typically result in a loss of effectiveness of the products containing them. As such the term 'reduced susceptibility' is used rather than resistance for increased cationic antiseptic MICs (Maillard *et al.*, 2013).

As with resistance to metals, reduced susceptibility to cationic antiseptics is frequently associated with the expression of efflux pumps. The RND transporter AcrAB-TolC has been implicated in conferring resistance to QACs in clinical *E. coli* strains (Buffet-Bataillon *et al.*, 2012). Indeed, the use of cationic antiseptics can select for mutations in MDR efflux transporters resulting in reduced susceptibilities. For example, Maseda *et al.* (2009) cultured *Serratia marcescens* in increasing concentrations of CPC and identified *S. marcescens* mutants with reduced susceptibility to CPC, chlorhexidine and other QACs. Using transposon mutagenesis, they demonstrated that mutations in the outer membrane protein HasF which associates with the SdeAB RND transporter were responsible for the observed reduced susceptibility (Maseda *et al.*, 2009).

Transporters of the MFS as well as the multidrug and toxic compound extrusion (MATE) and small multidrug resistance (SMR) superfamilies which are all proton dependent antiporters are involved in cationic antiseptic resistance, Table 1.5 (Poole, 2005).

Table 1.5 Transporter Families Involved in Reduced Quaternary Ammonium Compound or Chlorhexidine Susceptibility.

Transporter Family	Transporters	Antiseptic substrate	Organism
Resistance Nodulation Division (RND)	AcrAB-TolC	QAC*	<i>E. coli</i> , <i>S. enterica</i> serovar Typhimurium
	MexAB-OprM	CHX, QAC*	<i>P. aeruginosa</i>
	CmeABC	QAC*	<i>Campylobacter jejuni</i>
	OqxAB	CHX, QAC*	<i>E. coli</i> ,
Major Facilitator Superfamily (MFS)	QacA	CHX, QAC*	<i>S. aureus</i>
	QacB	QAC*	<i>S. aureus</i>
	NorA	CHX, QAC*	<i>S. aureus</i>
	NorB	QAC*	<i>S. aureus</i>
	MdeA	QAC*	<i>S. aureus</i>
	EmeA	QAC*	<i>E. faecalis</i>
	MdfA	QAC*, CHX	<i>E. coli</i>
Multidrug and toxic compound extrusion (MATE)	MepA	CHX, QAC*	<i>S. aureus</i>
	NorM	QAC*	<i>Neisseria</i> spp.
	PmpM	CHX	<i>P. aeruginosa</i>
Small multidrug resistance (SMR)	QacE		<i>K. pneumoniae</i> , <i>P. aeruginosa</i>
	QacEΔ1	QAC*	<i>Pseudomonas</i> sp., <i>E. coli</i> , <i>K. pneumoniae</i> , <i>S. enterica</i> serovar Typhimurium, <i>S. marcescens</i> , <i>Vibrio</i> spp.,

		<i>Campylobacter</i> spp.,
		<i>E. cloacae</i> ,
		<i>S. aureus</i> , <i>E. faecalis</i>
QacF	QAC*	<i>Enterobacter</i> spp.
QacG	QAC*	<i>P. aeruginosa</i> , <i>S. aureus</i>
QacH	QAC*	<i>S. aureus</i>
QacJ	QAC*	<i>Staphylococcus</i> spp.
Smr (QacC)	QAC*	<i>S. aureus</i>
SugE	QAC*	<i>E. cloacae</i> , <i>E. coli</i>

Amended from Poole *et al.* (Poole, 2005).

*Resistance to CTAB and/or CPC

Alterations in the cell envelope can also result in reduced susceptibility to cationic antiseptics and have been described in *E. coli*, *Serratia* spp. and *Pseudomonas* spp. (Lannigan and Bryan, 1985; Ishikawa *et al.*, 2002). For example, Ishikawa *et al.* (2002) isolated an *E. coli* mutant with reduced susceptibility to CTAB by growing the cells on stepwise increasing concentrations of the antiseptic. They showed that the mutant had a different LPS pattern to its parent strain when viewed by SDS-PAGE and that introduction of the mutant LPS into the parent strain could confer the reduced susceptibility phenotype (Ishikawa *et al.*, 2002). In an earlier study, Tattawasart *et al.* (2000) similarly showed that alterations in the LPS profile between CPC and CHX resistant *Pseudomonas stutzeri* mutants and their parent strains were responsible for the resistance phenotypes in these mutants (Tattawasart *et al.*, 2000).

1.3.9 The Importance of Antimicrobial Resistance Research

As novel antimicrobials are discovered it is important that we understand how resistance to them may arise and know what environments may act as reservoirs for such resistance genes. Thus, studies such as those discussed in this text are important in developing our understanding of how and where antibiotic resistance may arise and may give us the opportunity to prolong the life of our current antibiotics and those in development.

1.4 Bacteriocins as Alternatives to Antibiotics

In addition to understanding how and where resistance can arise to our currently used antibiotics, novel compounds must also be discovered and developed continuously to ensure we always have a viable treatment option for infection; this is currently not the case. In this respect, bacteriocins may offer a novel treatment option. Bacteriocins are ribosomally synthesised peptides produced by bacteria that inhibit the growth of closely related bacteria species, although some have broader spectra of activity (Cotter *et al.*, 2005). In this sense, they differ from antibiotics which themselves are secondary metabolites.

Genes involved in bacteriocin biosynthesis have been identified in nearly every bacteria species studied to date, however the main focus of bacteriocin research has been those produced by the lactic acid-producing bacteria (LAB) (Cotter *et al.*, 2005). This is due to LAB having generally regarded as safe (GRAS) status, meaning that these bacteria and their

metabolites, including bacteriocins, can be used in food and pharmaceutical products to prevent spoilage by inhibiting bacterial growth. For example, the bacteriocin nisin A produced by *Lactococcus lactis* was first discovered in 1928 and following its first use as a food preservative in 1969 is now used as such in over 50 countries (Delves-Broughton *et al.*, 1996; Hansen, 1994; Rogers and Whittier, 1928). Bacteriocins are now beginning to receive attention as potential clinical treatments, as will be discussed in this section.

1.4.1 Classification of Bacteriocins

Bacteriocins can be characterised based on whether they possess post translational modifications (PTMs) or not, as well as their size and whether they have enzymatic activity or not. Class I bacteriocins have modifications, and class II contain no modifications; both are less than 10 kDa and heat stable. The class III bacteriocins are larger than 10 kDa and are heat labile; their classification as bacteriocins is debated (Cotter *et al.*, 2005). Class I bacteriocins were originally referred to as the lantibiotics as they contained the amino acid residues lanthionine and β -methyl lanthionine (Guder *et al.*, 2000). However, as bacteriocins with PTMs other than those resulting in the synthesis of these residues have been described the class has broadened to account for them, Table 1.6 (Claesen and Bibb, 2010; Freeman *et al.*, 2012; Knappe *et al.*, 2010; Li and Kelly, 2010; Melby *et al.*, 2011; Murphy *et al.*, 2011; Sivonen *et al.*, 2010).

Similarly, class II bacteriocins are also subdivided into classes IIa-e and class III bacteriocins can be divided into lytic and non-lytic subgroups, detailed in Table 1.6.

Table 1.6 Classification of Bacteriocins.

Bacteriocin Class	Characteristics	Example	Producing Organism	Targets
Class I				
Lasso peptides	Have a lasso structure	MccJ25	<i>E. coli</i>	<i>E. coli</i> , <i>Salmonella</i> spp.
Lantibiotics	Lanthionine and β -methyl lanthionine Residues	Nisin Mutacin Mersacidin	<i>Lactococcus</i> spp. <i>Streptococcus mutans</i> <i>B. subtilis</i>	<i>Streptococcus</i> spp., MRSA, VRE, <i>Bacillus</i> spp.
Linaridins	Dehydrated amino acids	Cypemycin	<i>Streptomyces</i> spp.	<i>Micrococcus luteus</i>
Sactibiotics	Sulfur- α -carbon linkages	Thuricin CD	<i>Bacillus thuringiensis</i>	<i>C. difficile</i>
Thiopeptides	Central pyridine, dihydropyridine or piperidine ring	Thiostrepton	<i>Streptomyces</i> spp.	MRSA, VRE, <i>Bacillus</i> spp.
Botromycins	Macrocyclic amidine, decarboxylated C-terminal thiazole and methylated residues	Botromycin A2	<i>Streptomyces</i> spp.	MRSA, VRE
Glycocins	S-linked glycopeptides	Sublancin 168	<i>B. subtilis</i>	<i>B. subtilis</i> , <i>S. aureus</i>
Class II				
IIa (Pediocin)	Conserved a YGNGV motif	Pediocin PA-1	<i>Pediococcus acidilactici</i>	<i>Listeria monocytogenes</i> , <i>Lactobacillus</i> spp.
IIb	Two peptides are required for activity	Lactacin F	<i>Lactobacillus acidophilus</i>	
IIc	Cyclic peptides	Enterocin AS-48	<i>E. faecalis</i>	
II d	Unmodified, linear, non-pediocin, single peptide	Microcin V Lactococcin A	<i>E. coli</i> <i>L. lactis</i>	<i>L. monocytogenes</i> , <i>Bacillus</i> spp., <i>Staphylococcus</i> spp., <i>E. coli</i>
IIe	Serine rich carboxy terminal with a siderophore type modification	Microcin M	<i>E. coli</i>	<i>E. coli</i>
Class III				
Lytic	Cell wall degrading	Enterolysin A	<i>E. faecalis</i>	<i>L. lactis</i> , <i>Enterococcus</i> spp. <i>Staphylococcus</i> spp.
Non-lytic	Non-cell wall degrading	Helveticin J	<i>Lactobacillus helveticus</i>	<i>Lactobacillus</i> spp.

This table was amended from Cotter *et al.* (Cotter *et al.*, 2013).

1.4.2 Molecular Biology of Bacteriocins

Bacteriocins are typically expressed as pre-peptides that contain an N-terminal leader sequence making them inactive in the producing cell. These pre-peptide genes are found in gene clusters that contain other genes required for their expression (Jack *et al.*, 1995).

Bacteriocin gene clusters are present on the chromosome but many have been found on MGEs. The class II pediocin bacteriocin clusters are found on plasmids in LAB and the nisin gene cluster is carried on the CTn, Tn5276 (Cui *et al.*, 2012; Lubelski *et al.*, 2008).

The composition of the bacteriocin gene clusters depends on the bacteriocin type and some are more complex than others. For example, in *Carnobacterium divergen* the divergicin A pre-peptide gene is found in an operon with only one other gene encoding an immunity protein and likely represents the simplest bacteriocin operon structure. The larger nisin gene cluster contains 11 genes in four transcriptional operons. (Lubelski *et al.*, 2008; Worobo *et al.*, 1995). Figure 1.18 provides an illustration of the diversity in the size and genes present on a select number of bacteriocin gene clusters.

Figure 1.18

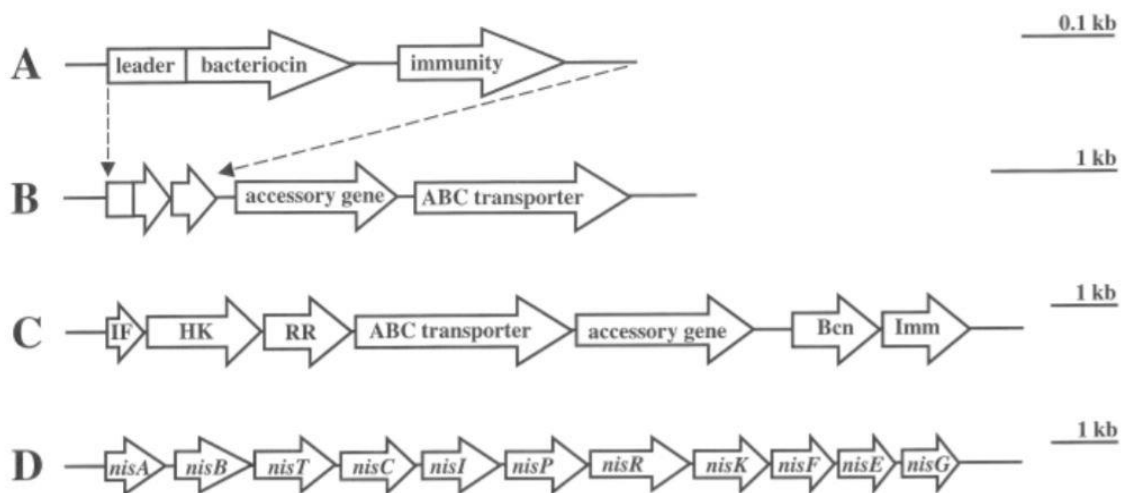


Figure 1.18 Bacteriocin Gene Cluster Organisation. Diagram of the genetic organisation of four bacteriocin gene clusters indicating the diversity in their structure; with a kilobase (kb) scale for each illustration on the right-hand side. (a) The gene organisation in the divergicin A operon. (b) Gene cluster based on that for pediocin PA-1 ACh, identifying its dedicated ABC transporter gene. (c) Gene cluster of carnobacteriocin B2 containing in addition to the equivalent genes for (b) above: the pre-induction peptide (IF); and the regulatory proteins HK (histidine kinase) and RR (response regulator); Bcn is the pre-peptide. (d) Genes required for the production of the lantibiotic, nisin A. *nisA* is the prepeptide, *nisBC* are PMT enzymes, *nisT* is the ABC transporter, *nisP* is the leader peptide protease, *nisRK* is involved in expression regulation, and *nisIFEG* are required for nisin immunity. Figure reproduced from van Belkum and Stiles (van Belkum and Stiles, 2000)

Bacteriocin gene clusters typically encode their own dedicated ABC transporters which in many cases also serve to cleave the leader peptide sequence via an N-terminal cysteine protease (Havarstein *et al.*, 1995). This has been demonstrated to be the case for class II bacteriocins such as pediocin PA-1 ACh, whereby the genes *papC* and *papD* encode an ABC transporter that cleaves the double-glycine type leader peptide sequence of the pre-peptide

bacteriocin. For lantibiotics, leader sequence cleavage may occur following export of the prepeptide; NisT transports the *L. lactis* nisin pre-peptide which is then cleaved by the NisP protease (van der Meer *et al.*, 1993).

The production of immunity proteins protects the cell from being effected by their own bacteriocins. In some cases immunity proteins are ABC transporters as is the case for the class IIc enterocin AS-48 as well as some lantibiotics (Diaz *et al.*, 2003).

Immunity proteins may also function to bind and sequester the bacteriocin. *B. subtilis*, for example, partly gains immunity to the subtilin lantibiotic it produces through the production of a surface-expressed lipoprotein, SpaI, which has been shown to bind the bacteriocin *in vitro* (Stein *et al.*, 2005).

Gene clusters may control their own expression by encoding two-component regulatory systems composed of a histidine kinase receptor and a regulator protein. Genes in lantibiotic operons for example are upregulated when their LanK receptors recognise a specific extracellular lantibiotic and activates its cognate regulator; this has been demonstrated for nisin and subtilin (Kleerebezem, 2004). Thus, any cell expressing the two-component system and encoding the lantibiotic immunity gene will survive in the presence of the bacteriocin. For some class II bacteriocins the receptor kinase recognises an induction factor that is co-transcribed with the bacteriocin; the binding of this molecule ultimately induces bacteriocin expression. This model of regulation has been described for enterocin A, Figure 1.19.

Figure 1.19

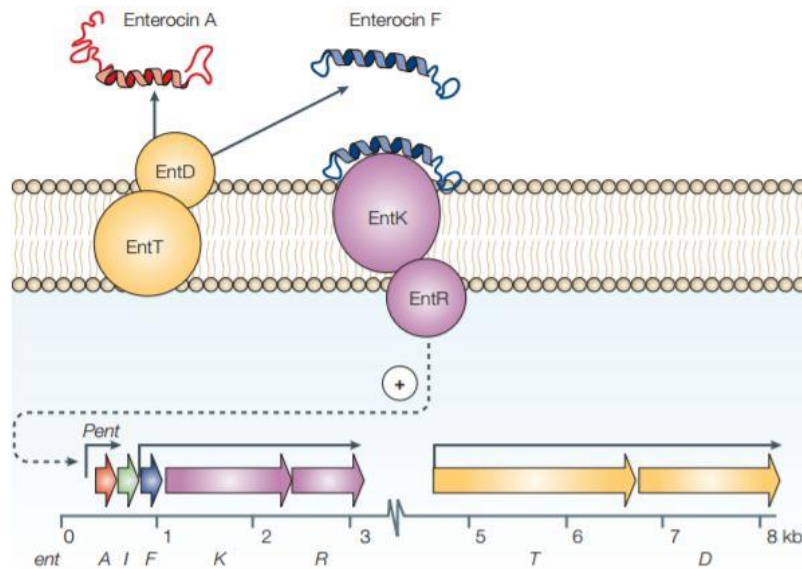


Figure 1.19 Regulation of Bacteriocin Gene Cluster Transcription. Shows the induction of enterocin A through the recognition of the induction factor enterocin F. Both enterocin A and enterocin F genes are co-transcribed and their products exported from the cell via the ABC transporter (EntA) and the accessory protein, EntD. The EntK histidine kinase recognises enterocin F, causing it to autophosphorylate and ultimately activate the regulator EntR that induces expression of the enterocin A and enterocin F genes. Lantibiotic autoinduction follows a similar mechanism, though the bacteriocin itself is recognised rather than an induction factor. This figure was reproduced from Cotter *et al.* (Cotter *et al.*, 2005).

Due to the presence of PTMs in their structure, class I bacteriocins are often found in larger operons than those that encode class II bacteriocins, as they also encode the enzymes required for these modifications. Lantibiotic operons for example encode the enzymes required to synthesise the non-proteinogenic amino acids present in their structure. In the nisin operon two enzymes, *nisB* and *nisC*, encode a dehydratase and cyclase respectively. Both enzymes are required for the conversion of serine and threonine residues to

lanthionine and β -methyl lanthionine in two steps (Lubelski *et al.*, 2008). In other lantibiotic operons a single bifunctional enzyme, *lanM*, is encoded to accomplish these conversions, as is the case for lactacin 481 (Xie *et al.*, 2004).

1.4.3 Mode of Action of Bacteriocins

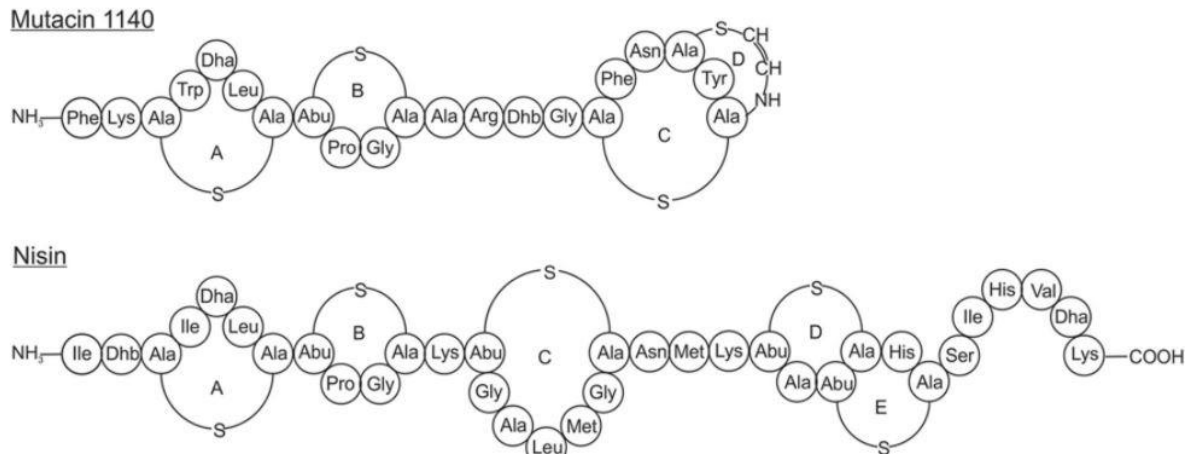
As illustrated in the previous sections, the structure, genetic organisation, regulation and export of bacteriocins is varied and subsequently so are their mechanisms of inhibition.

1.4.3.1 Inhibition by Lipid II Binding

Lantibiotics are surface-active antimicrobials that target Gram-positive bacteria. Linear lantibiotics, such as nisin and the mutacins, are able to bind to the phosphate moiety of the peptidoglycan precursor lipid II via their positively charged N-terminal A and B rings, Figure 1.20(a) (Bierbaum and Sahl, 2009; Smith *et al.*, 2008). This sequestering of lipid II inhibits cell wall synthesis. Nisin is also able to form pores in the target cell in a lipid II-dependent manner, and using a pyrene labelled lipid II model Bierbaum and Sahl (2009) deduced that the pores contain 4 lipid II molecules and 8 nisin molecules (Bierbaum and Sahl, 2009). The formation of these pores results in the loss of cell contents and ultimately cell death (AlKhatib *et al.*, 2014), Figure 1.20(b).

Figure 1.20

(a)



(b)

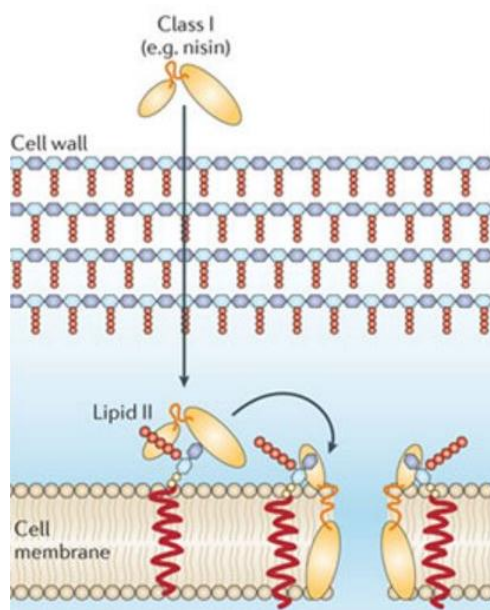


Figure 1.20 Structure and Mode of Action of Lipid II Binding Bacteriocins. (a) The structure of the lantibiotics mutacin 1140 and nisin highlighting their A and B rings involved in lipid II binding, reproduced from Escano *et al.* (Escano *et al.*, 2015). (b) A depiction of the mode of action of the lantibiotics with nisin as an example. The diagram illustrates the binding of lipid II that abrogates cell wall synthesis. The pores formed by the lipid II-nisin complexes that results in cell death is also illustrated above. This figure was reproduced from Cotter *et al.* (Cotter *et al.*, 2005).

The globular lantibiotics, such as mersacidin which is produced by *S. aureus*, also interact with lipid II to inhibit cell wall synthesis, although they do not form pores (Brotz *et al.*, 1995). Additionally, mersacidin is not positively charged and binds to the glucose residue of the precursor rather than the phosphate moieties (Brötz *et al.*, 1998).

1.4.3.2 Pore-Forming Bacteriocins

Class II bacteriocins are known to act through the formation of pores, permeabilising the target cell membrane. However, the mechanism of pore formation differs between bacteriocins.

The class IIa bacteriocin, lactococcin A, binds to mannose phosphotransferase (MPTs) proteins in the cell membrane before inserting itself into the cell membrane, Figure 1.21.

The deletion of the genes required for MPTs synthesis in a sensitive *L. lactis* strains resulted in resistance to lactococcin A (Diep *et al.*, 2007).

Figure 1.21

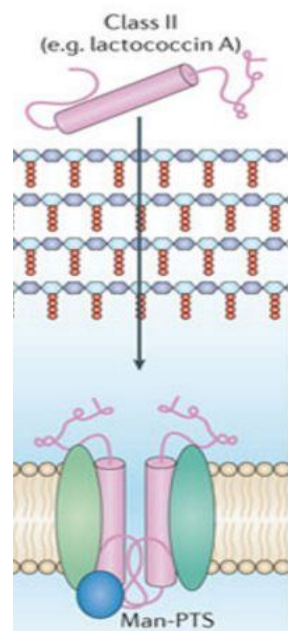


Figure 1.21 Mode of Action of Class IIa Bacteriocins. An illustration of the interaction of the class IIa, lactococcin A with cell membrane bound mannose phosphotransferase (Man-PTS) followed by its insertion into the cell membrane. This figure was reproduced from Cotter *et al.* (Cotter *et al.*, 2005).

The class IIe bacteriocin microcin E492, has been shown to recognise the *E. coli* siderophore receptors via its C-terminal, and following transport through the outer membrane in a TonB dependent manner, depolarises the cytoplasmic membrane (Destoumieux-Garzon *et al.*, 2006).

The class IIb and the class IIc bacteriocins form pores in an oligomeric manner. The two class IIb lactococcin G bacteriocin components contain conserved GxxxG-motifs. Nuclear magnetic resonance (NMR) analysis of wild-type and mutant forms of this bacteriocin has indicated that these motifs are present on transmembrane helices on each subunit that stabilise the transmembrane form (Rogne *et al.*, 2008). The circular bacteriocin AS-48 has been shown to oligomerise above pH 5, and NMR analysis of the AS-48 dimer indicate that

following membrane binding, hydrophobic residues become exposed allowing it to insert into the membrane (Abriouel *et al.*, 2001; Sanchez-Barrena *et al.*, 2003).

1.4.3.3 Enzymatic Bacteriocins

The antimicrobial activity of some bacteriocins is mediated through enzymatic activity, typically targeting cell wall components or cell nucleic acids.

For example, the enterolysin A contains an N-terminal metalloprotease domain with homology to the *Staphylococcus* spp. lysostaphins. Purified enterolysin has been demonstrated to rapidly lyse *L. lactis* cells and to be able to digest purified *L. lactis* peptidoglycan (Nilsen *et al.*, 2003).

1.4.3.4 Bacteriocins Targeting DNA, RNA and Protein Synthesis

A number of class I bacteriocins can also inhibit gene expression through the inhibition of translation. Bottromycin and the thiopeptide thiostrepton, both from *Streptomyces* spp., bind to the ribosomal 50S subunit preventing charged aminocyl-tRNA molecules from entering the translation complex (Bagley *et al.*, 2005; Kobayashi *et al.*, 2010). The microcin MccB17 targets DNA gyrase stabilising it in a complex with cleaved DNA; this results in DNA breakage and cell death (Thompson *et al.*, 2014). Microcin J25 (MccJ25) on the other hand can bind to RNA polymerase, preventing nucleotide triphosphates from accessing the active

site and being added to the growing mRNA polymer, inhibiting transcription (Vincent and Morero, 2009).

1.4.4 Clinical Applications of Bacteriocins

The diversity of antimicrobial activities of bacteriocins make them attractive alternatives to antibiotics and they offer a number of advantages in this regard. Most bacteriocins are generally non-toxic to humans which is evident from the fact that humans have been ingesting them from fermented food for millennia. The only lantibiotic that has demonstrated toxicity towards human cells is the *Enterococcus* spp. cytolysin (Coburn and Gilmore, 2003).

The majority of bacteriocins exhibit broad-spectrum inhibition; MccB17 for example inhibits *Escherichia* spp., *Citrobacter* spp, *Klebsiella* spp, *Salmonella* spp, *Shigella* spp and *Pseudomonas* spp; although some exhibit more targeted inhibition (Baquero and Moreno, 1984). In a human colon model the bacteriocin, thuricin CD, was demonstrated to have comparable activity to vancomycin against *C. difficile*. However, thuricin CD did not significantly disrupt the commensal microbiota as is observed in vancomycin treatment that leads to *C. difficile*-associated diarrhoea (Rea *et al.*, 2011).

As most bacteriocins do not share cell targets with currently used antibiotics they have activity against a number of antibiotic-resistant pathogens. Lantibiotics and the thiopeptide bottromycin can inhibit the growth of VRE, and further to this a number of lantibiotics are currently in pre-clinical trials (Kobayashi *et al.*, 2010; Piper *et al.*, 2009). Oragenics Inc.

currently have two mutacin derivatives, MU1140-S and OG716, in or entering into pre-clinical trials for the treatment of MRSA and *C. difficile* infections (BusinessWire, 2016). A second lantibiotic derivative, Deoxyactagardine B, has completed clinical trials for *C. difficile* treatment (Sandiford, 2015).

Bacteriocins and bacteriocin-producing strains are now found in a number of products: *S. salivarius* K12-producing salivaricins A and B has been included in mouth spray to treat throat infections and nisin is used in teat disinfection wipes (BLIS Technologies Ltd)(Wipe Out Immucell).

1.4.5 Resistance to Bacteriocins

Although bacteriocins show promise as alternative and adjunctive treatments for antibiotic-resistant pathogens, bacteriocin resistance could arise. With regard to nisin, some strains of *L. lactis* that do not produce nisin have been shown to produce proteolytic enzymes conferring resistance to it (Sun *et al.*, 2009). There is also the potential for susceptible strains to acquire the bacteriocin immunity gene from producer strains. In a recent study, Draper *et al.* (2012) demonstrated that the heterologous expression of the *spiFEG* immunity locus in *Streptococcus infantarius* increased its survival in subinhibitory concentrations of nisin U (Draper *et al.*, 2012). However, in nisin's 40 years of being used as a food preservative no, significant resistance to it has been found outside of lab conditions.

As previously mentioned, resistance to class IIa bacteriocins has been demonstrated in the lab for *L. lactis* strains producing MPTs (Diep *et al.*, 2007). It may be that clinical strains with

reduced expression of MPTs exhibit reduced susceptibility to class IIA bacteriocins.

Spontaneous mutations in *L. monocytogenes* resulting in decreased expression of MPTs have been shown to exhibit resistance to pediocin PA-1 (Gravesen *et al.*, 2002).

1.4.6 The Importance of Identifying Novel Bacteriocins

As discussed above, the bacteriocins represent a diverse group of antimicrobial proteins with different modes of action to our current arsenal of antibiotics and as such have activity against many antibiotic resistance pathogens. If bacteriocins are to find clinical use it is important that we continue to identify novel members that have activity against clinically important pathogens that can be developed as therapies. As every bacterial species studied to date has been found to encode at least one bacteriocin, the bacterial species that have yet-to-be cultured are likely to produce novel variants.

1.5 Metagenomics

1.5.1 The Beginning of Metagenomics

Microbiology as a science has its foundations in the observation of microorganisms, either through a lens or growing in a petri dish. However, it was noted that the cells under the microscope far outnumbered the colonies growing on agar. This discrepancy has since been coined the 'great plate count anomaly' (Staley and Konopka, 1985).

Estimates put the number of microbial cells on the planet at between 9.2×10^{29} and 3.17×10^{30} (Kallmeyer *et al.*, 2012). However, as approximately 95-99 % of these microorganisms have yet-to-be cultured there is a vast pool of untapped diversity and potentially useful biomolecules to be discovered (Ekkers *et al.*, 2012; Schloss and Handelsman, 2004; Torsvik and Ovreas, 2002).

The term metagenomics was first used by Handelsman *et al.* in 2004 to describe the field of study that utilises culture-independent approaches to analyse this uncultivable majority (Handelsman., 2004). Such studies negate the need to culture bacteria, instead analysing their genetic material using various genetic tools as will be discussed (Thomas *et al.*, 2012).

1.5.2 Approaches to Metagenomic Studies

Metagenomic studies can be defined as either sequence-based or functional. The analysis of the 16S rRNA gene to determine the composition of the microbiota in an environment is a

sequence-based approach; however, it provides no phenotypes for the microorganisms identified (Tringe and Hugenholtz, 2008). Gene functions can be inferred from sequence data by comparing with sequences of functionally characterised gene sequences.

Alternatively, functional activity of gene products can be directly observed by shotgun cloning DNA into a cultivable host, observing a change in the phenotype of interest of this host and subsequently sequencing its insert.

1.5.2.1 Sequence-Based Metagenomic Approaches

1.5.2.1.1 16S rRNA Gene as a Taxonomic Marker

The 16S rRNA gene is frequently used as a marker for determining the phylogenetic diversity in an environmental sample. It has been chosen as a gene marker for several reasons.

Firstly, it is universally distributed across bacteria and archaea; secondly it is functionally homologous across microorganisms allowing for meaningful comparisons to be made; and thirdly its primary sequence is relatively stable over time and contains both conserved and highly variable regions which allows for taxonomic relationships to be made (Chroneos, 2010; Tringe and Hugenholtz, 2008), Figure 1.22.

Figure 1.22

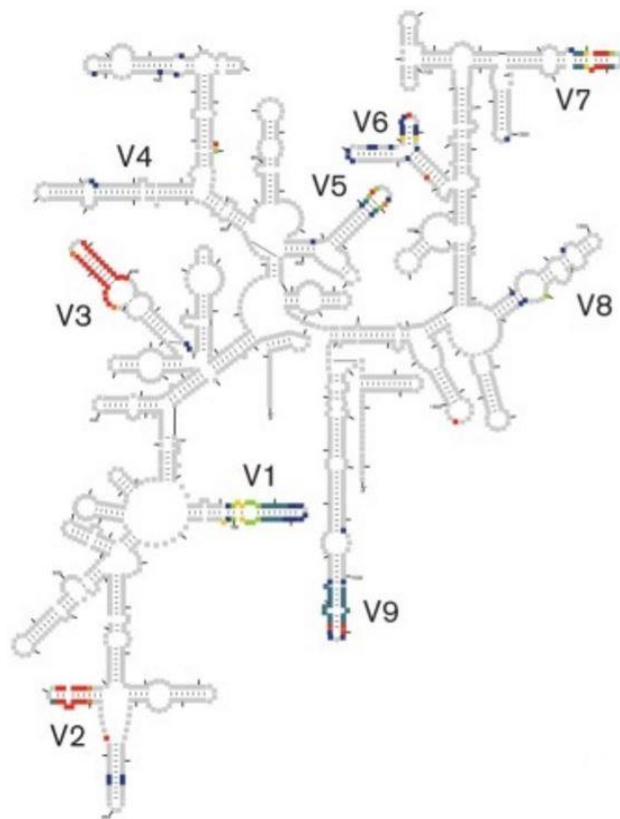


Figure 1.22 Structure of 16S rRNA. The above diagram illustrates the transcribed 16S rRNA structure. The 9 variable regions that can be used to characterise taxa are labelled V1-V9. Figure reproduced from Case *et al* (Case *et al.*, 2007).

16S rRNA gene analyses originally relied on the amplification of full length, 1.5 Kb, rRNA genes from a metagenomic sample followed by cloning of these amplicons into a cultivable host. The clones in the resulting 16S rRNA library are then Sanger sequenced and analysed. Such surveys are laborious and sequencing depth is limited by the number of clones analysed which may result in the omission of rare taxa (Elshahed *et al.*, 2008).

The use of next-generation sequencing such as the Illumina Miseq and Roche 454 platforms have offered access to these rare taxa. Although these systems can sequence only short

read lengths (250 bp in the case of Illumina Miseq; less than the full length 16S rRNA gene) they can generate millions of these reads in a single run giving a much greater coverage than full length 16S rRNA clone libraries. These studies have demonstrated that between 10 % and 74 % of sequence reads may correspond to unclassified taxa (Bartram *et al.*, 2011; Youssef *et al.*, 2015).

16S rRNA gene sequencing studies have greatly expanded our knowledge of the global diversity of bacteria. Genbank contains over 5 million full-length 16S rRNA gene sequences, and as of August 2014, 100 bacterial phyla were included in the curated 16S rRNA databases, GreenGenes and Silva. Of these phyla, only 30 are cultivable and 18 of these were originally identified by 16S rRNA sequencing (McDonald *et al.*, 2012; Quast *et al.*, 2013; Youssef *et al.*, 2015).

1.5.2.1.2 Gene Identification from Metagenomes

In addition to assessing metagenomes for their taxonomic composition, they can also be mined to identify novel genes of interest. Such studies can take a sequence-based approached whereby novel genes are identified based on their homology to already genetically and functionally characterised genes. Some of the techniques used in this regard are outlined in Table 1.7 (Culligan *et al.*, 2014).

Table 1.7 Techniques Used to Identify Genes from Metagenomic Samples and Data.

Technique	Requirements	Uses	Application Example	Reference
PCR/Metagenome Analysis	Design of degenerate primers to amplify genes of interest from metagenomic sample.	Identification of novel members of known gene families.	Identification of novel putative antibiotic synthesis gene clusters from soil metagenomes.	(Courtois <i>et al.</i> , 2003; Owen <i>et al.</i> , 2013)
Shotgun Metagenomic Sequencing	Sequencing and subsequent assembly of non-targeted metagenomic DNA.	Identification of novel members of known gene families.	Identification of pederin type toxin gene clusters from lichen metagenome.	(Kampa <i>et al.</i> , 2013)
Data Mining	Identification of genes of interest by mining existing metagenomic sequence data.	Identification of novel members of known gene families.	Identification of novel methyl halide transferase genes.	(Bayer <i>et al.</i> , 2009)
TRACA (Transposon-aided capture)	Tn5 transposon insertion into plasmid DNA from metagenomic samples and subsequent cloning into <i>E. coli</i> host.	Identification of plasmids from metagenomic samples and the genes they encode following sequencing from Tn5.	Plasmid encoded toxin-antitoxin genes from human plaque samples	(Warburton <i>et al.</i> , 2011)
Integron Capture	PCR amplification of integron captured gene cassettes using primers targeting conserved repeats in their sequence	Identification of gene cassettes that can identify novel genes without prior sequence information.	Identification of numerous toxin-antitoxin gene cassettes from human saliva integrons	(Tansirichaiya <i>et al.</i> , 2016)
Solution Hybrid Selection	Multiple 'explorative' and variant probes are designed and hybridized with metagenomic DNA fragments.	To enrich metagenomic DNA samples with fragments containing genes of interest that can then be sequenced.	Carbohydrate active and protease enzymes from soil.	(Manoharan <i>et al.</i> , 2015)

Table amended from Culligan *et al.* (Culligan *et al.*, 2014).

Sequenced-based approaches to identify genes from metagenomic samples have been aided in recent times by the development of online annotation tools that identify genes of interest based on primary sequence homology, gene cluster structure and predicted protein structure, Table 1.8.

Table 1.8 Tools Used to Identify Genes of Interest from Metagenomic Sequence Data.

Online Tool	Website	Application
BRENDA	http://www.brenda-enzymes.org/	To find the comprehensive enzyme information system in the metagenomic library
IMG/M	http://img.jgi.doe.gov/m	Provides comparative data analysis tools extended to handle metagenome data, together with metagenome-specific analysis tool
Megx.net	http://www.megx.net/	To predict gene functions of metagenome sequences
MetaBioME	http://metasystems.riken.jp/metabiome/	To find novel homologs for known commercially useful enzymes in metagenomic datasets and completed bacterial genomes
MG-RAST	http://metagenomics.nmpdr.org/	Provides a new paradigm for the annotation and analysis of metagenomes
EnGenIUS	http://engenius.software.informer.com/	Provides a comprehensive metagenome research toolset
BAGEL	http://bagel.molgenrug.nl/	To identify bacteriocin gene clusters from sequence data

*This table was amended from Krishnan *et al.* (Krishnan *et al.*, 2014).

1.5.2.2 Functional Metagenomics

Functional metagenomics, in contrast to sequence-based gene identification approaches, does not require any prior sequence information to identify genes of interest. Instead this approach requires the shotgun cloning of metagenomic DNA fragments using a suitable vector and cultivable host strain to create metagenomic libraries (Ekkers *et al.*, 2012). These metagenomic libraries can then be screened to identify clones with phenotypes of interest such as pigmentation, reduced antimicrobial susceptibility, antimicrobial production etc. Sequencing of these clones in conjunction with mutagenesis and subcloning allows for the identification of the genes involved in the altered phenotype (Arivaradarajan *et al.*, 2015; Craig *et al.*, 2010).

Although functional metagenomic studies do not suffer from PCR biases the cell lysis and DNA fragmentation protocols can introduce biases as they determine the diversity, composition and fragment size of the metagenomic DNA (Tanveer *et al.*, 2016). Additionally, the choice of host and vector will affect the outcome of functional metagenomic surveys as will be discussed (Ekkers *et al.*, 2012).

1.5.2.2.1 Design of Functional Metagenomic Surveys

As functional metagenomics requires the identification of a change in phenotype of the host to identify genes of interest, the cloned genes must be expressed in the host. Successful expression requires host transcription and translation factors to recognise signals in the

cloned DNA. Additionally, the host must use the same codons, produce the correct co-factors, provide conditions for proper folding, secrete the protein if necessary and not be inhibited by the gene products. Thus, it is not surprising that functional metagenomic surveys frequently have low hit rates, <2 from 10,000 screened clones (Vester *et al.*, 2015b).

1.5.2.2.1.1 Expression Vectors

When creating a metagenomic library, the desired insert size is an important consideration when choosing a vector. Plasmids are used to maintain small inserts (≤ 15 Kb) while larger inserts can be obtained using fosmids and cosmids (≤ 40 Kb) and bacterial artificial chromosomes (BACs; up to 200 Kb) (Ekkers *et al.*, 2012; Leis *et al.*, 2013). The choice of insert size greatly determines the outcome of a functional metagenomic screen.

Small inserts contain only a few genes which can be overexpressed by inducible promoters on multicopy plasmids increasing the chance of their identification. However, these small inserts will not contain full biosynthetic gene clusters and will also lack the genetic context which helps to identify the likely source of cloned DNA libraries (Taupp *et al.*, 2011).

Additionally, more clones must be screened in order to achieve comparable coverage to larger insert libraries.

Larger inserts can contain full biosynthetic gene clusters and their greater genetic context may provide more confidence on the source of the cloned DNA as well if genes of interest are located on MGEs. However, the genes present on large inserts likely rely on their native promoter sequences for transcription as the promoters on the vectors will not be able to

drive expression of all genes on the insert due to differences in gene orientation and the presence of premature transcription stop signals (Liebl *et al.*, 2014).

Vectors and cassettes utilising T7 RNA polymerase (RNAP)-promoter systems, anti-termination proteins and convergent promoters have been developed to increase the expression of genes in large inserts (Liebl *et al.*, 2014; Terrón-González *et al.*, 2013).

Additionally, the use of shuttle vectors that replicate in more than one host have been developed and used to increase the hit rate of functional metagenomic screens (Liebl *et al.*, 2014).

Table 1.9 is a non-exhaustive list of vectors that have been used in metagenomic studies or show promise as tools for functional metagenomic studies.

Table 1.9 Vectors and Cassettes as Tools for Functional Metagenomic Studies.

Vector/Cassette	Hosts	Traits	Reference/ Company
pCC1BAC™	<i>E. coli</i>	Single Copy, Inducible to multicopy, ≤200 Kb, T7 promoter*.	Epicentre®
pCC1FOS™	<i>E. coli</i>	Single Copy, Inducible to multicopy, ≤40 Kb, transfection, T7 promoter*.	Epicentre®
pWEB™ Cosmid	<i>E. coli</i>	Single Copy, Inducible to multicopy, ≤40 Kb, transfection, T7 promoters*.	Epicentre®
pMPO579	<i>E. coli</i>	pCC1FOS™ based shuttle vector, T7 promoter, oriT for conjugative transfer, <i>nutL</i> site for phage transcription anti-termination protein N assembly. Promoterless <i>gfp</i> for substrate induced gene expressions (SIGEX).	(Terrón-González <i>et al.</i> , 2013)
pJOE930	<i>E. coli</i>	Convergent inducible lac promoters flanking cloning site.	(Lämmle <i>et al.</i> , 2007)
pHT01	<i>E. coli</i> , <i>B. subtilis</i>	ColE1 replicon, inducible <i>groE</i> promoter	MoBiTec GmbH
pRS44	<i>E. coli</i> , <i>Pseudomonas fluorescens</i> , <i>Xanthomonas campestris</i>	pCC1FOS™ based shuttle vector, <i>oriT</i> for conjugative transfer, <i>parDE</i> stabilising elements	(Aakvik <i>et al.</i> , 2009)
pCT3FK	<i>E. coli</i> , <i>Thermus thermophilus</i>	pCC1FOS™ based shuttle vector, contains <i>pyrF</i> and <i>hyp</i> genes of <i>T. thermophilus</i> for chromosomal integration	(Leis <i>et al.</i> , 2015)
pJWC1	<i>Agrobacterium tumefaciens</i> , <i>Burkholderia graminis</i> , <i>Caulobacter vibrioides</i> , <i>E. coli</i> , <i>Pseudomonas putida</i> , <i>Ralstonia metalliduran</i>	RK2 replicon, pTR101 derived cosmid	(Craig <i>et al.</i> , 2010)
Transfer and Expression of biosynthetic pathways (TRES)	<i>E. coli</i> <i>P. putida</i> <i>Rhodobacter capsulatus</i>	Two cassette system that labels and mediates conjugative transfer of gene clusters to non- <i>E. coli</i> hosts. Convergent T7 promoters.	(Loeschcke <i>et al.</i> , 2013)

* These vectors and *E. coli* EPI300 T1^R strains do not encode the T7 RNA polymerase.

1.5.2.2.1.2 Metagenomic Library Hosts

E. coli is the most utilised host for creating metagenomic libraries as it is well characterised and amenable to genetic manipulation. However, using a single host will not allow for the expression of every cloned gene. Indeed, Gabor *et al.* (2004) estimated that from the genes of 32 different genera, *E. coli* would be able to express only 40 % of them (Gabor *et al.*, 2004). This figure is likely to be an overestimate as the authors only looked at expression signals and did not account for differing cofactors, chaperones etc.

The expression of alternative sigma factors and chaperones in *E. coli* has been demonstrated to increase the expression of cloned genes. For example, the use of *E. coli* EPI300 T1^R harbouring additional sigma factors from *Clostridium* and *Streptomyces* spp. had a 20 – 30 % increased hit rate in functional metagenomic screens to identify hydrolytic enzymes (Liebl *et al.*, 2014). In addition to altered *E. coli* hosts, alternative hosts may also be used to create metagenomic libraries, Table 1.10. In fact, the use of alternative hosts may be required when screening for certain functions such as the identification of cold enzymes, as *E. coli* does not grow well at low temperature (Strocchi *et al.*, 2006).

Table 1.10 Alternative Hosts Used in Functional Metagenomic Surveys.

Host	Metagenome	Novel Genes/Phenotypes	References
<i>T. thermophilus</i>	Hotspring sediment and water	Esterases	(Leis <i>et al.</i> , 2015)
<i>B. subtilis</i>	Deciduous forest Soil	Antimicrobial Production	(Biver <i>et al.</i> , 2013)
<i>P. putida</i>	Wheat Soil	Polyhydroxyalkanoate Synthases	(Cheng and Charles, 2016)
<i>S. lividans</i>	Alaskan Soil	Sigma Factors and Haemolysins	(McMahon <i>et al.</i> , 2012)
<i>A. tumefaciens</i>	Pennsylvanian soil	Pigmentation	(Craig <i>et al.</i> , 2010)
<i>Rhizobium leguminosarum</i>	Anaerobic Sludge	Alcohol/Aldehyde Dehydrogenase	(Wexler <i>et al.</i> , 2005)
<i>B. graminis</i>	Pennsylvanian soil	Antimicrobial Production	(Craig <i>et al.</i> , 2010)
<i>R. metallidurans</i>	Pennsylvanian soil	Pigmentation, Antimicrobial Production	(Craig <i>et al.</i> , 2010)

1.5.2.2.1.3 Functional Screen

Following the creation of a metagenomic library, appropriate functional screens to identify clones of interest must be designed. Functional screens can be grouped into three strategies: phenotypic insertion detection, modulated detection and reporter-based screens.

Phenotypic insertion detection involves the observation of a phenotype of interest. These phenotypes may be direct such as altered colony morphology or pigmentation, or indirect by the interaction of a clone's gene product(s) with a substrate or indicator organism. For example, protease activity can be detected by culturing metagenomic libraries on skimmed milk containing agar while antimicrobial producing clones can be identified by identifying halos in sensitive indicator organism overlays (Arivaradarajan *et al.*, 2015; Waschowitz *et*

al., 2009). Such screens are typically low tech and not very sensitive as poorly expressed genes may be missed. However, the coupling of such screens with microfluidic approaches can increase throughput and sensitivity. Scanlon *et al.* (2014) developed an antimicrobial screen whereby a metagenomic clone and indicator organism are immobilised in a gel droplet. Staining of the droplets with a fluorescent dye to identify lysed cells allowed for the screening of > 5 million clones in one day (Scanlon *et al.*, 2014).

Modulated detection involves the identification of genes of interest based on their expression allowing the host cell to survive under certain conditions. For example, a cold-sensitive *E. coli* mutant unable to grow at temperatures below 20°C has been used to identify DNA polymerases from a glacial ice metagenome that allowed the mutant to survive low temperatures (Simon *et al.*, 2009). Similar functional screens have been designed to identify genes that allow their heterologous host to survive on exogenous lysine and to utilise ethanol (EtOH) as a carbon and energy source (Chen *et al.*, 2009; Wexler *et al.*, 2005). The addition of antimicrobials to a media also allows for the discovery of clones expressing antibiotic resistance genes (Card *et al.*, 2014).

Reporter-based screens utilise a reporter gene such as *gfp* (green fluorescent protein) coupled to the activity of a cloned gene promoter or to a cloned gene's product. For example, SIGEX involves the cloning of genes upstream of a promoter-less *gfp*, linking their expression. Following induction by a substrate, induced genes can be identified by fluorescence associated cell sorting (FACS). SIGEX has been used to identify many genes including those induced by hydrocarbons (Meier *et al.*, 2015). Similarly, product-induced gene expression (PIGEX) utilises a system whereby *gfp* transcription is under the control of a

promoter sensitive to a gene product of interest. PIGEX has been used to identify a novel amidase enzyme involved in benzoate synthesis (Uchiyama and Miyazaki, 2010).

1.5.2.2.2 Bacterial Artificial Chromosomes

The BAC vector was first developed in 1992 by Shizuya *et al.* (1992) to clone large human DNA fragments with the aim of creating a high-resolution map of the human genome. It was created through the modification of the mini-F plasmid pMB0131 (Shizuya *et al.*, 1992).

The ability of BACs to stably maintain their large inserts is due to their ability to regulate their own replication and copy number. The former is accomplished by the presence of an F-factor replicon (*repE* and *Ori2* in pCC1BAC) that mediates the unidirectional replication of the vector, while copy number is maintained by the expression of the *par* operon (*parCBA* in pCC1BAC) which prevents the maintenance of multiple plasmids in the cell, Figure 1.23 (Easter *et al.*, 1998; Ishiai *et al.*, 1992; Wild *et al.*, 2002).

The cloning site of pCC1BAC is composed of bacteriophage A *cosN* and P1 *loxP* sites, three restriction sites (*HindIII*, *BamHI* and *EcoRI*) and flanking T7- and IPTG-inducible promoters. The vector also encodes a chloramphenicol resistance gene and a *lacZ* gene for blue/white screening, Figure 1.23 (Wild *et al.*, 2002).

In addition to *Ori2*, pCC1BAC encodes a second, high copy number origin of replication, *OriV*, which increases the vector's copy number to approximately 25 copies per cell when induced by the *trfA* gene product from *E. coli* EPI300 T1^R, Figure 1.23 (Wild *et al.*, 2002).

Figure 1.23

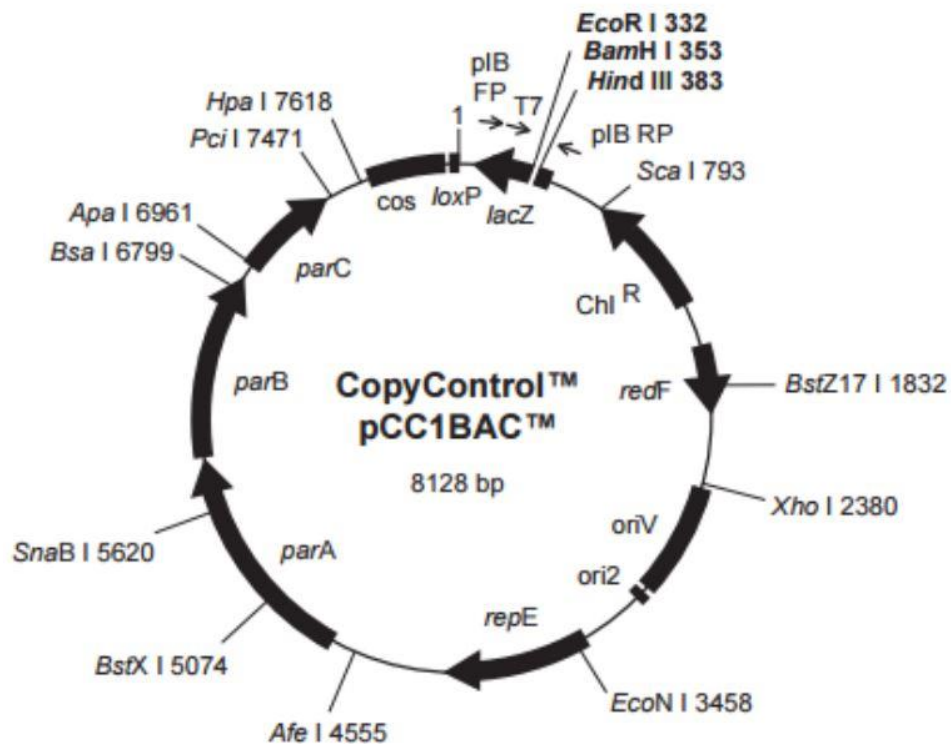


Figure 1.23 Map of the CopyControl™ pCC1BAC™ vector. Genes are indicated by black arrows, origin of replication sites and the cosN and loxP sites are indicated by black rectangles. Restriction sites at the multicloning site are highlighted in bold. This figure was reproduced from the Epicentre® CopyControl™ pCC1BAC™ Cloning kit manual.

Although various expression systems have been developed and multiple hosts can now be used to create metagenomic libraries, *E. coli* is still the most widely used host and in recent years the BAC system has been used to create libraries from various metagenomes, Table 1.11.

Table 1.11 Metagenomic Libraries Created Using BAC Vectors.

Metagenome	Novel Genes/Phenotypes/Compounds	Reference
Marine	Bacteriorhodopsin	(Beja <i>et al.</i> , 2000)
Silt Loam Soil	Kanamycin resistance	(Riesenfeld <i>et al.</i> , 2004)
Soil	Indirubis-like antimicrobial compound	(MacNeil <i>et al.</i> , 2001)
Soil	Turbomycin A and B antimicrobials	(Gillespie <i>et al.</i> , 2002)
Ikaite columns	Amylase	(Vester <i>et al.</i> , 2015a)
Ikaite columns	B-galactosidase	(Vester <i>et al.</i> , 2014)
Antarctic soil	Cellulase	(Berlemont <i>et al.</i> , 2009)
Human Plaque and Saliva	Antimicrobial Protein	(Arivaradarajan <i>et al.</i> , 2015)
Mouse Large Intestine	β -glucanases	(Walter <i>et al.</i> , 2005)
Yingtian Red Soil	β -glucanases	(Liu <i>et al.</i> , 2011)
Red Sea	Anti-Quorum Sensing Compounds	(Yaniv <i>et al.</i> , 2017)
Mouse Large Intestine	Enhanced Adherence	(Yoon <i>et al.</i> , 2013)
Yak Rumen	Cellulases, Xylanases, Esterases	(Dai <i>et al.</i> , 2012)
Cattle Rumen	Xylanase	(Gong <i>et al.</i> , 2013)
Gisburn Forest Soil	Monooxygenase Operon	(Dumont <i>et al.</i> , 2006)
Soil	Amylase, Lipase, Haemolysin, Antimicrobial, DNase	(Rondon <i>et al.</i> , 2000)
Cow Rumen	Amylase, Xylanase	(Zhao <i>et al.</i> , 2010)

1.6 Aims of this Study

The focus of the work described in this thesis was to identify ARGs and bacteriocin gene clusters from the human saliva and calf faecal metagenomes. We studied these environments because not only were samples easy to obtain (saliva was collected non-invasively and calf faeces was collected from the floor of the animal's holding at the APHA on-site farm) but they also harbour diverse populations of bacteria, the majority of which have yet-to-be cultured. Furthermore, these environments are known to harbour ARGs and bacteriocin producing bacteria. Thus, screening functional metagenomic libraries created from DNA extracted from these environments allows us to identify novel ARGs and bacteriocin producing genes from the yet-to-cultured bacteria from these environments.

To identify genes of interest we aimed to create large insert functional metagenomic libraries from human saliva and calf faecal metagenomic DNA using the pCC1BAC vector.

Following the creation of the human saliva and calf faecal metagenomic libraries we aimed to screen them for resistance to antibiotics (tetracycline and ampicillin), antimicrobials (CTAB, CPC and chlorhexidine), metals (copper and silver) and for antimicrobial activity against *Bacillus subtilis* CU2189 and *Micrococcus luteus*.

If clones of interest were identified our aim would then be to determine what cloned genes were responsible for the observed phenotypes of interest. If the genes were novel or in a novel genetic context, such as a MGE, we would then characterise them using molecular genetic, biochemistry and *in silico* protocols.

Studies that focus on the functional screening of metagenomic libraries to identify novel ARGs have the potential to identify ARGs that may become clinically important. As such, these studies also provide us with information on what environments may act as points of dissemination of ARGs to clinically relevant pathogens. Furthermore, functional metagenomic library screening also gives us access to antimicrobial production genes of the yet-to-be cultured bacteria of an environment, thus expanding our pool of potential antimicrobial therapies.

Chapter 2

Methods and Materials

The methods detailed in this chapter are essential for the work that is described in each chapter. Specific methods and materials are included at the beginning of each chapter.

2.1 Bacterial strains and culture

The bacterial strain used to create the human saliva and calf faecal metagenomic library as well as those used in the functional metagenomic screen to identify antimicrobial producing clones are indicated in Table 2.1. Further strain information is given at the beginning of each chapter.

Table 2.1 Bacterial Strains Used in this Work.

Strain	Information	Source
TransforMax™ EPI300™-T1R Electrocompetent <i>E. coli</i>	Functional Metagenomic Library Host	Epicentre® CopyControl™
<i>E. coli</i> α -Select Silver	Subcloning of genes of interest	Bioline©
<i>Bacillus subtilis</i> CU2189	Antimicrobial Indicator Organism	(Christie <i>et al.</i> , 1987)
<i>Micrococcus luteus</i>	Antimicrobial Indicator Organism	Provided by Dr. Jorge Gutierrez; University of Surrey, UK
<i>Streptococcus mutans</i> UA159	Cariogenic oral isolate	(Ajdić <i>et al.</i> , 2002)

The *E. coli* strains used in this study were grown on LB (Lennox L) agar (Life Technologies, Paisley, UK) or in LB broth (Oxoid Ltd, Basingstoke, UK). When required media was supplemented with tetracycline (5 $\mu\text{g/ml}$), chloramphenicol (12.5 $\mu\text{g/ml}$) or ampicillin (50 $\mu\text{g/ml}$). When blue/white screening was required to identify *E. coli* clones harbouring plasmids with inserts LB agar was supplement with 0.1 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) and 40 $\mu\text{g/ml}$ of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal). *Bacillus subtilis* CU2189 and *Micrococcus luteus* were grown on BHI (Brain Heart Infusion) agar and BHI broth (Sigma-Aldrich Company Ltd, Gillingham, UK).

Streptococcus mutans UA 159 was cultured in BHI agar and broth. BHI for *S. mutans* growth was supplemented with 5 µg/ml erythromycin when required.

All strains except for *S. mutans* UA 159 were grown at 37 °C with aeration by shaking at 200 RPM. *S. mutans* was grown at 37°C in 5% CO₂.

All antibiotics were sourced from Sigma-Aldrich Company Ltd.

2.2 Stock Solution Preparations

2.2.1 Antibiotic Stock Solutions

All of the antibiotics used to make stock antibiotic solutions in this study were purchased from Sigma-Aldrich. Ampicillin sodium salt was dissolved in 70 % EtOH to a final concentration of 10 mg/ml. Tetracycline hydrochloride was dissolved in 70 % EtOH to make a stock solution of 10 mg/ml. Tigecycline hydrate was dissolved in 70 % EtOH to make a solution of 5 mg/ml. Erythromycin was dissolved in 70 % EtOH to a final stock concentration of 20 mg/ml. Kanamycine sulfate was dissolved in 70% EtOH to a final concentration of 50 mg/ml. All stock solutions were sterilised by passing them through a 0.22 µm filters (Millipore, Watford, UK) and then stored at -20 °C.

2.2.2 Antimicrobial Stock Solution

All of the antimicrobials used to make stock antimicrobial solutions in this study were purchased from Sigma-Aldrich. Stock concentrations of copper sulphate, silver nitrate, CTAB, CPC and chlorhexidine digluconate were made using molecular grade water to final concentrations of 25 mg/ml, 20 mg/ml, 10 mg/ml, 20 mg/ml and 10 mg/ml respectively. All stock solutions were sterilised using 0.22 µm filters (Millipore) and stored at 4 °C.

2.3 Molecular Techniques

2.3.1 Polymerase Chain Reaction (PCR)

For standard PCR amplification and adding A-overhangs onto PCR products, MyTaq™ Red Mix (Bioline, London, UK) was used. Reactions contained 25 µl of 2X MyTaq™ Red Mix and 0.4 µM of primers (Sigma Aldrich). PCR programmes had a 2 min initial denaturing step at 98 °C, followed by 25-30 cycles of 30 second denaturing at 98 °C, 30 second annealing at a temperature specific to the primer pair and extension at 72 °C at 1 min per 1 Kb. The final step in the PCR reaction was a final extension step that was 1 min per 1 Kb plus 1 min. The reactions were then cooled to 4 °C.

For PCR reactions where the product was required for cloning, the Q5® High-Fidelity DNA Polymerase (New England Biolabs; NEB, Hitchin, UK) was employed. PCR reactions were set up according to the manufacturer's instructions containing 10 mM dNTP (NEB). PCR programmes were designed as they were for PCR reactions using MyTaq™ Red Mix.

PCR products were run on 0.9% agarose gels at 100 mV for 45-80 min. Agarose gels contained 0.2 µg/ml ethidium bromide (Life Technologies). Gels were imaged under ultraviolet (UV) light using Alphaimager hardware (AlphaInnotech, San Leandro, USA) with the Alphaview software (version 3.2.2.0).

Primers used in this work are detailed at the beginning of each relevant chapter.

2.3.2 Restriction Enzyme Digestion of DNA

Unless stated otherwise, DNA was digested in 10 µl reactions using restriction enzymes sourced from NEB. Digestions were carried out for 1 h at 37 °C. These reactions contained 1 µl of 10X buffer that was appropriate for the restriction enzyme used and 1 µl of restriction enzyme (10U). The reaction was then made up to 10 µl with the solution containing the DNA to be digested and molecular grade water (Sigma-Aldrich). For double restriction enzyme digestions, reactions were set up as described above with 1 µl of each restriction enzyme (10U) being used with a buffer that was appropriate for both enzymes.

2.3.3 Dephosphorylation of DNA Digests

Unless otherwise stated, digested DNA was dephosphorylated using Calf Intestine Alkaline Phosphatase, CIAP (Promega Corporation, Southampton, UK) in 10 µl reaction by incubating at 37 °C for 1 h. These reactions contained 1 µl of 10X CIAP buffer and 1 µl of CIAP enzyme (10U). The reaction was then made up to 10 µl by the solution containing the DNA to be dephosphorylated and molecular grade water (Sigma-Aldrich).

2.3.4 Purification of PCR and DNA Digest Products

PCR and DNA digest products were purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's guidelines.

2.3.5 Ligation Reactions

PCR products with A-overhangs were ligated into pGEM[®]-T Easy (Promega Corporation) in a 10 µl reaction using the 2X Rapid Ligation Buffer (Promega Corporation). 25 ng of pGEM[®]-T Easy was used in each reaction with a 3:1 molecular ratio of PCR product to plasmid being used, calculated using the following equation.

$$\text{Insert Mass in ng} = [\text{Insert Length in bp} / \text{Vector Length in bp}] \times \text{Vector Mass in ng}$$

Reactions were then incubated overnight at 4 °C.

For the ligation of restriction enzyme-digested DNA fragments into pHSG396 (Clontech Laboratories, Inc; Takara, Paris, France) T4 DNA ligase from NEB was used. 25 ng of plasmid was used in a ligation reaction with a 3:1 molecular ratio of insert to plasmid. The reactions were then incubated overnight at 16 °C. The T4 DNA ligase was inactivated by incubating at 70 °C for 15 min.

2.3.6 Electroporation of TransforMax™ EPI300™T1^R *E. coli*

Prior to electroporation ligation reactions were desalted in agarose cones (molecular grade water, 1.8% (w/v) glucose, 1% (w/v) agarose) on ice for 1 h.

The electrocompetent TransforMax™ EPI300™T1^R *E. coli* (Epicentre; *E. coli* EPI300 T1^R) cells were thawed on ice and 50 µl was transferred to an ice-cold 0.1 cm electroporation cuvette. Between 1 µl and 5 µl of DNA solution was added to the cells. Electroporation was conducted at 1.7 kV, 200 Ω and 25 µF. Unless otherwise stated, after electroporation 950 µl of SOC (NEB) was added to the cells in the electroporation cuvette. The cell suspension was then transferred to a sterile 25 ml tube and incubated at 37 °C with shaking at 200 RPM for 1 h. The cell suspension was then plated (100 µl per plate) on to LB agar containing the selective antibiotic. The plates were then incubated overnight at 37 °C.

2.3.7 Extraction of Plasmid from *E. coli* Strains

Plasmids were extracted from *E. coli* EPI300 T1^R and Alpha-Select Silver Efficiency (Bioline) using the QIAprep Spin Miniprep Kit (QIAGEN). For *E. coli* clones harbouring pHSG396 or pGEM[®]-T Easy 10 ml overnight cultures were centrifuged at 4200 g. Plasmids were extracted from the resulting cell pellet according to the manufacturer's instructions. For clones harbouring pCC1BAC, 1 ml of an overnight was used to inoculate 9 ml fresh media containing 10,000X CopyControl[™] Induction (1 µl in 10 ml). CopyControl[™] Induction solution induces expression of *trfA* in *E. coli* EPI300 T1^R, the product of which induces the pCC1BAC to replicate from the multicopy origin of replication, *oriV*. The CopyControl[™] induced subculture was incubated horizontally for 4 h at 37 °C with shaking at 200 RPM. This culture was then used for plasmid extraction as described above.

2.3.8 Sequencing of Plasmid Inserts and PCR Products

Sequencing of PCR products and plasmid inserts was conducted using primer extension Sanger sequencing undertaken by Beckman Coulter Genomics Inc. PCR products were sequenced using their amplifying primers. Plasmid inserts were end sequenced using primer pairs that flanked the multi cloning site, M13 primers (pHSG396 and pGEM[®]-T Easy) and pCC1 primers (pCC1BAC). Primer walking was employed to sequence longer inserts with primers being designed from the obtained sequence data.

2.4 *In Silico* Analysis of Sequence Data

2.4.1 Assembly of Contigs from Sanger Sequencing

Contigs were assembled using SeqMan Pro (Lasergene software, DNASTAR, Madison, WI, USA) and sequence gaps were closed using PCR and Sanger sequencing. Sequences were analysed using the tools on NCBI (<https://blast.ncbi.nlm.nih.gov/blast/Blast.cgi>).

2.4.2 Identification and Translation of Open Reading Frames (ORFs)

The NCBI BLASTN and BLASTX tools were used to identify similarities between nucleotide sequences generated during this project and nucleotide and amino acids sequences in the NCBI database. The later was used to identify putative ORFs. The online Fgenesb tool from Softberry© and the NCBI ORF finder tool were also used to putatively translate ORFs; the resulting amino acid sequences were then compared with those in the NCBI database using BLASTP.

2.5 Minimum Inhibitory Concentration / Antimicrobial Susceptibility

2.5.1 Microdilution

MIC determination using the broth microdilution method was completed according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (EUCAST,

2015). 16 h cultures for each clone grown in LB broth were adjusted to an OD₆₀₀ of 0.1. 10 µl of the adjusted cultures were used to inoculate 90 µl of LB broth containing varying concentrations of the antibiotic or antimicrobial in a round-bottom 96-well plate format (Corning® Costar®, Sigma-Aldrich). The plates were then incubated for 16 h at 37°C with shaking at 200 rpm. Growth was determined by spectrophotometry at OD₆₀₀. MIC and susceptibility levels were determined as the lowest concentration of antibiotic or antimicrobial that inhibited growth. Assays were conducted in technical and biological triplicates.

2.5.2 Agar Dilution

16 h cultures of each clone grown in LB broth were adjusted to an OD₆₀₀ of 0.1. 10 µl of these adjusted cultures were spotted onto the surface of LB agar containing varying concentrations of the antibiotic or antimicrobial and plates were incubated for 16 h at 37 °C. MICs and susceptibility levels were determined as the lowest concentration of the antibiotic or the antimicrobial that inhibited growth. Assays were conducted in technical and biological triplicates.

Chapter 3

Creation and Analysis of Human Saliva and Calf Faecal Metagenomic Libraries and Preparations

3.1 Introduction

3.1.1 The Human Oral Cavity

3.1.1.1 Microbiology and the Human Oral Microbiome

Microbiology was arguably born when Antonie van Leeuwenhoek first identified ‘animalcules’ from his own dental plaque. Since this initial observation, developments have been made in our ability to culture the fastidious organisms of the human oral cavity through our increased understanding of their metabolism. For instance, the inclusion of vitamin K in growth media is required for growth of some *Porphyromonas gingivalis* while fatty acids are important for *Treponema* spp., both of which are cultured in anaerobic conditions (Wyss, 1992). More recently our understanding of the human oral microbiome has broadened using culture-independent techniques including next-generation sequencing and microarray analysis (Zaura, 2012; Duran-Pinedo and Frias-Lopez, 2015). According to the Human Oral Microbiome Database (www.homd.org), 732 species have been identified in the human oral cavity (Dewhirst *et al.*, 2010; Chen *et al.*, 2010). However, only two-thirds have been cultured with the remaining being identified through 16S rRNA sequence analysis (Wade *et al.*, 2016).

3.1.1.2 Environments within the Human Oral Cavity

The human oral cavity is a part of the GI tract, which is the most colonised part of the human body (Sommer and Backhed, 2013). The human oral cavity contains multiple environments that are distinct in pH, dissolved oxygen, surface composition, fluid movement and availability of nutrients. These environments include the saliva, keratinised and non-keratinised mucosal surfaces and the sub- and supra- gingival tooth plaque, Figure 3.1. As a result of variations in the environmental conditions, these habitats harbour different bacterial communities that are adapted to the prevailing conditions in these niches (Mager *et al.*, 2003). The oral cavity is open to external conditions, meaning the microbial populations in it are sensitive to changes in temperature, hygiene, smoking and diet, as well as to colonisation by exogenous microorganisms (Sheiham and Watt, 2000; Darout *et al.*, 2002; Crielaard *et al.*, 2011; David, 2013; Wu *et al.*, 2016) . Intrinsic factors such as the state of the host immune system and gastric reflux can also impact these environments (Yang *et al.*, 2014; Scholz *et al.*, 2014). Inter-individual differences in the composition of the oral microbiome exist and distinct oral microbiomes have been identified from individuals based on their geography and host ethnicity (Zaura *et al.*, 2009).

Figure 3.1

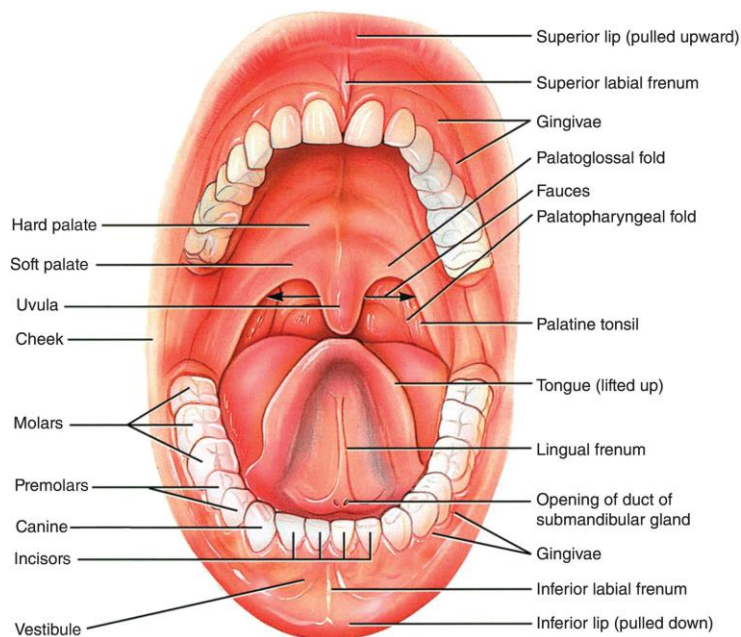


Figure 3.1 The Human Oral Cavity. A diagram showing the environments of the oral cavity that are colonised by microorganisms. The tooth surfaces are also colonised by bacteria including the sides of the tooth above and below the gingiva. This figure was reproduced from Tortora and Grabowski (Tortora and Grabowski, 2000)

3.1.1.2.1 The Saliva

Human saliva plays an important role in protecting the teeth and mucosa of the oral cavity and in maintaining the human oral microbiome. Saliva contains a number of inorganic components including Ca^{2+} and F^- ions as well as phosphates and carbonates. These ions are important for mineralising tooth enamel, and the bicarbonates present in saliva allow it to act as a buffer preventing acidification of the oral environment following the production of acid by bacteria when they metabolise dietary sugars (Larsen and Pearce, 2003; Dodds *et al.*, 2005).

Saliva also contains organic components including proteins involved in food digestion. For example, α -amylase and lingual lipase are two such enzymes that respectively convert starch to oligosaccharides and triglycerides to glycerides and free fatty acids that may be used by oral microbes (Hamosh and Scow, 1973; Kaczmarek and Rosenmund, 1977). A number of antimicrobial proteins are also present in the saliva, including cationic peptides such as histatins, statherin and lysozyme, IgA and IgG antibodies and chelating agents such as lactoferrin. These salivary components act to prevent harmful exogenous pathogens from colonising the oral cavity as well as controlling commensal microorganism populations (Rudney and Smith, 1985; De Sousa-Pereira *et al.*, 2013; Brandtzaeg, 2013).

Salivary components are the most important nutrient source for the majority of oral microbes, particularly in early plaque (polymicrobial dental biofilm) development (Marsh *et al.*, 2016). Glycoproteins including mucins and IgA are a source of fermentable carbohydrates while amino acids and DNA are also utilised by some oral bacteria (Kilian *et al.*, 1996; Wickstrom and Svensater, 2008; Palmer *et al.*, 2012; Edlund *et al.*, 2015). Saliva also contains serum components from the gingival crevicular fluid (GCF) that flows from the gingival margin (AlRowis *et al.*, 2014).

Analyses of the human saliva microbiome using 16S rRNA gene sequencing have been conducted to determine the diversity and stability of the human saliva microbiome. These studies have revealed that at the phylum level, Firmicutes typically dominate, followed by Bacteroidetes, Proteobacteria and Actinobacteria. Fusobacteria, TM7, Spirochaetes and the Synergistetes although prevalent are typically less abundant. At the genus level *Streptococcus*, *Neisseria*, *Veillonella* and *Prevotella* are often identified as being the most abundant, although inter-individual variations exist between healthy subjects (Segata *et al.*,

2012; Dassi *et al.*, 2014). A number of studies have also shown that there appears to be temporal stability over periods of weeks to years in the human saliva and the oral cavity in general (Costello *et al.*, 2009; Cameron *et al.*, 2015).

The human saliva microbiome has also been shown to be stable following exposure to antibiotics. A recent study conducted by Zaura *et al.* (2015) showed the human saliva microbiome to exhibit stability following a single course of clindamycin, amoxicillin, minocycline or ciprofloxacin, as microbial shifts were identified 1 week after treatment compared with baseline results (no antibiotic treatment) but at no other time points over a 12-month period. This indicated that the human saliva microbiome was able to rapidly return to its baseline composition following cessation of antibiotic use (Zaura *et al.*, 2015).

Although an earlier study conducted by Nasidze *et al.* (2009) showed no geographical clustering of saliva microbiomes, the survey used a 16S rRNA gene cloning and sequencing approach with relatively low sequencing depth (Nasidze *et al.*, 2009). Later studies using next generation 16S rRNA gene sequencing with greater sequencing depth have provided evidence for the contrary. For example, Li *et al.* (2014) used 454 sequencing to demonstrate clear geographic influence on the oral microbiota. They showed that the human saliva microbiota of Alaskan and German populations were more similar to each other (Firmicutes most dominant followed by Bacteroidetes and Proteobacteria) than African (Uganda, Sierra Leone and Democratic Republic of the Congo) populations (Proteobacteria most dominant followed by Firmicutes and Bacteroidetes) (Li *et al.*, 2014). In another study by Takeshita *et al.* (2014) the saliva microbiome of South Koreans was shown to be less diverse than that of Japanese individuals, indicating geographic location and diet as having an influence on the

human saliva microbiome as people from both countries have been shown to have similar genotypes (Abdulla *et al.*, 2009; Takeshita *et al.*, 2014).

3.1.1.2.2 The Teeth

Teeth are the only exposed non-shedding surfaces in the human body. They are made of enamel and represent approximately 20 % of the surface area of the mouth (Collins and Dawes, 1987). They are coated with an acquired enamel pellicle (AEP) which is a layer of proteins and glycoproteins from saliva, plasma and the oral microbiota and mucosa. The AEP protects teeth from acid degradation and abrasion, as well as acting as a platform for early microbial colonisers to interact with during plaque formation (Hannig and Joiner, 2006).

Plaque can reside above the gum line (gingiva) on the occlusal surfaces (fissure plaque) and the surfaces on the side of the tooth (supragingival). Subgingival plaque is on or below the gingiva. Due to its location, fissure plaque mainly harbours bacteria that can survive in aerobic conditions with *Streptococcus* spp. dominating, although some anaerobes such as *Veillonella* spp. are present due to the presence of anaerobic microenvironments (Wilson, 2005).

Compared with other environments in the oral cavity, greater diversity is seen in the supragingival and subgingival plaques. Primary colonisers of teeth such as *Streptococcus mitis*, *Streptococcus oralis*, *Neisseria mucosa*, *Veillonella parvum* and *Actinomyces* spp. are able to interact with the AEP glycoproteins through lectin-like proteins on their surfaces (Lu

and Levin, 2002; Teixeira *et al.*, 2006). Secondary colonisers interact with cells that are adhered directly to the AEP or with cells adhered to these primary colonisers. Later colonisers interact with the outer cell layers of the plaque and the extrapolymeric substances (EPS) that are produced by cells within the plaque, Figure 3.2 (Rosan and Lamont, 2000).

Figure 3.2

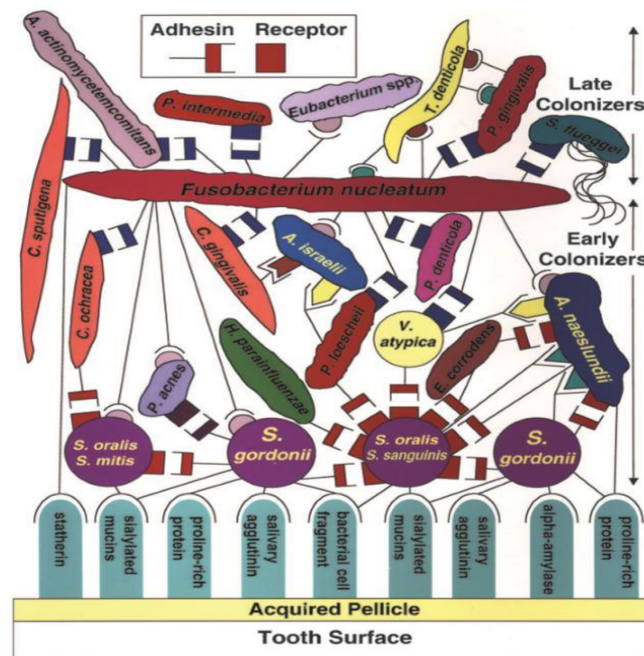


Figure 3.2 Dental Plaque Biofilm. A diagram showing the bacterial composition of dental plaque biofilms. The acquired enamel pellicle is bound by the primary *Streptococcus* spp. colonisers to which secondary colonisers interact. As the biofilm develops microenvironments are created including low oxygen zones (as oxygen is used by bacteria in the plaque or does not diffuse through the biofilm) that promote the growth of anaerobic bacteria including *Fusobacterium* spp. and *Prevotella* spp. This figure was reproduced from Kolenbrander *et al.* (Kolenbrander *et al.*, 2002).

The interaction of cells within the biofilm is primarily mediated by lectin-carbohydrate interactions and is called co-aggregation (Rosen and Sela, 2006; Schuler *et al.*, 2012). Co-aggregation results from cells coming into close contact and the creation of metabolic communication networks. An example of such a metabolic communication network is *P. gingivalis* producing free glycines that *Treponema denticola* metabolises to produce lactate that is utilised by *P. gingivalis* (Lewis *et al.*, 2009). Microenvironments also develop within the plaque where aerobes utilise oxygen creating zones of low oxygen and low redox potential allowing for the survival of anaerobes, Figure 3.2.

As the supragingival plaque matures, secondary colonisers including species of *Fusobacterium*, *Treponema*, *Prevotella* and *Corynebacterium* are incorporated into the biofilm, and the relative abundance of *N. mucosa* and *Streptococcus* spp. declines (Aas *et al.*, 2005; Uzel *et al.*, 2011; Segata *et al.*, 2012; Teles *et al.*, 2012). Compared with the saliva, Firmicutes are less dominant in the supragingival plaque although still abundant. As the subgingival plaque is in a more anaerobic environment than the supragingival plaque and receives its nutrients from the GCF, asaccharolytic anaerobic bacteria including species of *Fusobacterium*, *Porphyromonas*, *Prevotella*, *Tannerella*, *Parvimonas* and *Treponema* are more prevalent in this biofilm again at the expense of genera of Firmicutes (Aas *et al.*, 2005; Segata *et al.*, 2012; Ge *et al.*, 2013; Y. Li *et al.*, 2014).

Bacteria present in these oral biofilms have the potential to enter the saliva during biofilm dispersal. For example, dispersin B genes (*dspB*; encodes a protein involved in biofilm dispersion) are encoded by oral *Aggregatibacter* spp. and *S. mutans* produces a protein (surface-protein-releasing-enzyme; SPRE) that mediates its release from biofilm at low pH

(Kaplan, 2010). Thus, it is important to understand the bacterial composition of dental plaque when studying the saliva microbiota.

3.1.1.2.3 The Oral Mucosa

The oral mucosa is the outermost layer of squamous epithelia in the mouth and is a shedding surface. It is contiguous with the epidermis but differs in composition. The oral mucosa is well lubricated by saliva and the excretion of mucins by the cell layer. Mucin 5B and mucin 7 are the dominant mucins in the oral cavity. These mucins along with IgA and cystatin S contribute to the formation of the mucosal pellicle. As with plaque formation, interaction with this pellicle is the initial stage in colonisation of the oral mucosa (Bradway *et al.*, 1992; Gibbins *et al.*, 2014).

There are two compositionally different types of oral mucosa; keratinized and non-keratinized. Keratinized mucosa includes the hard palate (HP), keratinised gingiva (KG) and the tongue dorsum (TD). The non-keratinized mucosa includes the buccal mucosa (BM), tonsils, labial and alveolar mucosa as well as the lateral and ventral mucosa of the tongue. Interestingly, based on 16S analysis, the microbiota of the keratinized and non-keratinized mucosa did not group together. Using data obtained from the Human Microbiome Project (HMP) Segata *et al.* (2012) showed that the microbiota of the TD was more similar to that of the saliva and tonsils. The BM, KG and HP had more similar microbial communities (Segata *et al.*, 2012). Eren *et al.* (2014) conducted a higher-resolution study of the HMP data using oligotyping, which further indicated similarities between the BM and KG microbiota but

grouped the HP with saliva and the tonsils. At the phylum level, Firmicutes, particularly *Streptococcus* spp., are dominant across all oral mucosa sites particularly the BM, KG and HP. In the TD and tonsil communities *Streptococcus* spp. are less dominant while higher relative abundances of *Veillonella* spp. and *Prevotella* spp. 16S rRNA genes have been detected (Aas *et al.*, 2005; Eren *et al.*, 2014).

3.1.1.3 Diseases and Dysbiosis of the Human Oral Cavity

Imbalances or dysbioses in the oral microbiota have been associated with a number of conditions and in some cases, may be markers for disease. Bacteria of the oral microbiome or shifts in its composition have been associated with systemic diseases and conditions including cardiovascular infections, diabetes, pneumonia and irritable bowel disease (IBD) (Raghavendran *et al.*, 2007; Leishman *et al.*, 2010; Kampoo *et al.*, 2014; Fourie *et al.*, 2016). *S. aureus* and *P. aeruginosa* can be carried in the oral cavity and systemic infections caused by these pathogens may result from oral injury, surgery or during chronic periodontitis (McCormack *et al.*, 2015; Rivas Caldas *et al.*, 2015). In a recently published Japanese study 454 16S rRNA pyrosequencing was used to assess the microbiome of the saliva of 35 patients with Crohn's disease (CD) and ulcerative colitis (UC). These patients were found to have a higher relative abundance of Bacteroidetes and a lower abundance of Proteobacteria compared with the 24 healthy controls (HC). At the genus level, CD and UC patients had a significantly increased relative abundance of *Prevotella* spp. (25 % versus 10 % in HC) while *Streptococcus* spp. abundance was relatively lower (25 % versus 35 % in HC) (Said *et al.*, 2014).

Dental caries refers to the formation of cavities in tooth enamel caused by the production of organic acids in the oral cavity. The production of these acids is attributed to the breakdown of dietary sugars, in particular sucrose, by acidogenic bacteria including *S. mutans* and *Lactobacillus* spp. As the pH reduces acidogenic and acid-tolerant bacteria proliferate exacerbating the condition (van Houte, 1994; Ma *et al.*, 2015). Raner *et al.* (2014) used a DNA-DNA checker board technique to show that *Prevotella intermedia* and *Fusobacterium nucleatum* were more prevalent than *Streptococcus* spp. in the dental plaque of Thai tribes that had few incidences of caries. As these tribes had low sucrose diets and poor oral hygiene the authors hypothesised that the low caries rate was a result of increased dental plaque dominated by low acidogenic *Prevotella* spp. and *Fusobacteria* spp. that resulted from their poor oral hygiene while acidogenic *Streptococcus* spp. were less abundant as a result of their low sucrose intake (Raner *et al.*, 2014).

Gingivitis is a form of reversible periodontal disease that if left untreated can lead to periodontitis which may result in tooth loss and alveolar bone damage. Periodontitis is associated with dysbiosis in the oral bacterial microbiome (Darveau, 2010). Culture and 16S rRNA sequencing based studies have shown enrichment of *P. gingivalis* and *Treponema denticola* in the plaque of periodontitis patients (>10 %) compared with healthy individuals (<0.1 %) (Liu *et al.*, 2012; Griffen *et al.*, 2012; Tanner, 2015). However, discerning the health associated oral microbiota is difficult due to the presence of oral pathogens in the normal microbiota and inter-individual variations in their abundances in healthy individuals (Zaura *et al.*, 2009).

To conclude, intra-individual and inter-individual variation in the human oral cavity microbiome exists as a result of the varied environments of this part of the human digestive

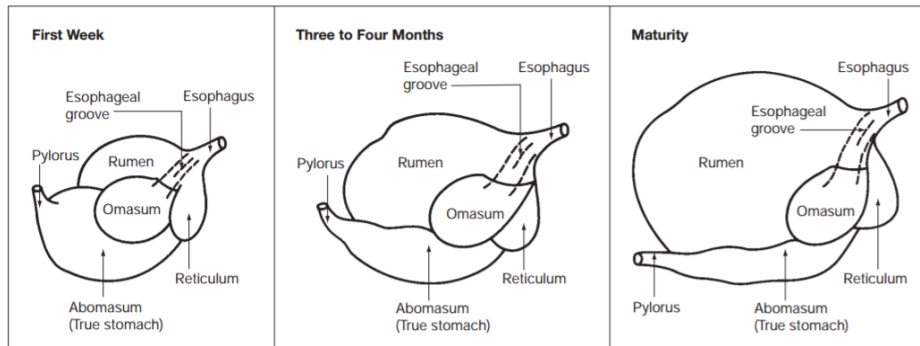
tract as well as health status, diet and geography, making the core oral cavity microbiome of healthy individuals difficult to define. This gives justification to pooling saliva samples when making a functional metagenomic library as a single sample would be less representative of the saliva microbiome.

3.1.2 The Calf Faecal Microbiome

Although the faecal microbiota are frequently studied with the aim of determining the composition of the bovine gastrointestinal (GI) tract microbiome there are large differences between the compositions of the microbiota of the faeces and different parts of the bovine GI tract, Figure 3.3 (a,b) (Frey *et al.*, 2010; Ross *et al.*, 2012). For instance, the rumen is an anaerobic environment suited to bacteria such as *Fibrobacter* spp. and *Ruminococcus* spp. that are less abundant in faeces. Additionally, following weaning Bacteroidetes abundance drops in the rumen but increases in the faecal microbiome, Figure 3.3 (a,b) (Dehority, 2003; Ross *et al.*, 2012; Meale *et al.*, 2016; Liu *et al.*, 2016). Faecal matter, however has passed through the abomasum (the fourth and final stomach of ruminants) and intestines of the animal resulting in further fermentation and lysis of cells due to low pH conditions and the presence of lytic enzymes (Van Winden *et al.*, 2002; Domínguez-Bello *et al.*, 2004; Gilbert *et al.* 2015). Further to this, compared with mature cows, the digestive tract of calves are underdeveloped and undergo a number of physiological and metabolic changes in the weeks and months following birth as its rumen develops, in response to microbial colonisation (Heinrichs, 2005; Uyeno *et al.*, 2010).

Figure 3.3

(a)



(b)

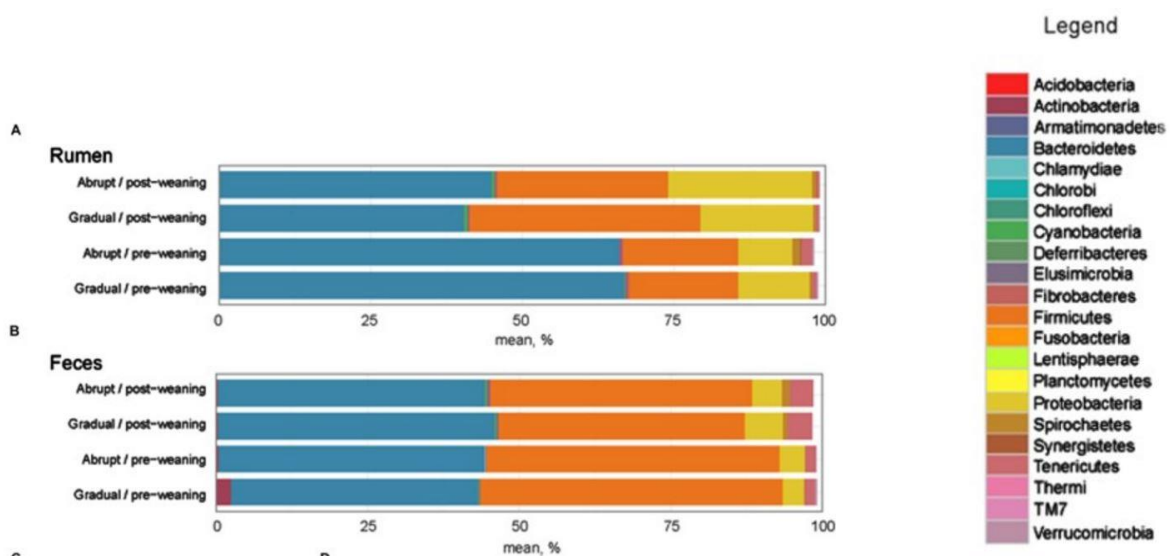


Figure 3.3 The Calf Gastrointestinal Tract and the Rumen and Faecal Microbiota. (a) A diagram depicting the rumen, omasum, reticulum and abomasum at the first week after birth and how it develops as the calf grows and its diet shifts from a mainly milk based to plant based diet. The pylorus continues on to the duodenum and the small and large intestines (Heinrichs and Jones, 2003). (b) Shows the relative abundance of the bacterial phyla present in the rumen and faeces of pre-weaned calves (36 days old) and weaned calves (54 days old). Bacteroidetes are indicated as dark blue on the graph and Firmicutes are shown in orange (Meale *et al.*, 2016).

Calf faecal microbiomes are dominated by three phyla, Bacteroidetes, Firmicutes and Proteobacteria which typically make up >90 % of the bacterial population while lower abundances of Actinobacteria (2.0 %) and Fusobacteria (0.76 – 5.67 %) and Spirochaetes (2.6 %) are also present (Ozutsumi *et al.*, 2005; Uyeno *et al.*, 2010; Oikonomou *et al.*, 2013). The age of the animal has been demonstrated to affect the relative abundance of these phyla however. For example, 16S rRNA pyrosequencing analyses have shown that Firmicutes dominate in calf faeces, while in older cattle dominance of Bacteroidetes has been described, although inter-individual differences exist (Shanks *et al.*, 2011; Jami and Mizrahi, 2012; Meale *et al.*, 2016). Durso *et al.* (2010) conducted a study to identify the core microbiome of rectal faecal samples from 6 dairy cows that were housed and fed together. By amplifying, cloning and sequencing the 16S rRNA genes from these samples they found that only 24 identified taxa were shared between all individuals out of a total of 1906 taxa identified (Durso *et al.*, 2010). Inter-individual differences may result from diet, for instance, a high starch diet has been associated with an increase in the relative abundance of Bacteroidetes genera including *Prevotella* spp. and the opposite trend for *Lactobacillus* spp. after weaning is observed (Shanks *et al.*, 2011). This makes defining the core microbiome difficult.

Additionally, the composition of the calf faecal microbiome changes dramatically within the first few weeks of the calf's life as its rumen develops. Oikonomou *et al.* (2013) used 16S rRNA pyrosequencing to study the faecal microbiomes of 61 calves up until weaning at 7 weeks. Firmicutes were shown to be dominant (64 – 82 % relative abundance) although levels of *Lactobacillus* spp. dropped after 4 weeks. This was observed for *Bifidobacterium* spp. also and is likely associated with a reduction in milk intake as the calves approach weaning age (Oikonomou *et al.*, 2013). This has been observed in other studies including

one by Uyeno *et al.* (2010) who also noted that *Ruminococcus* spp. and *Fibrobacter* spp. reached detectable levels post weaning (week 7 – 12) as fermentation in the anaerobic environment of the rumen increases as plant based food increases over milk (Uyeno *et al.*, 2010). From studies analysing weaned calf faeces using next generation 16S rRNA sequencing platforms (454 and illumina platforms), *Clostridium*, *Bacteroides*, *Prevotella*, *Ruminococcus* and *Succinivibrio* spp. have been shown to increase in relative abundance after weaning (Dowd *et al.*, 2008; Oikonomou *et al.*, 2013; Meale *et al.*, 2016).

The method of collection of the bovine faeces sample can impact the microbiome. Freshly deposited or rectally collected calf faeces contain an abundance of anaerobic bacteria from the *Clostridia* and *Bacteroidia* classes. However, exposure to aerobic and drying conditions results in a community shift towards more aerobic members of the Alpha- and Betaproteobacteria, Bacilli and Actinobacteria classes (Wong *et al.*, 2016).

3.2 Chapter Aims

The aim of the work presented in this chapter was to extract representative metagenomic DNA from human saliva and calf faecal microbiota that was of high molecular weight and quality in order to create large insert functional metagenomic libraries. Once the functional metagenomic libraries were created we aimed to analyse the libraries to determine their size and if they represented the human saliva and calf faecal metagenomes prior to storing them for future screening.

3.3 Methods and Materials

3.3.1 Human Saliva and Calf Faecal Sample Collection

Saliva samples were collected from 11 healthy individuals from different geographic locations who had not taken antibiotics within the previous three months. Saliva was expectorated into sterile tubes (approximately 5 ml per individual) and samples were pooled. The pooled samples were then split into 1.5 ml aliquots beside a Bunsen burner.

Ethical approval has been obtained to sample human saliva from volunteers and to use the saliva for this PhD project from the UCL Ethics committee, UCL Ethics Project ID Number 5017/001. The ethics form is included as an appendix, Appendix I.

Faeces were collected from a single weaned calf older than 7 weeks by collecting from the floor of its holding pen at the APHA on-site farm. Faeces was collected from a single calf only as high-quality DNA proved more difficult to reproducibly extract from this material. Faeces were stored in 5 g aliquots at -80 °C prior to extraction.

Bovine faeces was collected by picking up the material after the animal had excreted it. As this method of collection does not require direct extraction from the animal no ethical approval is needed.

3.3.2 Metagenomic DNA Extraction

Metagenomic DNA was extracted from bacterial cells using modified protocols for the Gentra Puregene Yeast Bact. Kit (Qiagen, Manchester, UK) as described below for human saliva and calf faeces (Seville *et al.*, 2009; Card *et al.*, 2014).

3.3.2.1 DNA Extraction from Human Saliva

The 1.5 ml saliva aliquots were centrifuged for 1 min at 15700 g. The resulting pellet was suspended in 1 ml of cell suspension solution (Qiagen) and 5 µl of lytic enzyme (contains lyticase at 4,000 U/ml; Qiagen) was added (Gentra Puregene). The suspension was incubated at 37°C for 45 mins. The supernatant was removed and the pellet was resuspended in 1 ml of cell lysis solution (Qiagen). The suspension was incubated for 20 mins at 80°C. The suspension was cooled to room temperature (RT) before addition of 0.4 mg proteinase K and incubating for 1 h at 55°C. The suspension was cooled to RT and 5 µl of RNase (Qiagen) was added before it was incubated at 37°C for 1 h. The suspension was cooled on ice and 500 µl of protein precipitation solution (high salt buffer; Qiagen) was added. It was incubated on ice for 30 min before centrifugation for 10 min at 15,700 g. The supernatant was removed and 10% (v/v) 3 M sodium acetate (NaOAc) and 600 µl of 100 % isopropanol was added. The supernatant was inverted 50 times and cooled on ice for 30 min before centrifugation for 10 min at 15,700 g. The resulting DNA pellet was washed with 700 µl of 70% EtOH by centrifuging at 15,700 g for 10 min. The EtOH was removed and the DNA

pellet air dried for 5 min before being resuspended in 100 µl of molecular grade water.

Human saliva metagenomic DNA solutions were stored at -20°C.

3.3.2.2 DNA Extraction from Calf Faeces

Calf faeces were thawed in 5 g aliquots from -80°C. The thawed faeces were washed 3 times with 35 ml PBS (0.1 M, pH 7.4) by vortexing vigorously, followed by centrifuging at 2,860 g for 2 min. Thus, for each 5 g calf faeces aliquot 3 X 35 ml of calf faeces washes were obtained. These washes were subsequently centrifuged at 4,200 g for 10 min to obtain bacterial pellets for metagenomic DNA extraction. The resulting three bacterial cell pellets were resuspended in 5 ml of cell suspension solution (Qiagen) and incubated with 5 µl of lytic enzyme (Qiagen) for 45 min at 37 °C. The 5 ml suspension was split into 4 X 1.25 ml aliquots which were then centrifuged for 1 min at 15,700 g. The supernatants were removed and the resulting pellets were processed in the same way as human saliva derived pellets up to the protein precipitation step (described in 3.3.2.1). However, following protein precipitation the calf faeces preparations were still visibly brown so a further CTAB and phenol clean was conducted prior to EtOH precipitation and DNA dissolving as follows. The supernatants obtained after protein precipitation were split into 500 µl aliquots and 100 µl of 5 M NaCl and 80 µl of extraction buffer (10% CTAB (V/V); 0.7 M NaCl) was added to each. These solutions were incubated at 65 °C for 10 min before adding 600 µl of phenol:chloroform:isoamylalcohol (25:24:1). The solutions were vortexed and then centrifuged for 10 min at 15,700 g. The resulting aqueous phases were transferred to new micro-centrifuge tubes and 600 µl of chloroform:isoamylalcohol (24:1) was added. The

samples were vortexed and centrifuged for 10 min at 15,700 g. The aqueous phases were transferred to clean tubes and 10% (v/v) 3 M sodium acetate (NaOAc) and 600 µl 100 % isopropanol were added. EtOH precipitation of the DNA was carried out in the same manner as for human saliva metagenomic DNA (described 3.3.2.1). Calf faecal metagenomic DNA solutions were stored at -20°C.

3.3.3 Analysis of Metagenomic DNA Preparations

3.3.3.1 Molecular Analysis

To determine the quantity and quality of the metagenomic DNA in the human saliva and calf faeces preparations the DNA solutions were analysed by spectrophotometry at 230 nm, 260 nm and 280 nm (Nanodrop). Additionally, the DNA samples were digested with *HindIII* to determine their sensitivity to restriction and as such if they were suitable for cloning.

Human saliva metagenomic DNA was digested with serial dilutions of *HindIII* (2 U – 0.03125 U; 1 U will digest 1 µg of DNA in 1 h at 37 °C) by incubating 100 ng of DNA with 1 µl of *HindIII* in a 20 µl reaction at 37 °C for 1 h. Calf faecal DNA was also digested with serially diluted *HindIII* (2 U to 0.125 U) in the same way.

3.3.3.2 Phylogenetic Analysis

3.3.3.2.1 Creation of 16S rRNA Dual-Indexed Illumina Libraries

The V5-V7 hypervariable regions of the 16S rRNA genes which have been used in previous human oral microbiome profiling studies, were amplified from human saliva and calf faecal metagenomic DNA preparations using the 785F and 1175R primer pair (Kraneveld *et al.*, 2012). These primers were modified so that adaptor and index sequences were introduced to the amplified products. Primer 785F introduced the i5 adaptor sequence (to immobilize it to the amplicon flow cell surface for sequencing) and primer 1175R introduced the i7 adaptor sequence (for immobilization of the amplicon on the flow cell surface). The human saliva V5-V7 library was indexed with the FWD08 and REV12 sequences while the calf faecal V5-V7 library was indexed with the FWD07 and REV12 sequences. The primers used in the construction of these libraries are included in Table 3.1.

PCRs were conducted using Moltaq 16S/18S DNA polymerase (VH Bio, Gateshead UK). PCR reactions contained 0.4 μ M forward and reverse primers, 200 μ M dNTPs, 2.5 μ l 10 X Moltaq buffer (1.5mM MgCl₂), 0.5 mM MgCl₂, 0.625 U of Moltaq enzyme and Moltaq H₂O to a volume of 25 μ l.

The PCR programme used included an initial 5 min denaturing step at 94 °C, followed by 30 cycles of 30 s denaturing at 94 °C, 40 s annealing at 54°C and extension at 72 °C for 1 min. The final step in the PCR reaction was a 10 min extension at 72 °C. The reactions were then cooled to 4 °C.

3.3.3.2.2 Clean-up of 16S rRNA Dual-Indexed Libraries

The Ampure XP beads kit (Beckman Coulter, Oakley UK) was used to clean up the 16S rRNA gene dual-indexed library PCR reactions following manufacturer's instructions. First, the elution buffer (Qiagen) was added to the PCR reactions to bring their volumes to 500 μ l. Then, 40 μ l Ampure XP beads were added, mixed by pipetting, incubated for 5 min at RT and pelleted using a magnet. The supernatants were discarded and the beads washed with 200 μ l of 80% EtOH before settling for 30 s. The EtOH was removed and a further two EtOH washes were conducted. Residual EtOH was removed using a 10 μ l pipette and the beads air dried for 5 min. The beads were removed from the magnet and washed thoroughly with elution buffer before incubating at RT for 2 min. Then, 40 μ l of binding buffer (20% polyethylene glycol, 2.5M NaCl) was added and mixed by pipetting before incubating for 5 min at RT. The supernatants were discarded, the beads pelleted for 2 min on a magnet followed by three EtOH washes as described above. The beads were then removed from the magnet and thoroughly washed with elution buffer. The beads were pelleted for 2 min on a magnet and the supernatants (containing cleaned library) were transferred to fresh tubes.

3.3.3.2.3 Sequencing and Analysis of 16S Libraries by Illumina MiSeq

Paired end sequencing of the human saliva and calf faecal 16S rRNA dual-indexed libraries was conducted using Illumina MiSeq at the UCL Institute of Child Health.

De-multiplexed sequence data was obtained and subsequently processed using QIIME, Appendix II (Caporaso *et al.*, 2010a). Forward and reverse reads were paired and sequences with a Phred score (base confidence score) of less than 20 were discarded. The resulting files were saved in fna file format and Operational Taxonomic Units (OTUs) were identified using the open reference method. This method included a closed reference step that identified OTUs by comparing the reads to sequences in the Greengenes core set, a 16S rRNA gene database (Caporaso *et al.*, 2010a). OTUs identified by the closed method were aligned using PyNAST (Caporaso *et al.*, 2010a). These aligned sequences were then quality filtered by lane masking and removal of chimeric sequences. The aligned and filtered OTUs were summarised and grouped based on taxonomy from phylum to genus level. The data was presented as a pie chart. The script used is shown in Appendix II (Caporaso *et al.*, 2010b).

Table 3.1 16S Amplification and pCC1BAC Sequencing Primers.

Name	Sequence (5'-3')	Information	Reference
P5_FWD0 7	AATGATACGGCGACCACCGAGATCTACACAAGGAGTAA CGTACGTACGTGGATTAGATACCCBRGTAGTC	V5-V7 amplification from calf faecal metagenome	(Kraneveld <i>et al.</i> , 2012)
P7_REV12	CAAGCAGAAGACGGCATAACGAGATTCCTCTACAGTCAGT CAGCCACGTCRTCCCCDCCTTCCTC	V5-V7 amplification from calf faecal metagenome	(Kraneveld <i>et al.</i> , 2012)
P5_FWD0 8	AATGATACGGCGACCACCGAGATCTACACCTAAGCCTAC GTACGTACGTGGATTAGATACCCBRGTAGTC	V5-V7 amplification from human saliva metagenome	(Kraneveld <i>et al.</i> , 2012)
P7_REV12	CAAGCAGAAGACGGCATAACGAGATTCCTCTACAGTCAGT CAGCCACGTCRTCCCCDCCTTCCTC	V5-V7 amplification from human saliva metagenome	(Kraneveld <i>et al.</i> , 2012)
pCC1-F	GGATGTGCTGCAAGGCGATTAAGTTGG	End sequencing of pCC1BAC	Epicentre®
pCC1-R	CTCGTATGTTGTGTGGAATTGTGAGC	End Sequencing of pCC1BAC	Epicentre®

3.3.4 Creation of Metagenomic Libraries

3.3.4.1 Preparation of Metagenomic DNA for Ligation

For construction of both human saliva and calf faecal metagenomic libraries in pCC1BAC the insert DNA was partially digested using *HindIII* in a 40 µl reaction volume made up of 22 µl of metagenomic insert DNA (50ng/µl), 4µl of 10x restriction enzyme buffer, 1 µl of *HindIII*

(2U; NEB) and 13 μ l of molecular grade water (Seville *et al.*, 2009; Card *et al.*, 2014). A flow diagram illustrating the general steps in the creation of a metagenomic library is depicted in Figure 3.4. The reaction was split into 2 x 20 μ l reactions; one aliquot was incubated for 1 min at 37°C and the other for 2 min. Immediately after digestion 2 μ l of 3M NaOAc and 200 μ l of 100% EtOH was added to the reactions and they were cooled on ice for 30 min before centrifugation for 20 min at 15,700 g. The EtOH and NaOAc were removed and pellets washed with 70% EtOH. The pellets were air dried for approximately 10 min until visibly dry and re-suspended in a total of 50 μ l of molecular grade water.

Figure 3.4

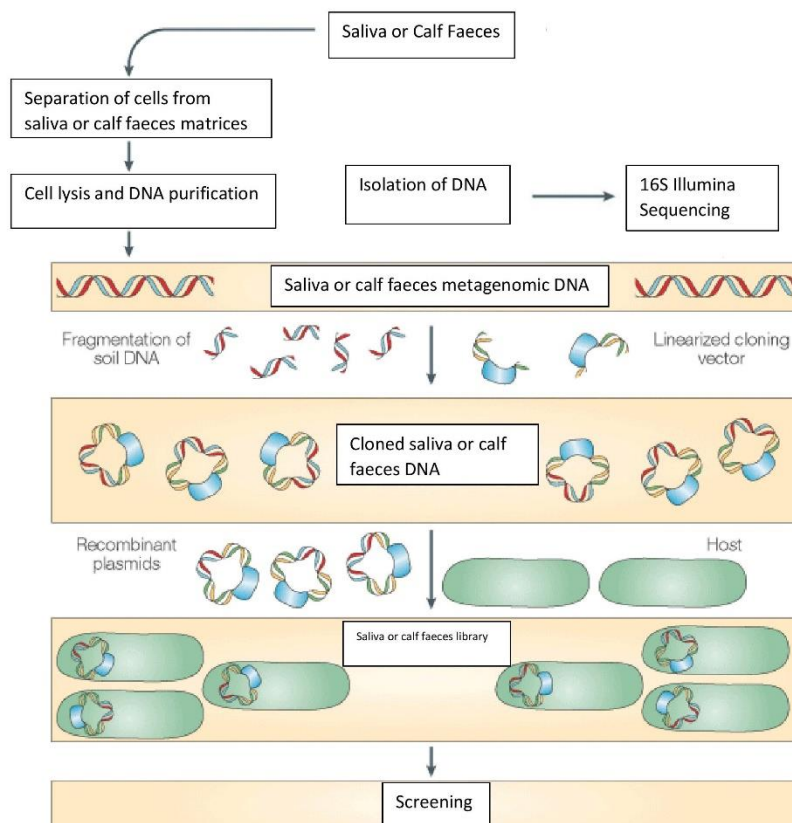


Figure 3.4 Functional Metagenomic Library Flow Diagram. A diagram showing the general steps involved in the creation of a functional metagenomic library. This diagram is amended from Daniel, 2005 (Daniel, 2005).

3.3.4.2 Ligation of Digested Metagenomic DNA into pCC1BAC

The pCC1BAC libraries were constructed following the Epicentre® CopyControl™ ligation kit protocol. First, 35 µl (30 ng/µl) of human saliva or 86 µl (50 ng/µl) of calf faeces *Hind*III digested insert DNA, 1 µl of pCC1BAC (25ng/µl, *Hind*III digested and ready for ligation from Epicentre) and molecular grade water to make the reaction volume up to 87 µl were incubated at 55°C for 10 min. The samples were cooled at RT and 10 µl of 10x Fast-Link ligase buffer (Epicentre), 1µl 10 mM ATP and 2 µl of Fast-Link ligase (4 U; Epicentre) were added making the final ligation reaction volume 100 µl. This was incubated overnight at 16°C.

3.3.4.3 Transformation of pCC1BAC Ligation Reactions

The human saliva and calf faecal metagenomic DNA pCC1BAC ligation reactions were transformed into *E. coli* EPI300 T1^R as described in 2.3.6.

3.3.4.4 Storage of Human Saliva Metagenomic Library

White colonies identified on LB agar plates supplemented with chloramphenicol (12.5 µg/ml), IPTG (0.1mM) and X-gal (40 µg/ml) were picked (27,000 clones) using sterile toothpicks and inoculated into 100 µl of LB broth containing chloramphenicol (12.5 µg/ml)

cultured in 96-well plates for 16 h at 37 °C with shaking at 200 rpm. After 16 h, 100 µl of sterile 40% glycerol was added to each well and the plates were stored at -80 °C.

3.3.5 Analysing the Metagenomic Libraries

3.3.5.1 Calculation of Ligation and Transformation Efficiency

The efficiency of the human saliva and calf faecal ligation reactions were estimated by calculating the percentage of colonies that were white on LB agar plates supplemented with chloramphenicol, IPTG (0.1 mM) and X-gal (40 µg/ml), following transformation of 2 µl or 5 µl human saliva and calf faecal ligation reactions into *E. coli* EPI300 T1^R. The following equation was used to determine the efficiency of the human saliva and calf faecal ligation reactions.

$$(\text{Number of White Clones} / \text{Total Number of Clones}) \times 100 = \text{Ligation Efficiency}$$

Human Saliva

Transformation of 5 µl of ligation reaction $(363/413) \times 100 = 88 \%$

Transformation of 2 µl of ligation reaction $(1180/1217) \times 100 = 97 \%$

Calf Faecal

Transformation of 5 µl of ligation reaction $(301/385) \times 100 = 78 \%$

Transformation of 2 µl of ligation reaction $(240/263) \times 100 = 91 \%$

Transformation efficiency was determined by calculating how many colony forming units (CFUs) were obtained per μg of DNA transformed. The following equation was used to determine the efficiency of transformation of the human saliva and calf faecal ligation reactions.

$$\text{Number of Colonies} / \mu\text{g of DNA transformed} = \text{Transformation Efficiency}$$

Human Saliva

$$\text{Transformation of } 5 \mu\text{l of ligation reaction} \quad 363 / 0.220 \mu\text{g} = 1.650 \times 10^3$$

$$\text{Transformation of } 2 \mu\text{l of ligation reaction} \quad 1180 / 0.088 \mu\text{g} = 1.341 \times 10^3$$

Calf Faecal

$$\text{Transformation of } 5 \mu\text{l of ligation reaction} \quad 301 / 0.2 \mu\text{g} = 1.505 \times 10^3$$

$$\text{Transformation of } 2 \mu\text{l of ligation reaction} \quad 240 / 0.08 \mu\text{g} = 3.0 \times 10^3$$

3.3.5.2 Determining the Source of Cloned DNA

To determine the source of the cloned DNA in the human saliva metagenomic library, and estimate what percentage was human and bacterial in origin, plasmids were extracted from 96 random clones and end sequenced using PCC1F and PCC1R primers, and the sequences obtained were analysed using BLASTN (megaBLAST and discontinuous megaBLAST) and BLASTX, Table 3.1. This method was also used to determine the source of the DNA cloned in the calf faecal metagenomic library using 10 randomly selected clones. Insert DNA was deemed concatemeric if BLASTN or BLASTX identified the end sequences as coming from

organisms of different phyla. Any concatemers identified were assumed to contain an equal amount of DNA from both sources.

3.3.5.3 Estimating the Size of the Metagenomic Libraries

To determine the size of the human saliva metagenomic library, the 96 random pCC1BAC plasmid extracts were digested using *HindIII* and analysed by gel electrophoresis. The 1 Kb Extended Ladder (NEB) was used as a size marker. This method was also used to analyse the 10 randomly extracted pCC1BAC vectors from the calf faecal metagenomic library.

The average bacterial DNA insert size was estimated by determining the total insert size for the bacterial DNA containing clones identified in 3.3.5.2 and dividing by the number of bacterial DNA containing clones analysed;

$$\text{Average Bacterial DNA Insert Size} = \frac{\text{Total Bacterial DNA Insert Size}}{\text{No. of clones with bacterial DNA}}$$

<u>Human Saliva</u>	$345.45 \text{ Kb} / 41 = 8.42 \text{ Kb}$
---------------------	--------------------------------------------

<u>Calf Faecal</u>	$237 \text{ Kb} / 10 = 23.7 \text{ Kb}$
--------------------	-----------------------------------------

The average insert size for the bacterial DNA containing clones and the number of clones containing bacterial DNA identified in 3.3.5.2 were assumed to be conserved over the whole library and were used to estimate how much bacterial DNA was present in the 27,000-clone library using the following equation.

Total Bacterial DNA cloned = (Average Insert Size X Number of Clones in Library) X

Proportion of Clones harbouring bacterial DNA

Human Saliva

From 96 clones

40 - Bacterial, 39 - Human, 1 - Bacterial/Human, 16 - Empty

41/96 contain bacterial DNA = 0.427 (Proportion of Clones harbouring bacterial DNA)

$$(8.42 \text{ Kb}) \times (27,000) \times (0.427) = 97,074 \text{ Kb}$$

Calf Faecal

Total Bacterial DNA cloned = Average Insert Size X Number of Clones in Library

$$(23.7 \text{ Kb}) \times (2,840) = 67,308 \text{ Kb}$$

3.4 Results

3.4.1 Molecular Analysis of Metagenomic DNA Preparations

The modified Genra Puregene Yeast Bact. Kit (Qiagen) protocol described in this chapter was used to obtain high concentration and high molecular weight DNA from the pooled human saliva and calf faeces, Figure 3.5 (a) and (b). The human saliva metagenomic DNA preparations showed some smearing when analysed by agarose gel electrophoresis indicating that smaller DNA fragments were also present. Nanodrop analysis showed that the human saliva and calf faecal metagenomic DNA had concentrations between 50 – 100 ng/ μ l. The 260/280 nm ratios were typically around 1.8 (a ratio of 1.8 is accepted as pure DNA) and 260/230 nm ratios ranged from 0.7 – 1.8 (a ratio of 2.0 is accepted as pure for DNA). Digestion of the saliva metagenomic DNA preparation with serial dilutions of *HindIII* resulted in a shift from high molecular weight fragments to smaller fragments as the restriction enzyme concentration increased, Figure 3.5 (a).

When viewed on agarose gels the calf faecal metagenomic DNA preparations showed high molecular weight with some smearing. The preparations were examined by Nanodrop which showed DNA concentrations between 75 - 450 ng/ μ l. The 260/280 nm and 260/230 nm ratios ranged from 1.75 – 2.0 and 0.9 – 1.9, respectively. A reduction in high molecular weight DNA bands was observed when the calf faecal metagenomic DNA was digested with serially diluted concentrations of *HindIII*, Figure 3.5 (b).

As the DNA obtained from human saliva and calf faeces were of high molecular weight and amenable to *HindIII* digestion, metagenomic libraries could be created.

Figure 3.5

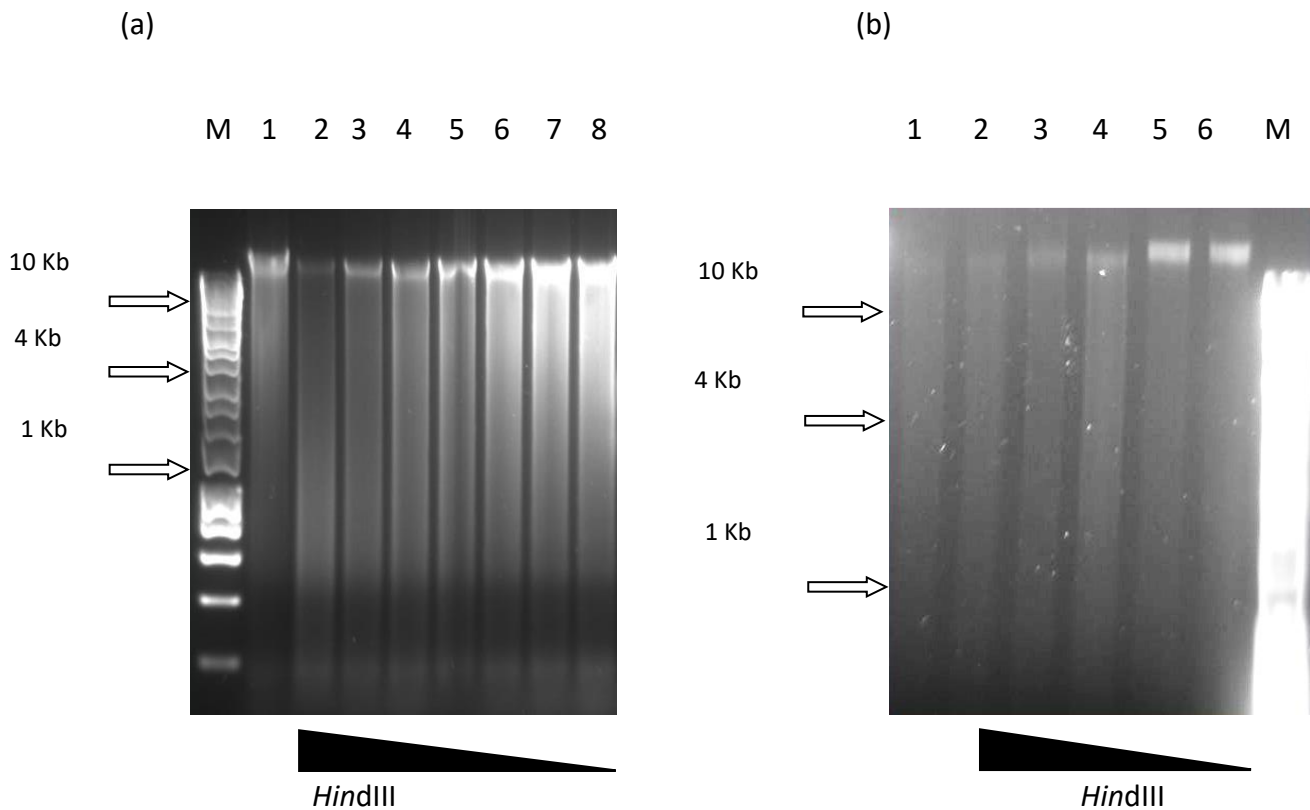


Figure 3.5 Partial *Hind*III Digest of Human Saliva and Calf Faecal Metagenomic DNA. In (a) and (b) M indicates Bioline Hyper Ladder I as a marker (a) Lane 1 contains the high molecular metagenomic DNA obtained from human saliva. Lanes 2 to 8 show the effect of digesting the human saliva metagenomic DNA with serially decreasing concentrations of *Hind*III (2U - 0.03125U). (b) Lane 5 contains the high molecular metagenomic DNA obtained from calf faeces. Lanes 1 to 5 show the results of digesting the calf faecal metagenomic DNA with decreasing concentrations of *Hind*III (2U – 0.125 U). The black triangles beneath each gel indicate the direction of increasing *Hind*III concentration.

3.4.2 Phylogenetic Analysis of Metagenomic DNA Samples

3.4.2.1 Phylogenetic Analysis of Human Saliva Metagenomic DNA

Illumina MiSeq next generation sequencing was used to identify the diversity of microorganisms present in the Human Saliva metagenomic DNA preparations by sequencing the V5-V7 hyper variable regions of the 16S rRNA gene present in the samples. 198,946 reads were generated during the sequencing run of which 143,351 passed quality filtering. 98.6 % of these reads (141,349) could be assigned to a phylum and 93.27 % (133,700) could be classified at the genus level. Two phyla were shown to dominate the human saliva metagenomic DNA sample, Bacteroidetes and Firmicutes representing 43.2 % and 42.1 % of the OTUs at this level. Proteobacteria was the third most abundant (9 %) while the remaining phyla (Actinobacteria, Unclassified phyla, Fusobacteria, Cyanobacteria, Tenericutes and Spirochaetes) represented less than 10 %, Figure 3.6 (a).

At the level of genus two members, again, appeared to dominate, *Prevotella* and *Veillonella* with 38.6 % and 21.5 % of the OTUs identified at this level being assigned to these genera respectively. 8.9% of the OTUs were identified as being of *Streptococcus*, 6.8 % were designated other while another 6 % were unclassified. The remaining 19.2 % represented 13 different genera including *Neisseria* and *Propionispora*, Figure 3.6 (b).

3.4.2.2 Phylogenetic Analysis of Calf Faecal Metagenomic DNA

455,475 reads were generated during the Illumina MiSeq sequencing run with 63.1 % (287,568) passing the quality filter. 99.51 % of these reads (286,151) could be assigned to a phylum and 91.07 % (261,887) could be classified at the genus level. Proteobacteria dominated the calf faecal metagenomic DNA sample with 98.7 % of OTUs identified as being from this phylum. 0.5 % and 0.1 % were designated unclassified or other, respectively with the remaining 0.7 % comprising Firmicutes, Bacteroidetes and Chlorobi, Figure 3.6 (c).

75.8 % of OTUs were designated as coming from *Escherichia*, 9.2 % from *Enterobacter* while 8.1 % were unclassified and 0.9% were assigned as other. 14 different genera represented the remaining 6 % of OTUs, Figure 3.6 (d).

Figure 3.6

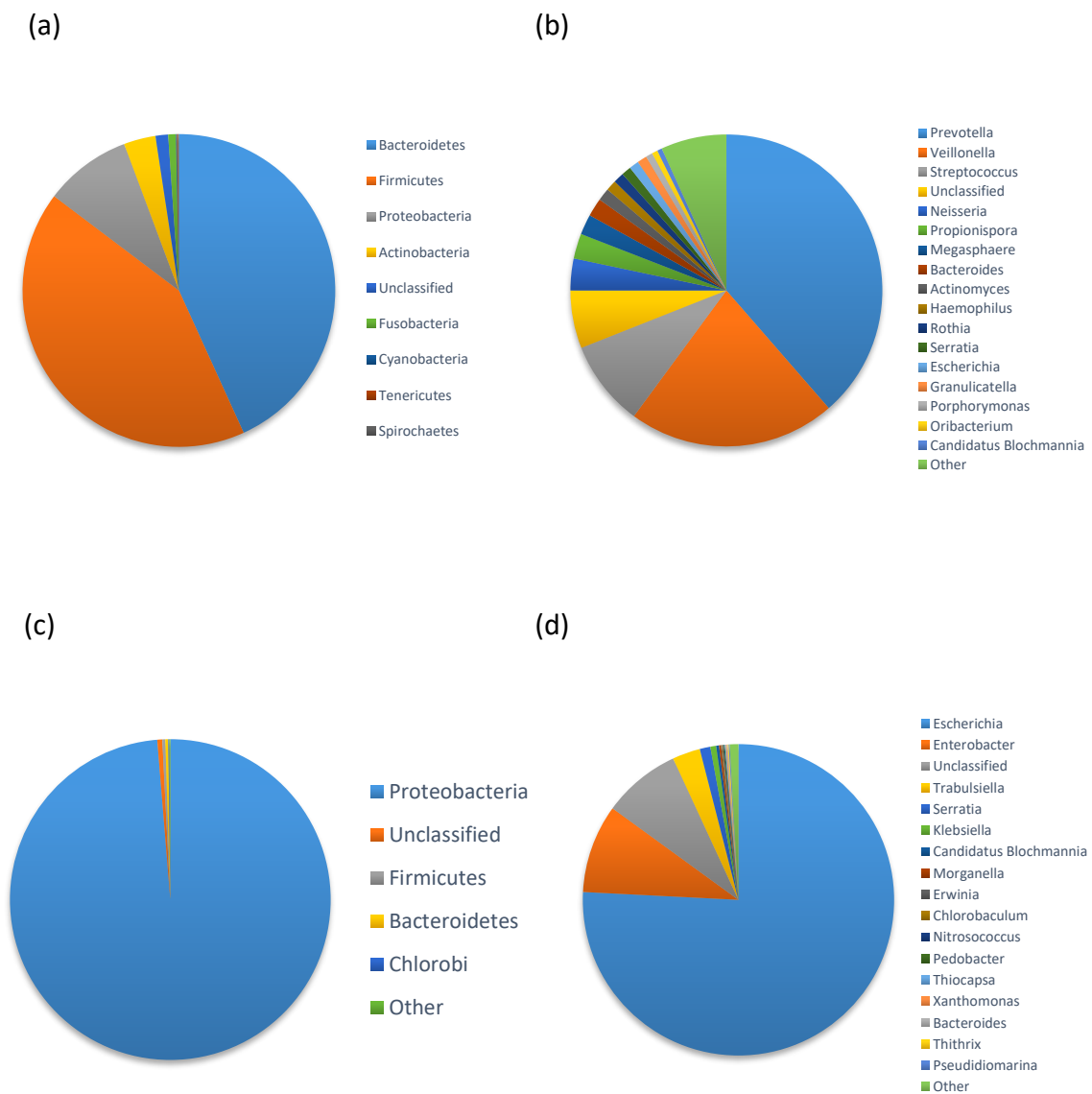


Figure 3.6 16S Composition of Human Saliva and Calf Faecal Metagenomic DNA Extractions. Pie charts were generated from the taxa identified from the Illumina pyrosequencing reads generated using the QIIME script described in Appendix II. (a) The relative abundances of the different phyla present in the human saliva metagenomic DNA. (b) The relative abundances of the different genera present in the human saliva metagenomic DNA (c) Pie chart depicting the respective relative abundances of the phyla Identified in the calf faecal metagenomic DNA (d) The relative abundances of the genera present in the calf faecal metagenomic DNA.

3.4.3 Analysis of Metagenomic Libraries

3.4.3.1 Efficiency of Transformation and Ligations

The transformation and ligation efficiencies of electroporating 2 – 5 µl of the human saliva – pCC1BAC and calf faecal – pCC1BAC ligation reactions into *E. coli* EPI300 T1^R are detailed in Table 3.2.

Table 3.2 Transformation and Ligation Efficiencies.

Ligation Reaction	Electroporation	Number of White Transformants (in 1000µl of recovered SOC)	Ligation *Efficiency	Transformation Efficiency (cfu/µg)
Human Saliva::pCC1BAC	5 µl of ligation 50µl EP1300	363	88 %	1.65 X 10 ³
	2 µl of ligation 50µl EP1300	1180	97 %	1.341 x 10 ⁴
Calf Faecal::pCC1BAC	5 µl of ligation 50µl EP1300	301	78 %	1.505 x 10 ³
	2 µl of ligation 50µl EP1300	240	91 %	3.0 X 10 ³

3.4.3.2 Determining the Size of the Metagenomic Libraries

A human saliva metagenomic library consisting of 27,000 clones was created by transforming 46 µl of a 100 µl ligation reaction into *E. coli* EPI300 T1^R. To estimate how much bacterial DNA was contained in the library 96 random clones were analysed by end sequencing using the pCC1 primers, Table 3.1. BLASTN and BLASTX analysis of the 96 pCC1BAC vector sequences from clones of the human saliva metagenomic library, showed

that 16 (16.67 %) contained no inserts, 39 (40.63 %) contained DNA of human origin only and 40 (41.67 %) contained DNA of bacterial origin only. A single clone was found to have a concatemeric insert as it contained DNA originating from both human and bacteria, thus 41 clones contained bacterial DNA (42.7 %) and 40 contained human DNA (41.67 %). The 41 bacterial DNA containing clones contained a total 345.45 Kb of DNA which indicated the average bacterial insert size for the library was 8.42 Kb, Figure 3.7(a). This average was assumed to be consistent across the entire library. Thus, the total amount of bacterial DNA in the library was 97,074 Kb ($8.42 \text{ Kb} \times 27,000 \text{ clones} \times 0.427$). As only 46 μl of the 100 μl ligation reaction was transformed initially, more clones could be obtained from transforming more of the reaction.

Figure 3.7

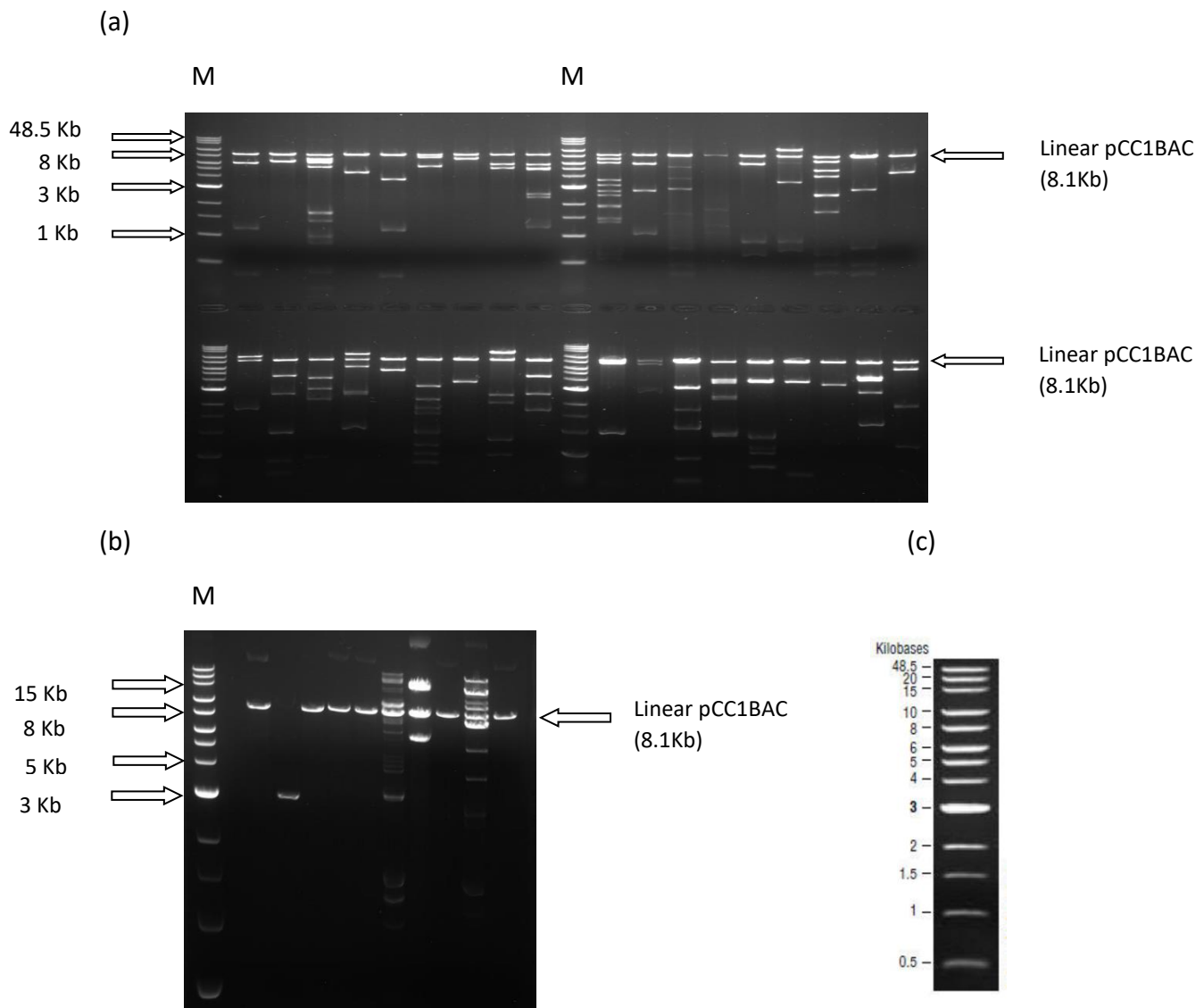


Figure 3.7 Estimating the Size of the Human Saliva Metagenomic Library. (a) Lanes marked M in this two-storyed gel contain the NEB 1 Kb extended ladder. The remaining 36 lanes contain *Hind*III digested pCC1BAC::insert vectors from randomly selected clones from the human saliva metagenomic library. (b) Lane M contains the 1 Kb extended ladder. The remaining 10 lanes contain randomly selected *Hind*III digested vectors from the calf faecal metagenomic library. (c) An image showing the size of the DNA fragments in the NEB 1 KB extended ladder.

Ten clones from the calf faecal metagenomic library were picked and 3 contained inserts. The three inserts contained a combined 237 Kb of DNA, thus over the 10 clones the average insert size was 23.7 Kb, Figure 3.7(b). Approximately 2,840 clones from the calf faecal metagenomic library were screened. Thus, assuming this average is conserved across the library, a total of 67,308 Kb (23.7 Kb X 2,840) of DNA was screened. However, based on these results 70 % of clones were empty so the 67.308 Mb of DNA would have been maintained in only 852 (2,840 X 0.3) clones.

3.4.3.3 Source of Cloned Bacterial DNA in the Metagenomic Libraries

3.4.3.3.1 Human Saliva Metagenomic Library – Source of Cloned DNA

In order to determine if our cloning protocol resulted in a loss of diversity in our metagenomic libraries compared with our metagenomic DNA extractions BLASTN and BLASTX analysis of 96 randomly selected human saliva metagenomic clones was conducted. Forty-one clones contained bacterial DNA; 18 contained DNA from the phylum Bacteroidetes only, 15 from Firmicutes only, 3 from Proteobacteria, 3 from Actinobacteria and 1 from an unclassified phylum, Figure 3.8 (a), Table 3.3. One clone contained DNA from the phyla Firmicutes and Bacteroidetes. At the genus level the majority of clones contained DNA from *Prevotella* spp. (17 clones), and *Veillonella* spp. and *Streptococcus* spp. were the second most abundant with six clones each, Table 3.3 and Figure 3.8 (b).

Two of the bacterial DNA containing clones contained an insert that when end sequenced showed one side of the insert to be from a different organism to the other. One clone

contained DNA that had 84 % nucleotide identity (100 % cover) to *Prevotella melaninogenica* when sequenced with the PCC1F primer and 36 % nucleotide identity (73 % cover) to *V. parvum* when sequenced with the PCC1R primer, indicating the insert DNA was a concatemer. A second bacterial DNA containing clone was also identified as a concatemer as its insert contained *Megasphaera* spp. and human DNA.

The bacterial composition of the human saliva metagenomic library was also determined by calculating how much DNA was cloned from each phylum identified. In the 41 clones that contained bacterial DNA, 188.9 Kb originated from Bacteroidetes, 112.85 Kb from Firmicutes, 11.2 Kb from Proteobacteria, 23 Kb from Actinobacteria and 9.5 Kb from an unclassified phylum (Candidate), Figure 3.8 (c). The average insert sizes for Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria and the unclassified Phylum were 9.94 Kb, 7.52 Kb, 3.7 Kb, 7.6 Kb and 9.5 Kb respectively.

Figure 3.8

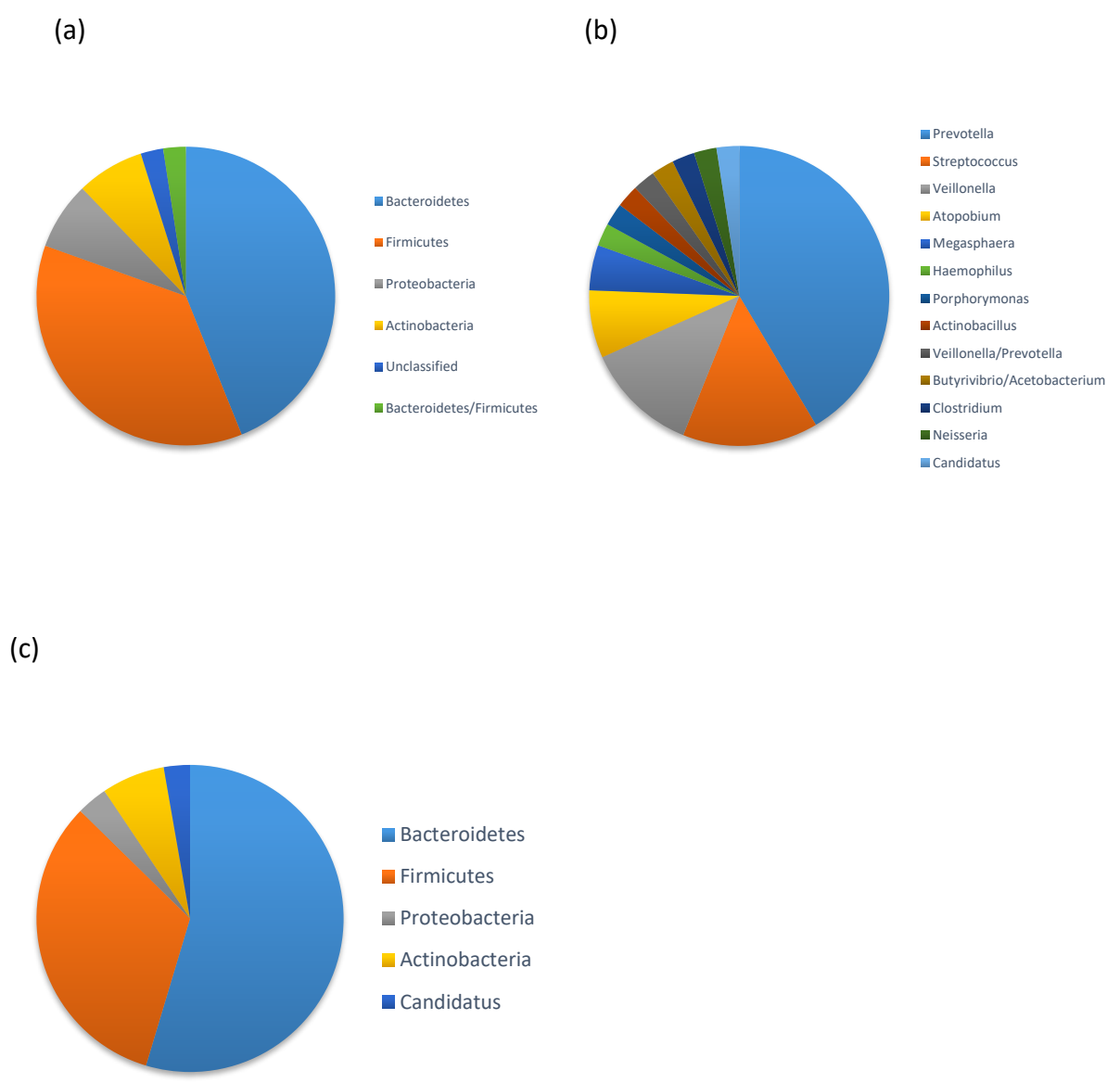


Figure 3.8 The Bacterial Composition of the Human Saliva Metagenomic Library. (a) Pie chart showing the relative abundance of each phyla in the human saliva metagenomic library based on clone number. (b) Pie chart depicting the relative abundance of each genus in the human saliva metagenomic library based on clone number. (c) Pie chart depicting the relative abundance of each phyla in the human saliva metagenomic library based on the amount of DNA cloned.

Table 3.3 BLAST Results for 41 Randomly Selected End Sequenced Human Saliva

Metagenomic Clones.

Clone	Forward Reaction	Reverse Reaction
1	<i>Prevotella melaninogenica</i> 99 % Cover, 78 % Identity	<i>Prevotella melaninogenica</i> 79 % Cover, 76 % Identity
2	<i>Prevotella melaninogenica</i> 99 % Cover, 94 % Identity	<i>Prevotella</i> spp. Oral Taxon 299 100 % Cover, 91 % Identity
3	<i>Prevotella melaninogenica</i> 87 % Cover, 84 % Identity	<i>Prevotella melaninogenica</i> 88 % Cover, 81 % Identity
4	<i>Prevotella melaninogenica</i> 100 % Cover, 99 % Identity	No Sequence
5	<i>Prevotella melaninogenica</i> 99 % Cover, 85 % Identity	<i>Prevotella melaninogenica</i> 98 % Cover, 70 % Identity
6	Uncultured Bacteria 97 % Cover, 69 % Identity	<i>Prevotella melaninogenica</i> 99 % Cover, 84 % Identity
7	No Result	<i>Prevotella melaninogenica</i> 41 % Cover, 83 % Identity
8	<i>Prevotella melaninogenica</i> 94 % Cover, 80 % Identity	<i>Prevotella melaninogenica</i> 52 % Cover, 76 % Identity
9	<i>Prevotella melaninogenica</i> 96 % Cover, 81 % Identity	<i>Prevotella melaninogenica</i> 78 % Cover, 91 % Identity
10	<i>Prevotella melaninogenica</i> 100 % Cover, 85 % Identity	No Sequence
11	<i>Prevotella melaninogenica</i> 97 % Cover, 76 % Identity	<i>Prevotella melaninogenica</i> 32 % Cover, 71 % Identity
12	<i>Prevotella intermedia</i> 100 % Cover, 79 % Identity	<i>Prevotella melaninogenica</i> 99 % Cover, 78 % Identity
13	<i>Prevotella denticola</i> 100 % Cover, 87 % Identity	<i>Prevotella melaninogenica</i> 63 % Cover, 78 % Identity
14	<i>Prevotella melaninogenica</i> 99 % Cover, 76 % Identity	<i>Prevotella melaninogenica</i> 98 % Cover, 80 % Identity
15	<i>Prevotella melaninogenica</i> 100 % Cover, 86 % Identity	<i>Elizabethkinga</i> spp. 32 % Cover, 71 % Identity
16	<i>Prevotella melaninogenica</i> 99 % Cover, 72 % Identity	No Result
17	<i>Prevotella denticola</i> 21 % Cover, 81 % Identity	<i>Prevotella melaninogenica</i> 99 % Cover, 90 % Identity
18	<i>Porphorymonas asaccharolytica</i> 44 % Cover, 87 % Identity	<i>Persicobacter</i> spp. 21 % Cover, 71 % Identity
19	<i>Veillonella parvum</i> 92 % Cover, 91 % Identity	<i>Veillonella parvum</i> 96 % Cover, 94 % Identity
20	<i>Veillonella parvum</i> 94 % Cover, 75 % Identity	<i>Veillonella parvum</i> 91 % Cover, 81 % Identity
21	<i>Veillonella parvum</i> 99 % Cover, 83 % Identity	<i>Veillonella parvum</i> 100 % Cover, 76 % Identity
22	<i>Veillonella parvum</i> 99 % Cover, 90 % Identity	<i>Veillonella parvum</i> 100 % Cover, 91 % Identity
23	<i>Veillonella parvum</i> 100 % Cover, 98 % Identity	<i>Veillonella parvum</i> 99 % Cover, 100 % Identity

24	<i>Prevotella melaninogenica</i> 100 % Cover, 84 % Identity	<i>Veillonella parvum</i> 12 % Cover, 78 % Identity
25	<i>Paenibacillus spp.</i> 9 % Cover, 79 % Identity	<i>Streptococcus salivarius</i> 76 % Cover, 74 % Identity
26	<i>Streptococcus pneumoniae</i> 99 % Cover, 78 % Identity	<i>Streptococcus mitis</i> 99 % Cover, 87 % Identity
27	<i>Streptococcus pneumoniae</i> 100 % Cover, 84 % Identity	<i>Streptococcus spp.</i> 99 % Cover, 91 % Identity
28	<i>Streptococcus oralis</i> 99 % Cover, 100 % Identity	<i>Streptococcus oralis</i> 98 % Cover, 99 % Identity
29	<i>Streptococcus pneumoniae</i> 99 % Cover, 83 % Identity	<i>Streptococcus mitis</i> 99 % Cover, 100 % Identity
30	<i>Streptococcus mitis</i> 98 % Cover, 90 % Identity	<i>Streptococcus mitis</i> 100 % Cover, 93 % Identity
31	<i>Butyrvibrio proteoclasticus</i> 79 % Cover, 71 % Identity	<i>Acetobacterium woodii</i> 52 % Cover, 72 % Identity
32	<i>Clostridium acetobutylicum</i> 55 % Cover, 70 % Identity	No Result
33	<i>Megasphaera elsednii</i> 52 % Cover, 82 % Identity	No Sequence
34	<i>Megasphaera micronuciformis</i> 100 % Cover, 99 % Identity	Human DNA
35	<i>Actinobacillus succinogens</i> 99 % Cover, 65 % Identity	<i>Haemophilus parainfluenzae</i> 40 % Cover, 95 % Identity
36	<i>Haemophilus parainfluenzae</i> 98 % Cover, 95 % Identity	No Sequence
37	<i>Nesseria mucosa</i> 97 % Cover, 100 % Identity	No Sequence
38	<i>Atopobium parvula</i> 100 % Cover, 82 % Identity	<i>Atopobium parvula</i> 100 % Cover, 81 % Identity
39	<i>Atopobium parvula</i> 99 % Cover, 91 % Identity	<i>Atopobium parvula</i> 99 % Cover, 91 % Identity
40	<i>Atopobium parvula</i> 99 % Cover, 93 % Identity	<i>Atopobium parvula</i> 99 % Cover, 91 % Identity
41	<i>Candidatus sacchari</i> 99 % Cover, 78 % Identity	<i>Candidatus sacchari</i> 99 % Cover, 79 % Identity

3.4.3.3.2 Calf Faecal Metagenomic Library – Source of Cloned DNA

The three calf faecal metagenomic library clones that contained an insert were end sequenced. One of the clones contained a potential concatemeric insert. The forward reaction sequence showed that the insert had low similarity to an *Enterobacter* species. When sequenced from the reverse end, sequence identity could only be achieved using

discontiguous megablast revealing similarity to a species of *Paenibacillus*, Table 3.4. The remaining clones had inserts with sequence similarity to genera of the class *Enterobacteriaceae*, Table 3.4

Table 3.4 BLASTN Results for End Sequenced Calf Faecal Metagenomic Clones.

Clone	Forward Reaction	Reverse Reaction
Clone 1	<i>Enterobacter</i> spp 33 % Cover, 82 % Identity	<i>Paenobacillus</i> spp. 14 % Cover, 85 % Identity
Clone 2	<i>Shigella</i> spp. 91 % Cover, 100 % Identity	<i>E. coli</i> 97 % Cover, 100 % Identity
Clone 3	<i>E. coli</i> 95 % Cover, 100 % Identity	<i>E. coli</i> 96 % Cover, 100 % Identity

3.5 Discussion

The aim of the work described in this chapter was to create two metagenomic libraries using pCC1BAC; one from human saliva and the other from calf faeces. These environments are known to harbour antibiotic resistance genes and as the majority of bacteria species from these environments have yet-to-be cultured, novel resistance genes can be identified from screening these functional metagenomic libraries. To create a metagenomic library suitable for functional screening it is vital that the metagenomic DNA be of high concentration, high molecular weight and of high-enough quality for restriction enzyme digestion and subsequent ligation into a suitable vector. The modified Gentra Puregene Yeast/Bact. protocol proved sufficient in this regard as previously demonstrated by Seville *et al.* (Seville *et al.*, 2009). This method relies on separating the cell content from the sample matrix (saliva components and calf faecal matter in our samples) prior to isolating the metagenomic DNA. This minimises contamination of the DNA extracts with metal ions, salts and carbohydrates but may also introduce biases in the preparations as not all cells will be easily separated from the sample matrix (Delmont *et al.*, 2011). Our human saliva and calf faecal metagenomic DNA preparations contained DNA of high molecular weight with fragments greater than 10Kb in size observable by agarose gel electrophoresis, although smearing indicated smaller fragments were also present. Furthermore, although the 260/230 nm ratios were low for the human saliva and calf faecal metagenomic preparations (indicative of contamination with phenols or carbohydrates), the high molecular weight DNA was amenable to digestion by *Hind*III indicating that the DNA was of sufficient quality for cloning (ThermoScientific, 2011).

At the phylum and genus levels 16S rRNA analysis of the human saliva metagenomic preparation was in agreement with previous studies in that Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria (97.6 %) represented the four most abundant phyla and *Prevotella*, *Veillonella*, *Streptococcus* and *Neisseria* (72.3 %) were the dominant classified genera (Dassi *et al.*, 2014; Segata *et al.*, 2012). Our results showed Bacteroidetes to be the most abundant phylum and *Prevotella* to be the dominant genus in our metagenomic preparation. Although studies have demonstrated that Firmicutes are the dominant phyla in saliva, individuals with higher proportions of Bacteroidetes and lower relative abundances of Firmicutes and Proteobacteria have also been observed, exemplifying the diversity of this environment (Mager *et al.*, 2003).

The only prerequisite for individuals to be involved in this study was that they had not taken antibiotics in the previous three months and were not at the time taking medication. Thus, health status, diet, oral hygiene or ethnicity of the individuals providing saliva were not taken into account, but likely contributed to the diversity observed in our metagenomic preparation. For instance, increased relative abundances of *Prevotella* spp. have been associated with gingivitis, periodontitis and poor oral hygiene and this may explain the high relative abundances of *Prevotella* spp. in our human saliva metagenomic DNA (Darveau, 2010; Liu *et al.*, 2012). Differences in the core oral microbiomes may arise due to dietary or ethnic differences. For instance, Li *et al.* (2014) have shown that Alaskan and German oral microbiomes were dominated by Firmicutes while Proteobacteria were the most abundant in African oral cavities (Li *et al.* 2014). The saliva samples used in this study originated from 11 individuals from different geographic locations that may harbour higher proportions of *Prevotella* spp. in their oral cavities. The method by which individuals expectorated may also have contributed to the high relative abundances of the anaerobic genera. Some individuals

may have dislodged dental, tongue dorsum or tonsil plaque, which have higher proportions of *Prevotella* spp. and *Veillonella* spp., when generating saliva, which may have contributed to the 16S rRNA results observed.

The abundance and proportion of each bacterial genera and species observed may also have been an artefact of the protocols used. Brooks *et al.* (2015) noted an increased abundance of *Enterococcus* spp. and lower *Bacillus* spp. and *Pseudomonas* spp. 16S rRNA levels when they used a Qiagen kit rather than the Powersoil Kit when analysing a mock community of 21 archaeal and bacterial species (Brooks *et al.*, 2015). *Streptococcus* represented 8.9 % of the genera present in our human saliva metagenomic DNA. Although comparable levels of *Streptococcus* spp. have been identified in the saliva of some individuals, it is possible that the lytic enzyme used in the extraction did not lyse *Streptococcus* spp. cells effectively resulting in an underrepresentation of this genus and potentially other genera in our sample (Segata *et al.*, 2012). The lytic enzyme may have failed to lyse other Gram-positive bacteria in the saliva samples collected as only 15 % of the genera identified were Gram-positive. PCR has also been shown to introduce bias in 16S rRNA studies as a result of the polymerase and conditions used for amplification or from the choice of primers and hypervariable region amplified (Ahn *et al.*, 2012; Lee *et al.*, 2012). For example, Kumar *et al.* (2011) showed by 454 sequencing that when using V1-V3 amplifying primers *Prevotella* spp. were the most abundant (28.1 %) in the subgingival plaque of smokers with periodontitis whilst *Streptococcus* spp. represented 8.3 % of total species. Whereas, using V4-V6 primers, they showed *Streptococcus* spp. to be the dominant species (25.2 %) compared with *Prevotella* spp. (8.2 %) (Kumar *et al.*, 2011).

Although Bacteroidetes represented the most abundant phyla in our metagenomic DNA extraction, it was the least diverse, with *Prevotella* making up approximately 94 % of genera. Firmicutes was the most diverse phylum with five genera identified; Proteobacteria followed with four identified genera and Actinobacteria and Bacteroidetes each had three. Six percent of the 16S sequences could not be classified to the genus level. This may be because these sequences were not in the Greengenes dataset or that they are completely novel sequences representing unidentified bacterial genera. At the genus level, bacteria with a relative abundance of less than 0.5 % were grouped into the 'other' category and represented 6.8 % of the diversity at this level. This group may contain genera of the TM7 and Synergistetes phyla that are also present in human saliva. Interestingly, *Candidatus Blochmannia* represented 0.5 % of the genera present in the sample; *Candidatus Blochmannia* is an endosymbiotic bacteria associated with carpenter ants (Sauer *et al.*, 2000).

Transformation of the Human Saliva-pCC1BAC and Calf Faecal-pCC1BAC ligation reactions into *E. coli* EPI300 T1^R had maximum efficiencies of 1.341 X 10⁴ CFU/ml and 1.066 X 10⁴ CFU/ml, respectively. These were below the estimated transformation efficiency for *E. coli* EPI300 T1^R by a factor of approximately 10⁶. This is not surprising as the reported efficiencies were calculated using pUC19 which is smaller and likely more stable than the pCC1BAC vectors containing large DNA fragments from different host backgrounds that we cloned into *E. coli* EPI300 T1^R (Epicentre, 2011). The library transformations discussed here were conducted using ligation reactions containing plasmids of different sizes with different levels of supercoiling that likely also contained unligated DNA fragments from the metagenomic DNA extractions which can reduce transformation efficiency (Zhu and Dean,

1999). A higher transformation efficiency was observed for both HS-pCC1BAC and CF-pCC1BAC when 2 μ l of the ligation reactions were used rather than 5 μ l. Plateauing and reductions of efficiency have previously been observed with increasing DNA concentrations (Chung *et al.*, 1989; Dorella *et al.*, 2006). Greater volumes of ligation reaction contain more DNA which can have a toxic effect on the competent cells. Higher volumes may also mean more impurities including salts and ligase in the ligation reaction which reduces efficiency of transformation (Wu *et al.*, 2010; Brambach *et al.*, 2013).

A total of 27,000 clones were created from the transformation of 46 μ l out of a 100 μ l ligation reaction. If the total ligation reaction had been transformed an estimated 59,696 clones would have been obtained, and more ligation reactions could be conducted and transformed to expand the human saliva library. Of the 96 clones selected, 16.67 % were empty and 41.67 % contained human DNA. Metagenomic preparations from human saliva are prone to contamination with human DNA as mucosa cells present in the samples lyse with the bacterial cells. For example, Seville *et al.* (2009) found that 61 % of their human saliva metagenomic library contained human DNA, which is a greater proportion than our library (Seville *et al.*, 2009). The bacterial inserts present in the human saliva metagenomic library ranged from 100 bp to 31 Kb with an average insert size of 8.42 Kb. An average insert size of 8.42 Kb is small for pCC1BAC which can maintain inserts greater than 100 Kb.

Functional metagenomic surveys of the human saliva metagenome conducted by Card *et al.* (2009) and Seville *et al.* (2014) using this vector obtained average insert sizes of 20 Kb and 30 Kb, respectively (Seville *et al.*, 2009; Card *et al.*, 2014). Although small by BAC standards, 8.42 Kb is large enough to contain antibiotic resistance genes and operons involved in bacteriocin production. For instance, *Prevotella nigrescens* encodes a bacteriocin and

immunity gene, *nigAB*, on a 4, 868 bp region of their chromosome, while mutacin II is encoded by an 8 - 9 Kb operon (Qi *et al.*, 1999; Kaewsrichan *et al.*, 2005).

Minimal loss of diversity between the metagenomic DNA and the functional metagenomic library was observed as both had similar phylum and genus level profiles. End sequencing of 96 random clones revealed 41 bacterial DNA containing clones that, as with the 16S rRNA sequencing data, contained inserts dominated by Bacteroidetes followed by Firmicutes, Proteobacteria and Actinobacteria. At the genus level as with the 16S rRNA data, *Prevotella*, *Veillonella* and *Streptococcus* inserts were the most abundant. This suggests that digestion of the human saliva metagenomic DNA with the AT rich (5'-AAGCTT-3') targeting *HindIII* enzyme did not introduce a strong bias in our library. To further assess any bias that may have been introduced into the library by *HindIII*, the contribution of each phylum to the library was determined based on insert size. Bacteroidetes represented 46.3 % of the clones analysed but accounted for 54.7 % of the cloned DNA. Whereas, 39 % of the clones contained DNA from Firmicutes and this phylum contributed to 32.7 % of the cloned DNA. This may suggest that *HindIII* introduced a bias towards larger Bacteroidetes inserts. For instance, *Veillonella* and *Streptococcus* spp. which made up the bulk of the Firmicutes have GC contents of approximately 37 % while *Prevotella* spp., which was the most abundant genus in the Bacteroidetes phyla are more GC rich with a GC content greater than 40 %. As the *Streptococcus* and *Veillonella* spp. genomic material is more AT rich than that of *Prevotella* spp. they may have had more *HindIII* sites that were cleaved to generate smaller fragments when compared with the Bacteroidetes fragments, and thus were easier to clone. In a recent paper, Lam and Charles (2015) determined that a reduction in abundance of Firmicutes was observed between their human faecal metagenomic preparations and the cosmid library they created. They determined that this was a result of instability and loss of

inserts containing Firmicutes DNA due to constitutive transcription from *rpoD* promoters that are typically more abundant in AT rich genomes (Lam and Charles, 2015). This may explain why Firmicutes is less abundant in our human saliva library library (32.7 %) compared with our 16S rRNA sequence data (42.1 %). It should be noted that these results come from only a small number of clones and unlike the 16S rRNA analysis phylogenetic annotation was conducted using non-comparable sequences of varying size. Additionally, for some cloned inserts MegaBLAST was used for annotation while discontinuous MegaBLAST or BLASTX were used for others. Thus, although these tools have been used previously to quantify diversity in metagenomic libraries the use of various tools to assign taxonomy make the data less robust (Manichanh *et al.*, 2008; Seville *et al.*, 2009). In conclusion, the extraction protocol used in this study to extract metagenomic DNA from human saliva gave a sample with a 16S rRNA profile that was in agreement with previous human saliva microbiome studies.

16S rRNA analysis of the calf faecal metagenomic samples revealed it to be dominated by bacteria of the phylum Proteobacteria (98.7 %). The next most abundant phyla belonged to the unclassified phyla representing 0.6 % of all phyla followed by Firmicutes and Bacteroidetes at 0.3 %. Although previous phylogenetic studies have shown that these three phyla typically make up > 90 % of the calf faecal microbiome, Proteobacteria are over represented in our metagenomic preparation. Previous studies are in agreement that Firmicutes and Bacteroidetes are the two most abundant phyla in calf faeces with Proteobacteria representing between 1.4 and 9.75 % (Ozutsumi *et al.*, 2005; Uyeno *et al.*, 2010; Oikonomou *et al.*, 2013). A study conducted by Xu *et al.* (2104) found that even in faeces from super-shedder cattle (cattle that shed > 10⁴ CFU of *E. coli* O157:H7 per gram of faeces) Proteobacteria accounted for approximately 1.7 % of the microbiome (Xu *et al.*,

2014). *Escherichia* and *Enterobacter* accounted for 75.8 % and 9.2 % of the genera respectively in our calf faecal metagenomic DNA which is much higher than previously observed levels. Meale *et al.* (2016) described *E. coli* as having a relative abundance of 1.2 % in weaned calf faeces while another study that used an rRNA based approach found that *Enterobacteriaceae* dropped below detectable levels following weaning (Uyeno *et al.*, 2010; Meale *et al.*, 2016). The high abundance of Proteobacteria may be a result of the handling and processing of the faeces. Prior to freezing at -80°C calf faeces was stored in a sterile closed pot at ambient temperature for approximately one hour. Exposure of faecal samples to an aerobic environment has been shown to result in a shift in community structure including an increase in gamma-proteobacteria. However, even under such conditions this class of bacteria still represented only about 8 % of the microbiota (Wong *et al.*, 2016). Exposure to RT also alters the bacterial composition of faeces including a reduction in the abundance of Firmicutes including *Ruminococcus* spp. (Choo *et al.*, 2015). Although our 16S rRNA results showed high abundance of Proteobacteria and low levels of Firmicutes, the above studies exposed their samples to RT or aeration for periods of days to weeks so it is unlikely that the short period our calf faeces was exposed to ambient temperature and aeration would have resulted in such a shift in the bacterial community profile. A loss of diversity may have occurred as bacterial cells were separated from the faecal matter prior to extraction. Although *ex situ* extraction protocols produce higher molecular weight and purer DNA compared with *in situ* methods, they do so at the expense of diversity as lower abundance members are often lost in the process (Delmont *et al.*, 2011). Additionally, DNA released by lysis of bacterial cells during freezing may not have pelleted during cell separation resulting in a loss of this DNA from the metagenomic samples. As already discussed with regard to saliva extraction, our protocol may also have introduced bias by

preferentially lysing gram negative bacterial cells. However, as previous studies have used this protocol to achieve more representative extractions it is likely that contamination of the sample occurred (Seville *et al.*, 2009). All reagents and tools used to process the faecal samples were sterilised, as was the case when processing the saliva samples. Likely contamination sources present during the faecal processing were the calves environment or from the CTAB reagent used to further clean the calf faecal metagenomic DNA. The latter was prepared, sterilised by autoclaving and kept at 60°C however, it may still have contained *E. coli* DNA that subsequently contaminated our sample resulting in our sample not representing the metagenome of calf faeces.

Of the 10 clones analysed from the faecal metagenomic library 7 of the clones contained no insert although the colonies were white, which be a result of mutations in the host strain resulting in a loss of *lacZ* expression. The remaining 3 colonies contained large inserts resulting in a combined 237 Kb. Thus, the average insert size for these 3 clones was 79 Kb, large enough to maintain large gene clusters including bacteriocin producing operons. Two of the clones contained DNA with high similarity to *Enterobacteriaceae* while the third had low similarity to *Enterobacter* and *Paenobacillus* spp. These results are in agreement with the 16S rRNA data showing that *Enterobacteriaceae* make up bulk of the metagenomic DNA. However, the analysis of only three clones makes any meaningful conclusion impossible to make in this regard. No further clones were analysed from this library due to the high number of clones apparently containing no insert even when they were white. The high proportion of clones containing no insert may have resulted from contamination of the metagenomic DNA with phenol inhibiting the ligation reaction. Although phenol is used to remove proteinaceous material that may interfere with downstream molecular processing, it is also known to inhibit enzymes including ligase and restriction enzymes. Although *HindIII*

digestion and PCR were not inhibited, the phenol may have denatured the T4 DNA ligase resulting in the creation of few plasmid::insert constructs (Schrader *et al.*, 2012).

To conclude, a library containing mostly empty clones was created using calf faecal metagenomic DNA. The high proportion of empty clones was likely a result of phenol contamination that inhibited the ligation reaction. A human saliva library of 27,000 clones with an average insert of 8.42 Kb, representing 97,074 Kb of bacterial DNA was also created. This library was suitable for functional screening to identify genes of interest as will be discussed in chapter 4.

Chapter 4

Screening the Human Saliva and Calf Faecal Metagenomes

4.1 Introduction

4.1.1 Antimicrobial Resistance in the Human Oral Cavity

4.1.1.1 Antibiotic Resistance in the Human Oral Cavity

The 'reservoir hypothesis' posits that the commensal bacteria act as a reservoir of resistance genes (Salyers *et al.*, 2004). There is contention over what constitutes a resistance gene and indeed a reservoir of resistance genes. Martínez *et al.* (2015) suggest that housekeeping genes identified as conferring antibiotic resistance to a host in a functional metagenomic screen should not be described as such. The term 'reservoir' is still used however and the human oral cavity microbiome is often referred to as such. Genes conferring resistance to tetracyclines and β -lactams are frequently identified from the oral cavity resistome (the collection of resistance genes present in both pathogenic and non-pathogenic bacteria) studies (Ready *et al.*, 2003; Gaetti-Jardim *et al.*, 2010; Kim *et al.*, 2011; Moraes *et al.*, 2015; Martínez *et al.*, 2015).

The oral microbiota harbouring resistance genes have the potential to cause infections, as described in chapter 3. These ARG-harboured bacteria may also transfer their resistance genes via conjugation to other human pathogens that are transiently present in the oral cavity (Li *et al.*, 2000; Lockhart *et al.*, 2009). As such the human oral cavity has been the focus of studies that have aimed to identify and characterise the resistome of this environment (Lancaster *et al.*, 2003; Seville *et al.*, 2009; Card *et al.*, 2014; Rashid *et al.*, 2015).

Antibiotics use can alter the oral microbiome resulting in an increased abundance of commensal bacteria harbouring ARGs. In a study conducted by Rashid *et al.* (2015), individuals that took a 10-day course of ciprofloxacin (500 mg dose) or clindamycin (150 mg dose) had proportionally more ciprofloxacin-resistant strains of *Prevotella*, *Veillonella* and *Lactobacillus* spp. or clindamycin resistant *Prevotella* spp. respectively in their saliva after 12 months compared with individuals who took a placebo for 10 days (Rashid *et al.*, 2015). In another survey, Zaura *et al.* (2015) used shotgun metagenomic sequencing to show that although antibiotic use didn't increase the abundance of antibiotic resistance genes, minocycline and clindamycin use did select for multidrug efflux genes (Zaura *et al.*, 2015). Additionally, even when antibiotics have not been taken, antibiotic resistance in the oral cavity has been described (Lancaster *et al.*, 2005; Zaura *et al.*, 2015). The acquisition of such genes may occur when they are passed down vertically from mother to child when the mother's commensal bacteria and resistome are transferred during gestation and birth (Dominguez-Bello *et al.*, 2010; Aagaard *et al.*, 2014).

4.1.1.2 Oral Biofilms and Antibiotic Resistance

Intrinsic mechanisms of resistance also exist within the oral microbiota. For instance, cells growing in biofilms including plaque can be up to 1000-fold more resistant to antimicrobials than planktonically growing cells (Hoyle and Costerton, 1991; Moskowitz *et al.*, 2004; Bjarnsholt *et al.*, 2007). The EPS (extrapolymeric substance) produced by biofilm cells provides a barrier against antimicrobial diffusion resulting in lower concentrations of the inhibiting compound within the structure (Corbin *et al.*, 2011).

4.1.1.3 Metabolic Activity Can Alter Antibiotic Susceptibility

As discussed in chapter 3, there is a heterogeneity in biofilms, including dental plaque, in terms of nutrient and dissolved oxygen availability and distribution of signalling factors. This results in metabolic differences between different cells in the biofilm and in some cells being less metabolically active than their planktonic counterparts (Gilbert *et al.*, 1990; Anderl *et al.*, 2003). A loss of metabolic activity may induce a compositional change to the cell's surface resulting in modified lipid and polysaccharide structures and altered surface protein expression (Sutherland, 1982; Chalabaev *et al.*, 2014). For example, the OmpF porin involved in antimicrobial uptake is downregulated by the cells in an *E. coli* biofilm while OmpC expression increases (Simonet *et al.*, 2000; Bredin *et al.*, 2003; Freire *et al.*, 2006).

4.1.1.4 Tetracycline Resistance in the Human Oral Cavity

A number of studies have been conducted to characterise the resistome of the cultivable and non-cultivable microorganisms of the oral cavity in order to define the oral tetracycline resistome and identify novel tetracycline resistance determinants. These studies have shown that tetracycline resistant genes are abundant and prevalent in this environment. Gaetti-Jardim *et al.* (2010) surveyed the cultivable microbiome of the oral cavity (saliva, mucosa, supragingival and subgingival plaque). Of the 304 isolates tested 101 (33.2%) were resistant to tetracycline (Gaetti-Jardim *et al.*, 2010).

The RPP gene, *tet(M)* is the most frequently identified and most abundant tetracycline resistant gene in the human oral cavity, typically followed by *tet(Q)*, *tet(W)* and *tet(O)* (Villedieu *et al.*, 2003; Lancaster *et al.*, 2003; Lancaster *et al.*, 2005; Seville *et al.*, 2009; Kim *et al.*, 2011) . In a survey of the oral cavity of 54 Greeks, *tet(M)* and *tet(Q)* were found in between 70 % and 82 % of all subjects, respectively, using PCR (Ioannidis *et al.*, 2009). Functional screening of human saliva and plaque metagenomic libraries and microarray analyses have provided further evidence for RPP genes being prevalent in these environments (Diaz-Torres *et al.*, 2003; Diaz-Torres *et al.*, 2006; Seville *et al.*, 2009). For instance, Diaz-Torres *et al.* (2006) found that *tet(M)*, *tet(Q)*, *tet(w)* and *tet(O)* expressing clones represented 37 % of the identified tetracycline-resistant clones and were present in all of their libraries (Diaz-Torres *et al.*, 2006).

The prevalence of RPP genes in the oral cavity is aided by their association with mobile genetic elements (MGEs) including conjugative plasmids and transposons (Ciric *et al.*, 2011; Brenciani *et al.*, 2014). Tn916 is the archetype of a family of broad host range conjugative transposons that carry *tet(M)*, although in Tn916S a *tet(S/M)* mosaic is present (Roberts and Mullany, 2011; Novais *et al.*, 2012). Tn916-like elements have been identified in over 30 genera including oral *Streptococcus*, *Neisseria*, *Veillonella* and *Fusobacterium* spp. from the oral cavity (McKay *et al.*, 1995; Olsvik *et al.*, 1995; Rossi-Fedele *et al.*, 2006; Ready *et al.*, 2006; Ciric *et al.*, 2012; Brenciani *et al.* 2014). *tet(Q)* is mainly associated with Gram-negative organisms including *Prevotella* and *Porphyromonas* spp. and has been shown to be more prevalent in patients with periodontitis (Okamoto *et al.*, 2001; Kim *et al.*, 2011). The *in vitro* transfer of *tet(Q)* from these species to others has been demonstrated and it has been found to be associated with non-conjugative IS21 transposable elements in *Prevotella* spp. (Chung *et al.*, 1999; Tribble *et al.*, 2010).

As well as RPP genes, tetracycline transporters of the MFS family have also been isolated from the human oral cavity, although less frequently than RPPs. Villedieu *et al* (2003) found that *tet(L)* was found in 2.8 % of the cultivable tetracycline resistant isolates in their study while *tet(A)* and *tet(K)* were encoded by 1 % of isolates (Villedieu *et al.*, 2003). *tet(A)* and *tet(B)* have also been identified from the human oral cavity using functional metagenomic and microarray approaches (Diaz-Torres *et al.*, 2006; Card *et al.*, 2014).

Novel tetracycline resistance determinants have also been identified from the human oral cavity including a novel ABC transporter, TetAB(46) from *Streptococcus australis* and a tetracycline degrading enzyme, Tet(37), identified from a functional metagenomic library (Diaz-Torres *et al.*, 2003; Warburton *et al.*, 2013). As tetracycline resistance genes are frequently isolated from the oral cavities of individuals, even in the absence of tetracycline use, and novel tetracycline resistance genes continue to be identified from this environment, screening of human saliva metagenomic libraries has the potential to identify more novel tetracycline resistance genes.

4.1.1.5 β -lactam Resistance in the Human Oral Cavity

The human oral cavity is also a source of β -lactam resistance genes. In Gaetti-Jardim's 2010 study, 178 of 304 (58.6 %) oral isolates were resistant to at least a single β -lactam of which 75.8 % were ampicillin resistant and 62.9 % were β -lactamase producers (Gaetti-Jardim *et al.*, 2010). In the oral cavity Ambler class A serine β -lactamases are the most abundant and as these genes are mainly found in Gram-negative bacteria, they are more prevalent in periodontitis patients (Gaetti-Jardim *et al.*, 2010; Darveau, 2010; Card *et al.*, 2014). In an oral biofilm study, Kim *et al.* (2011) used PCR to show that bla_{TEM} was present in more than 90 % of healthy individuals and in all periodontitis patients while a later survey conducted by Koukos *et al.* (2016), also employing a PCR approach, showed bla_{TEM} to be more prevalent in tongue mucosa samples than plaque regardless of periodontal health status (Kim *et al.*, 2011; Koukos *et al.*, 2016). In the same study, Kim *et al.* (2011) found $cfxA$ in approximately 70 % and 80 % of the subjects of the healthy and periodontal groups respectively (Kim *et al.*, 2011). β -lactamase genes that have been isolated from oral bacteria or from clinical isolates that are known oral inhabitants are listed in Table 4.1.

Table 4.1 β -lactamase genes Identified in Oral Bacteria.

B-lactamase gene	Class	Source	Reference
<i>bla_{SHV}</i>	Class A	Enteric and <i>Pseudomonas</i> spp. isolates from the oral cavity	(Ramos <i>et al.</i> , 2009)
<i>bla_{TEM}</i>	Class A	<i>E. coli</i> from the oral cavity	(Handal <i>et al.</i> , 2005)
		<i>Eikennella corridens</i> on a Tn3-like transposon	(Lacroix and Walker, 1992)
		<i>Neisseria</i> spp.	(Handal <i>et al.</i> , 2005)
		<i>Capnocytophaga ochrace</i> , plasmid encoded	(Rosenau <i>et al.</i> , 2000)
<i>bla_{CTX-M}</i>	Class A	Enteric species. isolates from the oral cavity	(Ramos <i>et al.</i> , 2009)
<i>cfiA</i>	Class B	Clinical <i>Bacteroides fragilis</i> isolates	(Love <i>et al.</i> , 1989; Roh <i>et al.</i> , 2010)
<i>ampC</i>	Class C	<i>Citrobacter freundii</i> from the oral cavity	(Handal <i>et al.</i> , 2005)
		Oral <i>Enterobacteriaceae</i>	(Leão-Vasconcelos <i>et al.</i> , 2015)
<i>bla_{OXA}</i>	Class D	<i>Fusobacterium nucleatum</i>	(Voha <i>et al.</i> , 2006)

Intrinsic mechanisms of β -lactam resistance are also present in the oral cavity including efflux mechanisms. For instance an ampicillin resistant clone harbouring *acrRAB* genes was isolated from a human saliva metagenomic library (Card *et al.*, 2014). Commensal *Streptococcus*, *Neisseria* and transient *Enterococcus* spp. that may be found in the oral cavity can be intrinsically resistant to penicillin due to the production of penicillin binding proteins that have low affinities for penicillin (Rice *et al.*, 2004; Wang *et al.*, 2012; Jensen *et al.*, 2015). Mutations of the PBP5 gene can result in resistance to ampicillin and some cephalosporins. β -lactam resistant *Staphylococcal* spp. including *mecA* positive *S. aureus* have been found in the oral cavity although they are not typically prevalent or highly abundant (Cuesta *et al.*, 2011; Koukos *et al.*, 2015). In a study of the saliva and oral mucosa from 37 healthy individuals and 46 cancer patients, 71 *Staphylococcus* spp. were isolated

from 28 individuals of which 9 were MRSA and 2 were oxacillin resistant coagulase negative *Staphylococcus* spp. (Yamashita *et al.*, 2013).

4.1.1.6 Decreased Susceptibility to Antiseptics and Metals in the Oral Cavity

Although research has focused less on antiseptic resistance in the oral cavity compared with antibiotic resistance; antiseptic resistance genes have been described for some oral bacteria. For instance, Wang *et al.* (2013) showed that mutation of the bacteriocin immunity genes *immA* and *immB* resulted in decreased chlorhexidine susceptibility in *S. mutans* and that their expression was increased following chlorhexidine treatment (Wang *et al.*, 2013). In another study, *P. gingivalis* was shown to have reduced chlorhexidine susceptibility as a result of vesicle production and release. These vesicles were shown to protect other oral microbes including *P. intermedia*, *S. mutans* and *Capnocytophaga ochracea* as they contained LPS that bound and sequestered the antiseptic, preventing it from targeting the bacterial cells (Grenier *et al.*, 1995). Antiseptic resistance genes have also been found on MGEs in oral bacteria. Ciric *et al.* isolated a CTAB-resistant strain of *S. oralis* from the oral cavity and found it to be carrying a CTAB efflux gene of the SMR family on a novel Tn916-like conjugative transposon, Tn6087 (Ciric *et al.*, 2011b).

Antiseptics, including chlorhexidine and QACs such as CTAB and CPC, are used in oral hygiene products including mouthwashes and in the treatment of periodontitis and their use is creating a selection pressure for the maintenance of such resistance genes (Costa *et al.*, 2015; Liu *et al.*, 2016). Antiseptic use may also select for resistance mutations. For

instance, Kulik *et al.* (2015) showed that successive culturing of *P. gingivalis* in sub-MIC concentrations of chlorhexidine *in vitro* generated strains that were 2- to 4-fold more resistant to chlorhexidine than their parent strains, although no characterisation of the resistant isolates was carried out (Kulik *et al.*, 2015).

As mercury is commonly used in dental amalgams, studies on heavy metal resistance in the oral cavity have mainly focused on this metal (Bates, 2006). Cultivable mercury-resistant bacteria have been shown to be prevalent in the oral cavity and variants of the mercury reductase encoding *merA* gene have been identified in oral Gram-negative and *Streptococcus* spp. (Ojo *et al.*, 2004; Stapleton *et al.*, 2004). Efflux and mercury methylation mechanisms of resistance have also been observed (Stapleton *et al.*, 2004; Nygren *et al.*, 2014).

Copper and silver resistance have received less attention, although bacteria resistant to these metals have been cultured from the human oral cavity. For instance, a copper regulated P-type ATPase gene, *copA*, has been identified in a strain of *S. mutans* (Vats and Lee, 2001). In another study, two silver-resistant *Enterobacter cloacae* strains were isolated from teeth and were shown to harbour the *silE* gene, encoding a periplasmic silver binding protein, on a 48.5 Kb plasmid (Gupta *et al.*, 1999; Davis *et al.*, 2005).

Since these metals are used in dental amalgams and as potential oral disinfectant agents they put a selective pressure on oral bacteria to maintain metal resistance (Bharti *et al.*, 2010; Peng *et al.*, 2012).

4.1.2 Antagonism and Antimicrobial Production in the Oral Cavity

Although co-aggregation of cells can lead to interspecies cooperation in the oral cavity, antagonistic relationships have also been described as species compete for nutrients and substrates. Competition has been well studied between different *Streptococcus* spp. as many of these species have similar nutritional requirements and occupy the same oral environments. For instance, *S. gordonii*, *S. sanguinis* and *S. oligofermentans* produce H₂O₂ through the expression of pyruvate, lactate and L-amino acid oxidases (Tong *et al.*, 2007; Kreth *et al.*, 2008; Tong *et al.*, 2008). The production of H₂O₂ by these *Streptococcus* spp. has been shown to inhibit the growth of *S. mutans* (Krzeminski and Raczynska, 1993; Kreth *et al.*, 2005; Kreth *et al.*, 2008; Bao *et al.*, 2015). Clinical studies have shown that there is a negative correlation between the isolation of H₂O₂ producing *Streptococcus* spp. and *S. mutans*, with the former being more abundant in caries-free individuals (Becker *et al.*, 2002; Giacaman *et al.*, 2015). Periodontal pathogens including *P. gingivalis* and *Prevotella intermedia* are also inhibited by the production of H₂O₂ by *Streptococcus* spp. (Herrero *et al.*, 2016).

S. mutans and *Lactobacillus* species are known acidogenic and aciduric bacteria and have been shown to inhibit other oral bacteria including *P. intermedia*, *Aggregatibacter actinomycetemcomitans* and various *Streptococcus* spp. through the conversion of pyruvate to lactic acid (Loesche, 1986; Terai *et al.*, 2015; Kozlovsky *et al.*, 2015).

As LAB (lactic acid-producing bacteria) species, much focus has been put on oral *Streptococcus* species with regards to bacteriocin production. For example, *Streptococcus salivarius* produces the lantibiotics, SalA and SalB (Upton *et al.*, 2001; Barbour *et al.*, 2016).

Mutacin I and IV produced by *S. mutans* are class I and IIb bacteriocins respectively with activity against *S. gordonii* and *S. sanguinis* (Hale *et al.*, 2005; Kreth *et al.*, 2005; Hossain and Biswas, 2011). *Streptococcal* enzymatic bacteriocins have also been isolated from the oral cavity such as the *S. mutans* cell wall hydrolysing zoocin A (Simmonds *et al.*, 1995).

Bacteriocin-like inhibitory substances (BLIS) with activity against *Streptococcus* spp. and periopathogens including *P. gingivalis* and *A. actinomycetemcomitans* have also been purified from oral *Lactobacillus* species (Pangsomboon *et al.*, 2006; Wannun *et al.*, 2016).

LAB microorganisms are not the only bacteriocin producers in the oral cavity. In fact a recent analysis of the HMP dataset revealed the human oral cavity to have a high abundance and diversity of bacteriocin class I, II and III genes and estimates from shotgun metagenomic data suggest that 35 % of enzymatic bacteriocin genes in the oral cavity are of *Actinomyces* and *Prevotella* origin (Zheng *et al.*, 2015).

BLIS have been identified, and/or purified from species of *Prevotella nigrescens*, *Porphyromonas gingivalis*, *Eikenella corrodens* and *F. nucleatum* (Teaupaisan *et al.*, 1998; Kaewsrichan *et al.*, 2004; Apolônio *et al.*, 2007; Ribeiro-Ribas *et al.*, 2009). Genetic characterisation of these BLIS is lacking and further work is required to determine if these antimicrobial peptides are true bacteriocins. More recently a novel antimicrobial peptide with activity against *Prevotella*, *Veillonella* and *Fusobacterium* spp. was identified from a human plaque and saliva metagenomic library. The antimicrobial peptide had greater than 90 % identity to a hypothetical protein of *Neisseria subflava* (Arivaradarajan *et al.*, 2015).

Therefore, studies of the antibiotic resistome using culture based and metagenomic methods have provided information on its composition and they continue to reveal novel resistance determinants. Furthermore, this environment may harbour resistance genes to metals and antimicrobials commonly used in dental therapies and may reveal more novel antimicrobials. As most of the species from oral environments including the saliva have yet-to-be cultured, taking a functional metagenomic approach we can identify genes from the uncultivable oral microbiome.

4.1.3 Antibiotic-Resistant Bacteria and Antibiotic Resistance Genes are Shed in Calf Faeces

ARGs have been detected in faecal matter and in faecal isolates from calves and cattle (Durso *et al.*, 2011; Thames *et al.*, 2012). Additionally, age, health status and diet can affect the resistome of calf faeces. Studies have shown that antibiotic-resistant *E. coli* are shed in higher abundance from calves than from older cattle particularly from herds experiencing diarrhoea (Hoyle *et al.*, 2004; Khachatryan *et al.*, 2004; Khachatryan *et al.*, 2006; de Verdier *et al.*, 2012; Barlow *et al.*, 2015). These antibiotic resistance genes may enter the environment and contaminate food products through the spreading of slurry and during slaughter (European Food Safety Authority and European Centre for Disease Prevention and Control, 2016; Graham *et al.*, 2016).

The clinical use of antibiotics and their presence in waste milk fed to calves has been shown to result in an increase in shedding of antibiotic-resistant *E. coli*, although these changes are typically transient (Kaneene *et al.*, 2008; Thames *et al.*, 2012; Aust *et al.*, 2013). The use of

ceftiofur as a treatment (2.2 mg/Kg daily for five days) in calves and cattle can lead to a transient increase in the shedding of *bla*_{CMY-2} and *bla*_{CTX-M} positive *E. coli* and *bla*_{CMY-2} positive *E. faecium* (Jiang *et al.*, 2006; Boyer and Singer, 2012; Kanwar *et al.*, 2014). It is worrying that following antibiotic use, selection for resistance to related and unrelated antibiotic classes has been demonstrated (Berge *et al.*, 2006). For instance, Bosman *et al.* (2014) found that the administration of tetracyclines, as well as the dose administered (measured as the animal daily dose per production cycle) was associated with increased amoxicillin and tetracycline resistant *E. coli* being identified in veal calf faecal samples (Bosman *et al.*, 2014). Furthermore, reports have shown that treatment of cattle with ceftiofur can result in an increased abundance of *E. coli* harbouring *bla*_{CMY-2} and *bla*_{CTX-M} and *tet*(A) genes (Mann *et al.*, 2011; Kanwar *et al.*, 2013).

Tetracycline resistance genes and resistant *E. coli* are frequently identified from calf and cattle faeces with efflux proteins encoded by *tet*(A) and *tet*(B) are most commonly associated with these (Kanwar *et al.*, 2013; Kanwar *et al.*, 2014; Shin *et al.*, 2015). RPP and enzymatic mechanisms of tetracycline resistance have also been identified in calf faeces (Thames *et al.*, 2012). Using PCR, Santamaría *et al.* (2011) found that *tet*(Q) and *tet*(O) were the most prevalent tetracycline resistance genes in DNA isolated from the faeces of Colombian Andes grass fed cows (Santamaría *et al.*, 2011).

The mobile nature of tetracycline resistance in bovine faeces has been demonstrated phenotypically and genotypically. MDR faecal *E. coli* isolates with reduced susceptibility to tetracycline, sulphonamides and ampicillin/amoxicillin are commonly identified in bovine faeces suggesting a genetic association between these genes (Bosman *et al.*, 2014; Duse *et al.*, 2015). Tetracycline efflux genes have also been found on conjugative plasmids. *tet*(Y)

was identified from cow faeces using an exogenous plasmid isolation method and *tet(A)* and *tet(B)* have been identified in faecal *E. coli* isolates (Shin *et al.*, 2015; Kyselkova *et al.*, 2016). In a study conducted by Shin *et al.* (2015), of 146 tetracycline resistant *E. coli* isolates studied, 121 were able to transfer their resistance gene to another *E. coli* strain, most frequently via *incFIB* plasmids (Shin *et al.*, 2015).

Other than *bla_{CYM-2}*, the study of β -lactam resistance in bovine faeces has mainly focused on the *bla_{CTX-M}* genes and *bla_{CTX-M}* positive *E. coli* due to their association with food products and infection (Ahren *et al.*, 2010; Overdevest *et al.*, 2011; Calbo *et al.*, 2011; Egervarn *et al.*, 2014). CTX-M positive *E. coli* are prevalent among dairy and beef cattle farms in many countries and *bla_{CTX-M}* is the predominant genes in food animals in Europe (Horton *et al.*, 2011; Liebana *et al.*, 2013; Diab *et al.*, 2016). For instance, Schmid *et al.* (2015) found that of the 45 farms in their study CTX-M positive *E. coli* could be found in 39. This study also showed that shedding of CTX-M positive *E. coli* was more prevalent in calves which was in agreement with earlier studies (Schmid *et al.*, 2013; Hordijk *et al.*, 2013).

The association of CTX-M genes with various conjugative plasmids including *IncN*, *IncI1* and *IncF* types likely contributes to their prevalence (Zheng *et al.*, 2012; Cottell *et al.*, 2013). *bla_{CTX-M}* has also been isolated from bacteriophages present in cattle faeces, as has the *mecA* gene, suggesting that these vectors play a role in HGT of β -lactam resistance genes (Colomer-Lluch *et al.*, 2011). Other β -lactamase genes including *bla_{TEM}*, *bla_{SHV}* and *bla_{OXA}* have been observed in bovine faeces and are found in Gram-negative pathogens including *E. coli* and *Salmonella* species that may be present in faeces (Ahmed *et al.*, 2009; Ibrahim *et al.*, 2016).

Although studies of antibiotic resistance in bovine faeces typically focus on antibiotic resistance of *E. coli* it is likely that this approach does not tell the full story. Kanwar *et al.* (2013) showed that a single dose of ceftiofur administered to cattle resulted in an increase in ceftiofur-resistant *E. coli* shed in the faeces. This study also revealed that ceftiofur resistance gene, *bla*_{CMY-2}, is co-selected with tetracycline resistance in *E. coli*, via *tetA* expression, as tetracycline resistant *E. coli* strains were also more abundant following ceftiofur administration (Kanwar *et al.*, 2013). In fact, subsequent treatment with chlortetracycline was able to maintain this increased abundance of ceftiofur-resistant *E. coli*. However, in a later study using quantitative PCR to monitor *tetA* and *tetB* genes in the faeces of calves receiving ceftiofur a reduction in their abundance was observed, likely as a result of non-*E. coli* bacteria harbouring *tetA* and *tetB* being ceftiofur susceptible (Kanwar *et al.*, 2014). This example shows the importance of looking at the total faecal microbiome when conducting these resistance studies.

As well as identifying novel resistance genes and generating information on resistance from the uncultivable microbiome, metagenomics can give an insight into how antimicrobial use can select for antibiotic resistance genes. A recent metagenomic survey conducted by Chambers *et al.* (2015) showed that following a single ceftiofur injection, dairy cow faeces contained a higher number of gene sequences related to HGT including phages, plasmids and transposable elements (Chambers *et al.*, 2015). This increase is a result of a selection for bacteria harbouring antibiotic resistance genes, including those on mobile elements, and it indicates the importance of such mobile elements in the transmission of antibiotic resistance genes in calf faeces.

4.2 Chapter Aims

The aim of the work presented in this chapter was to screen the human saliva metagenomic library for antibiotic and antiseptic resistance, and antimicrobial production, as well as to screen the calf faecal metagenomic library for antibiotic resistance.

4.3 Methods and Materials

4.3.1 Minimum Inhibitory Concentration Determination

4.3.1.1 Antibiotic MICs for *E. coli* EPI300 T1^R::pCC1BAC

The susceptibility of *E. coli* EPI300 T1^R::pCC1BAC to tetracycline and ampicillin was determined by broth microdilution as described in 2.5.1. The concentrations of tetracycline and ampicillin tested were 0.25 – 32 µg/ml and 2 – 64 µg/ml respectively.

4.3.1.2 Susceptibility of *E. coli* EPI300 T1^R::pCC1BAC to Antimicrobials

The susceptibility of *E. coli* EPI300 T1^R::pCC1BAC to copper, silver, CTAB, CPC and chlorhexidine was determined using the broth microdilution as described in 2.5.1. The concentration ranges tested for copper, silver, CTAB, CPC and chlorhexidine were 0.28 – 2.25 mg/ml, 3.6 – 36 µg/ml, 0.365 – 14.58 µg/ml, 0.45 – 9 µg/ml and 0.1125 – 9 µg/ml respectively. Copper sulfate and silver nitrate salts were used to make stock solutions, however the concentrations listed above refer to the metal ion concentration and not the concentration of the metal salt.

4.3.2 Functional Screening of the Metagenomic Libraries

4.3.2.1 Human Saliva Metagenomic Library Antibiotic Resistance Screen

To screen the human saliva metagenomic library for tetracycline- and ampicillin-resistant clones, the library was cultured on LB agar supplemented with 12.5 µg/ml chloramphenicol and either 5 µg/ml tetracycline or 50 µg/ml ampicillin. These antibiotic concentrations represented 2.5 times the MIC of tetracycline (2 µg/ml) and ampicillin (20 µg/ml) for *E. coli* EPI300 T1^R::pCC1BAC. Clones from this library were gridded, 96 at a time, from the wells of a 96-well plate onto the surface of the agar (in 140 mm diameter petri dishes) using a 96-pin replica plater, which was sterilised with 70% EtOH between 96-well plates. The clones were incubated for 16 h at 37 °C. Plates were then checked for any clones that may have grown. If any clones exhibited a resistance phenotype they were subcultured in LB broth and agar containing chloramphenicol with and without the selecting antibiotic to determine if the clone was indeed showing a resistance phenotype. The plates containing 96 gridded clones were incubated for a further 24 h at 37 °C and analysed the following day in case the clones harboured large inserts or produced components that slowed the growth of the clone.

4.3.2.2 Human Saliva Metagenomic Library Antimicrobial Resistance Screen

A small fraction of the human saliva metagenomic library (1,248 clones) was screened for clones that were resistant to a number of antimicrobials. Clones were picked from 96-well plates using a 96-pin hedgehog, inoculated in 100 µl LB broth supplemented with

chloramphenicol and incubated for 16 h at 37 °C with shaking at 200 RPM. Ten µl of each 16 h culture was then used to inoculate 90 µl of LB broth supplemented with chloramphenicol (12.5 µg/ml) and copper, silver, CTAB, CPC or chlorhexidine at final concentrations of 1.72 mg/ml, 18 µg/ml, 3.7 µg/ml, 3.6 µg/ml and 1.8 µg/ml, respectively. The plates were then incubated for 16 h at 37°C with shaking at 200 RPM. Growth was determined by spectrophotometry by measuring the OD₆₀₀.

4.3.2.3 Human Saliva Metagenomic Library Antimicrobial Production Screen

The human saliva metagenomic library was screened for the presence of clones that could inhibit the growth of *Bacillus subtilis* CU2189 using a method described previously by Arivaradarajan *et al.* (Arivaradarajan *et al.*, 2015). The human saliva library was gridded onto LB agar containing chloramphenicol (12.5 µg/ml) and incubated overnight at 37 °C for 16 h. The clones were then incubated for a further 48 h at RT. Ten µl of a *B. subtilis* CU2189 16 h culture was sub-cultured in 10 ml of fresh BHI and incubated at 37 °C with shaking at 200 RPM until an OD₆₀₀ of 0.3 was reached. This subculture was diluted 1 in 10 using 0.75% BHI agar and poured over the gridded library. The plates were incubated for 16 h at 37 °C followed by 48 h at RT in the dark. The plates were examined daily for zones of inhibition in the *B. subtilis* overlay.

The library was also screened for clones producing antimicrobials against a sensitive strain of *Micrococcus luteus*. An overnight culture of *M. luteus* was diluted to an OD₆₀₀ of 0.15 or 0.24 in sterile PBS (0.1M, pH 7.4) and spread on to the surface of BHI agar using a sterile

swab. Clones from the human saliva metagenomic library were then gridded onto the agar and the plates were incubated for 16 h at 37 °C followed by two days at RT. The plates were checked daily for any zones of inhibition in the *M. luteus* lawn.

4.3.2.4 Screening of the Calf Faecal Library

After electroporation of the calf faecal metagenomic DNA pCC1BAC ligation reaction into *E. coli* EPI300 T1^R cells were recovered in 2.95 ml of SOC (NEB) by incubating at 37 °C with shaking at 200 RPM for 3 h. The recovered cells were then plated as follows; 10 X 100 µl on LB agar supplemented with chloramphenicol, IPTG (0.1 mM) and X-gal (40 µg/ml); 10 X 100 µl on LB agar supplemented with chloramphenicol and tetracycline (5 µg/ml); and 10 X 100 µl on LB agar supplemented with chloramphenicol and ampicillin (20 µg/ml). Plates were incubated for 16 h at 37 °C. Following incubation any clones found to be growing on tetracycline or ampicillin containing plates were noted and the plates were incubated for a further 24 h at 37 °C before checking them again.

4.3.3 PCR Screen to Identify β -lactam Resistance Genes from the Human Saliva and Calf Faecal Metagenomes

The human saliva and calf faecal metagenomic DNA were used as templates for PCR reactions to identify if ampicillin resistance genes were present in these preparations. PCRs to amplify *bla*_{TEM} (using RH605/606 primers), *bla*_{ROB}, *bla*_{OXA}, *bla*_{SHV} and *bla*_{CTX-M} were

performed using MyTaq™ Red Mix as described in 2.3.1. Primer information is included in Table 4.2.

Table 4.2 Primers for Clone Sequencing and Amplification of β -lactamase Genes.

Name	Sequence (5'-3')	Purpose	Source/Reference
pCC1-F	GGATGTGCTGCAAGGCGATTAAGTTGG	End sequencing of pCC1BAC	Epicentre®
pCC1-R	CTCGTATGTTGTGTGGAATTGTGAGC	End Sequencing of pCC1BAC	Epicentre®
RH605	TTTCGTGTCGCCCTTATTCC	692 bp amplicon from <i>bla</i> _{TEM}	(Bailey <i>et al.</i> , 2010)
RH606	CCGGCTCCAGATTTATCAGC	692 bp amplicon from <i>bla</i> _{TEM}	(Bailey <i>et al.</i> , 2010)
Bla_ROBF	ATCAGCCACACAAGCCACCT	692 bp amplicon from <i>bla</i> _{ROB}	(Tenover <i>et al.</i> , 1994)
Bla_ROBR	GTTTGCGATTTGGTATGCGA	692 bp amplicon from <i>bla</i> _{ROB}	(Tenover <i>et al.</i> , 1994)
Bla_OXAF	TTCAAGCCAAAGGCACGATAG	702 bp amplicon from <i>bla</i> _{OXA}	(Briñas <i>et al.</i> , 2002)
Bla_OXAR	TCCGAGTTGACTGCCGGGTTG	702 bp amplicon from <i>bla</i> _{OXA}	(Briñas <i>et al.</i> , 2002)
Bla_SHVF	CACTCAAGGATGTATTGTG	885 bp amplicon from <i>bla</i> _{SHV}	(Briñas <i>et al.</i> , 2002)
Bla_SHVR	TTAGCGTTGCCAGTGCTCG	885 bp amplicon from <i>bla</i> _{SHV}	(Briñas <i>et al.</i> , 2002)
Core CTX-MF	AACCGTCACGCTGTTGTTAG	766 bp amplicon from <i>bla</i> _{CTX-M}	(Chen <i>et al.</i> , 2004)
Core CTX-MF	TTGAGGCGTGGTGAAGTAAG	766 bp amplicon from <i>bla</i> _{CTX-M}	(Chen <i>et al.</i> , 2004)

4.4 Results

4.4.1 Minimum Inhibitory Concentrations for Functional Screens

In order to design appropriate functional screens to identify antibiotic-resistant clones and clones with reduced susceptibility to antimicrobials from the metagenomic libraries, MIC and susceptibility assays were conducted to determine the sensitivity of *E. coli* EPI300 T1^R and *E. coli* EPI300 T1^R::pCC1BAC to a number of antibiotics and antimicrobials. The MICs of the antibiotics, metals and antimicrobials tested against *E. coli* EPI300 T1^R::pCC1BAC are listed in Table 4.3.

Table 4.3 MICs of Antibiotic and Antimicrobials for *E. coli* EPI300 T1^R::pCC1BAC.

Antibiotic/Antiseptic/ Metal	Concentration ($\mu\text{g/ml}$)
Tetracycline	2 $\mu\text{g/ml}$
Ampicillin	20 $\mu\text{g/ml}$
Silver*	18 $\mu\text{g/ml}$
Copper*	1.7 $\mu\text{g/ml}$
CTAB	3.7 $\mu\text{g/ml}$
CPC	3.6 $\mu\text{g/ml}$
Chlorhexidine	1.8 $\mu\text{g/ml}$

* Values refer to the metal concentration only and not to the concentration of the salt of the metal.

Having shown that *E. coli* EPI300 T1^R and *E. coli* EPI300 T1^R::pCC1BAC were susceptible to the above antimicrobials, functional screens could be devised and carried out.

4.4.2 Results of Functional Screening

4.4.2.1 Screening of Human Saliva Metagenomic Library

Two tetracycline resistant clones were identified when the 27,000 clones from the human saliva metagenomic library were screened. No ampicillin resistant clones were identified from the screening of the library. 1,248 clones from the library were also screened for susceptibility to CTAB, CPC, chlorhexidine, copper and silver, and a single clone with decreased susceptibility to CTAB in comparison to EPI300 T1^R::pCC1BAC was identified. No clones were isolated that could inhibit growth of *B. subtilis* CU2189 or *M. luteus*. The clones of interest identified from the screening of the human saliva metagenomic library are included in Table 4.4.

Table 4.4 Clones Identified from the Human Saliva Metagenomic Library

Clone	Phenotype	Discussed in Chapter
PS9	Tetracycline Resistance	5
TT31	Tetracycline Resistance	6
A10(F2)	Reduced CTAB Susceptibility	7

4.4.2.2 Screening of Calf Faecal Metagenomic Library

None of the 2,840 clones from the calf faecal metagenomic library screened for resistance to tetracycline and ampicillin were found to be resistant.

4.4.3 PCR Screen to Identify β -lactamase genes

As no ampicillin resistant clones were identified from the human saliva or calf faeces metagenomic libraries we next used PCR to determine if β -lactam resistance genes were present in our metagenomic DNA preparations. PCR products were obtained from both the human saliva and calf faecal metagenomic DNA samples using the RH605/606 primers. These primers amplified a 692 bp region of *bla*_{TEM} as expected. Figure 4.1(a). The core CTX-M primer pair amplifies a 766 bp region of *bla*_{CTX-M}. However, using these primers resulted in the amplification of 500 bp products from both the human saliva and calf faecal metagenomic samples, Figure 4.1(b)

Figure 4.1

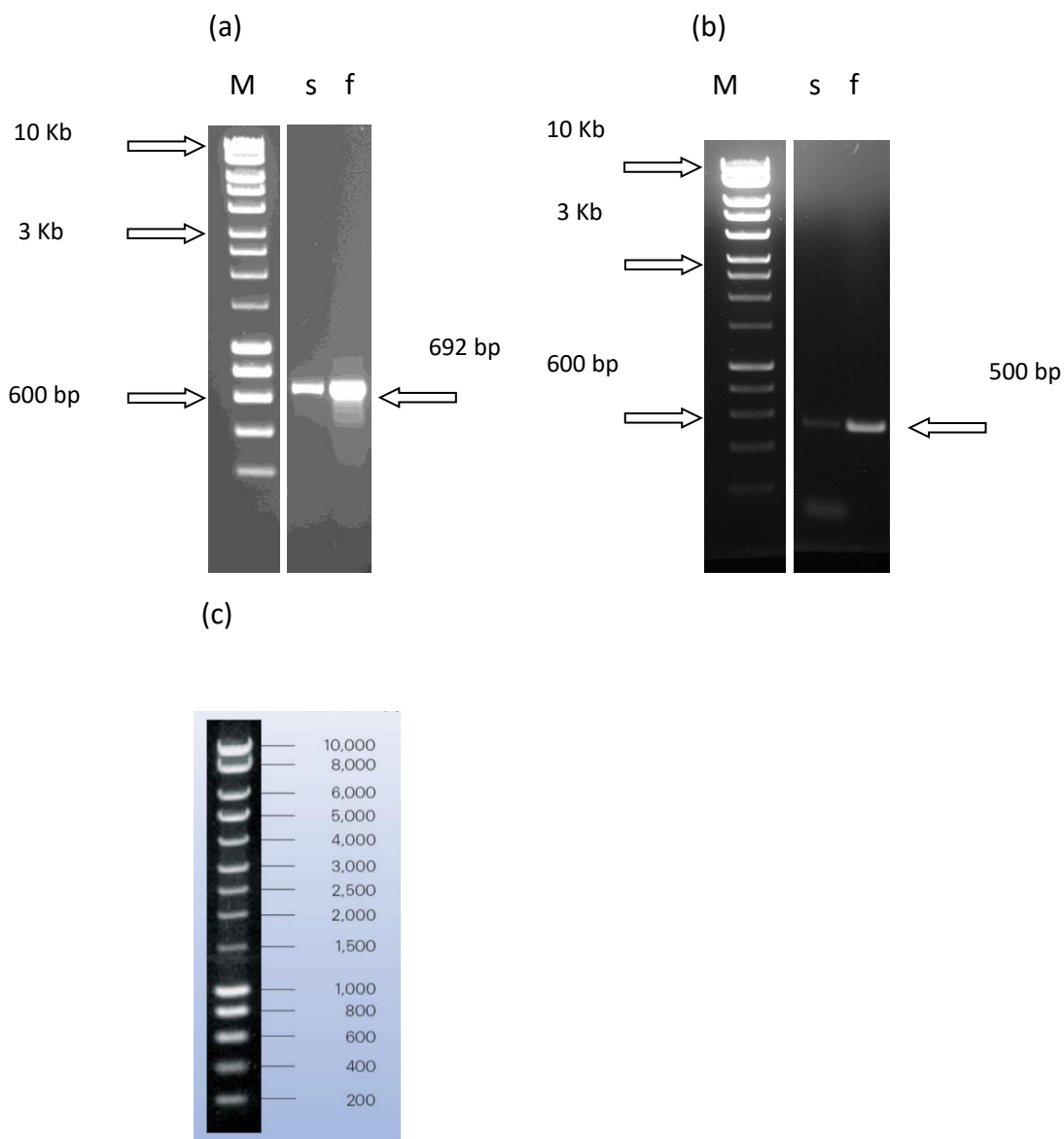


Figure 4.1 *bla*_{TEM} and *bla*_{CTX-M} Amplicons in Human Saliva and Calf Faecal Metagenomic DNA Extractions. The lanes marked M contain Hyper Ladder I; the lanes marked 's' indicate that human saliva metagenomic DNA was used as a template and lanes marked 'f' indicate calf faecal metagenomic DNA was used as a template. (a) Amplicons of the expected size were detected from both the saliva and faecal preps using the *bla*_{TEM} amplifying RH605/606 primers. (b) A smaller than expected PCR product was obtained from both samples using the *bla*_{CTX-M} primer pairs. (c) An image depicting the Bioline Hyper Ladder I with size labels.

Sequencing and subsequent BLASTN analysis of the 596 bp and 602 bp of the RH605/606 amplicons from saliva and faeces, respectively, revealed them to be identical to *bla*_{TEM} genes from a number of bacterial species, Table 4.5.

Sequencing of the human saliva *bla*_{CTX-M} PCR product resulted in two non-overlapping sequences (166 bp and 177 bp) that had 81-82 % identity to human DNA. Sequencing of the calf faecal *bla*_{CTX-M} amplicon also resulted in two non-overlapping sequences (167 bp and 174 bp) that had no sequence similarity to anything in the NCBI data base according to BLASTN. BLASTX, however, indicated that the putative product of the 174 bp sequence had 58 – 78 % identity to CTX-M proteins, Table 4.5. A number of conditions were used in an attempt to amplify PCR products from the human saliva and calf faecal metagenomic DNA preparations using the *bla*_{ROB}, *bla*_{OXA} and *bla*_{SHV} primer pairs; however, the various conditions used resulted in no products, multiple products or products of the incorrect size.

Table 4.5 BLASTN and BLASTX Hits for β -lactamase PCR Amplicons.

Sample	Primer	Length of Amplicon Sequenced	BLASTN	BLASTX
Saliva	RH605/606	596 bp sequence	Multiple* <i>bla</i> _{TEM} hits (100 % cover/ID)	Multiple** β -lactamases TEM-1 and KPC Hits (99 % cover/ 100 % ID)
	Bla_CTX-MF	166 bp sequence	<i>Homo sapiens</i> (82 % cover / 95 % ID)	No Hits
	Bla_CTX-MR	177 bp sequence	<i>Homo sapiens</i> (81 % cover / 99 % ID)	No Hits
Faeces	RH605/606	602 bp sequence	<i>E. coli bla</i> _{TEM} and multiple plasmids (100 % cover/ ID)	Multiple*** β -lactamase TEM-1 Hits (99 % cover / 100 % ID)
	Bla_CTX-MF	167 bp sequence	No Hit	No Hit
	Bla_CTX-MR	174 bp sequence	No Hit	CTX-M <i>E. coli</i> (55 % cover/ 78 % ID) and CTX-M uncultured bacterium (77 % cover/ 58 % ID)

**E. coli*, *Enterobacter* spp. and *Klebsiella* spp. ** *E. coli* and *Klebsiella* spp. and KPC from *Serratia marcescens*
 ****S. marcescens*, *E. coli*, *Burkholderia*, *Pseudomonas*, *Klebsiella* and *Citrobacter* spp.

4.5 Discussion

Using the EUCAST clinical breakpoints, *E. coli* EPI300 T1^R::pCC1BAC was sensitive to both tetracycline and ampicillin as their MICs were found to be 2 µg/ml and 20 µg/ml respectively (EUCAST, 2015). Since the strain was sensitive to both antibiotics it could be used as a host strain to identify cloned, functional tetracycline and ampicillin resistance genes in a functional metagenomic screen. In order to identify genes from the human saliva and calf faecal metagenomes conferring high-level resistance to tetracycline and ampicillin, screening concentrations of 5 µg/ml and 50 µg/ml, respectively were used. These concentrations have been used in previous functional screening projects to identify tetracycline and ampicillin resistance clones from metagenomic libraries (Diaz-Torres *et al.*, 2003; Fouhy *et al.*, 2014). Screening of the human saliva metagenomic library resulted in the identification of two tetracycline-resistant clones. No ampicillin resistant clones were identified. Screening of a subset of the library (1,248 clones) identified a clone with reduced susceptibility to CTAB and no clones resistant to CPC, chlorhexidine, silver or copper. No clones producing antimicrobials against *B. subtilis* or *M. luteus* were identified.

As described in chapter 3, the high number of clones containing either human DNA or no insert may partly explain why only a small number of clones were identified from the screening of the human saliva metagenomic library. It is possible that more genes of interest were cloned and thus present in the library but that they were not expressed during screening, indeed successful heterologous expression is one of the biggest hurdles of a functional metagenomic screen. Expression of genes within the screened clones was controlled by their native promoters, as IPTG induction was not employed in any of the

screening protocols. Thus, culturing on the nutrient-rich conditions of LB or BHI, under pH neutral conditions at 37 °C, may not provide the optimum conditions for the expression of all antimicrobial resistance and production genes. Conditions of stress including nutrient availability and pH have been shown to alter resistance phenotypes. For example, Kubicek-Sutherland *et al.* (2015) showed that MICs for colistin and ciprofloxacin were increased by more than 256- and 8-fold respectively in *S. enterica* serovar Typhimurium when it was cultured in low phosphate media at pH 5.5 compared with when it was grown in non-limiting phosphate conditions at pH 7.2 (Kubicek-Sutherland *et al.*, 2015).

Furthermore, differences in promoter site recognition and codon usage, the presence of transcription terminators as well as differing translational and post translational processing and export pathways between the host and source organism may effect gene expression and thus the success of the screen (Gabor *et al.*, 2004; Kudla *et al.*, 2009; Terrón-González *et al.*, 2013). Biver *et al.* (2013) screened a forest soil metagenomic library, created using a shuttle vector capable of replicating in an *E. coli* and *B. subtilis* host. A *B. subtilis* clone with antimicrobial activity was identified, however when its plasmid was cloned into *E. coli* no activity was observed (Biver *et al.*, 2013). Other hosts including *Streptomyces lividans*, *Burkholderia graminis* and *Pseudomonas putida* have also been used to create metagenomic libraries (Martinez *et al.*, 2004; Craig *et al.*, 2010; McMahon *et al.*, 2012). The bacteriocin exporter gene is not always located adjacent to the structural gene as is the case with mutacins where a single transporter, NImTE, is responsible for exporting all mutacins produced by *S. mutans* (Hale *et al.*, 2005). Thus, it is possible no bacteriocin producers were found from this library because a structural bacteriocin gene was cloned without its transporter. In fact, bacteriocin genes such as those involved in salivaricin A production have been identified in isolates of *Streptococcus pyogenes* that do not or cannot express them

(Wescombe *et al.*, 2006). The expression of some antimicrobials from oral bacteria species is controlled by different environmental conditions. For instance, the production of bacteriocins such as the mutacins is controlled by aeration and cell density (Kreth *et al.*, 2007). The production of hydrogen peroxide by mitis group *Streptococcus* spp. is also increased by aeration (Herrero *et al.*, 2016).

Additionally, Kreth *et al.* (2005) demonstrated that the sequence of inoculation can alter the outcome of an antimicrobial screen as *S. sanguinis* was shown to inhibit *S. mutans* only when it was inoculated first (Kreth *et al.*, 2005). In our anti-*B. subtilis* screen, clones were cultured on agar for 64 h before overlaying with *B. subtilis* while our anti-*M. luteus* screen involved inoculating the clones on a plate swabbed with this indicator strain; culturing them at the same time. These differences in methods may have had an effect on the outcome of our functional screens. The choice of indicator strain and host can also impact on the results of a metagenomic functional screen. For instance, an antimicrobial may be produced and secreted by a clone but the indicator strain is not sensitive and so the clone is not identified. On the other hand, if after cloning the host strain is sensitive to an antimicrobial it now produces it won't survive and so won't be screened. Thus, a single functional screen will not accommodate every condition necessary for the identification of every antimicrobial producing clone in a metagenomic library. As we have stored the human saliva metagenomic library, future screening using different media, growth conditions and indicator organisms can be conducted. In fact, following my departure from the Eastman Dental Institute, further functional screening of the human saliva metagenomic library identified four clones with alpha haemolytic activity. This demonstrates that clones capable of causing cell lysis are present in the library. These clones were shown to contain genes encoding pyruvate oxidases that produce hydrogen peroxide. As discussed above, hydrogen

peroxide can also inhibit bacterial cells, although our screen failed to identify these hydrogen peroxide-producing clones. It would be of interest to determine if our indicator strains have reduced susceptibility to hydrogen peroxide, by producing a catalase for example, or if using alternative screening protocols, such as those described by Kreth *et al.* (2005), with these indicator organisms would cause them to be inhibited by these alpha haemolytic clones (Baureder *et al.*, 2012; Kreth *et al.*, 2005).

The higher-than-MIC concentrations of tetracycline and ampicillin used in these screens may have resulted in genes conferring lower level resistance to *E. coli* EPI300 T1^R not being identified. This is unlikely to have been an issue during the antiseptic and metal functional screens as the lowest concentrations required for inhibition of *E. coli* EPI300 T1^R were used. Culturing the library clones on minimum inhibitory concentrations of ampicillin or tetracycline may identify clones with lower levels of resistance that were missed in the initial screen.

No clones of interest were identified from the calf faecal metagenomic library. As mentioned with regard to the human saliva metagenomic library, heterologous expression as well as conditions of the screen may have prevented the identification of genes of interest that were present in the library. However, the library was also limited by its size and diversity. In terms of the number of clones screened, the calf faecal metagenomic library was almost 10-fold smaller than the human saliva library (2, 840 vs 27, 000). Additionally, the library lacked diversity, as the source 'metagenomic DNA' was comprised mainly of *E. coli* DNA according to 16S analysis discussed in chapter 3, which likely contributed to the lack of clones of interest identified from this library. Although *E. coli* are often used to determine resistance levels in calf faeces, resistome studies have shown that they make up

only a small proportion of the faecal microbiota and not all isolates exhibit resistance to tetracycline and ampicillin (Uyeno *et al.*, 2010; Meale *et al.*, 2016).

No ampicillin-resistant clones were identified from the screening of either library so the metagenomic DNA from both environments was screened using PCR to identify β -lactam resistance genes. *bla*_{TEM}, *bla*_{CTX-M}, *bla*_{SHV}, *bla*_{ROB} and *bla*_{OXA} primers were used because they have been associated with oral microbiota and they represent some of the most common ESBLs associated with *Enterobacteriaceae* including *E. coli* which made up the vast majority of the calf faecal 'metagenomic' DNA extractions (Voha *et al.*, 2006; Ramos *et al.*, 2009; Ahmed *et al.*, 2009; Ibrahim *et al.*, 2016). *bla*_{TEM} genes were identified from both the human saliva and calf faecal metagenomic preps using both primer sets which is in accordance with previous studies. *bla*_{CTX-M} PCR products were identified from both samples, although they were smaller than expected. The product identified from human saliva had low sequence identity to human DNA indicating that non-specific binding occurred with contaminating human DNA. The *bla*_{CTX-M} PCR product was also smaller than expected and had no sequencing similarity to anything in the NCBI database at the nucleotide level; although the reverse PCR product had similarity to CTX-M proteins from *E. coli* and uncultured bacteria according to BLASTX. This primer pair has previously been used to identify *bla*_{CTX-M} bacterial isolates and so it may be that they are not suitable for identifying *bla*_{CTX-M} genes from more complexed DNA extractions. Although multiple reaction conditions were used *bla*_{SHV}, *bla*_{ROB} and *bla*_{OXA} gave either no products or non-specific products. The inconclusive PCR results does not mean these genes were not present as these genes have been identified from commensal bacteria and DNA preparations from oral and faecal/GI environments.

Although screening of the calf faecal metagenomic library did not result in the identification of clones of interest, the calf faecal DNA preparation may harbour novel β -lactamase genes that we failed to clone or express in our functional screens. Screening of the human saliva metagenomic library resulted in the identification of novel tetracycline resistance genes. Additionally, the screening of a small fraction of this library for metal and antimicrobial resistance identified a CTAB resistant clone.

Screening the remainder of the library as well as screening for different phenotypes may yet identify more novel genes from both the human saliva metagenome. Additionally, transformation of the remaining ligation reaction and conducting further ligation reactions would allow us to expand the human saliva metagenomic library and identify more clones of interest. However, as we identified three clones of interest from our functional screens we decided to characterise these clones further rather than continuing to screen the library.

Chapter 5

A Novel Tetracycline and Tigecycline ABC Transporter is Encoded by PS9

PS9 was identified when clones from the human saliva metagenomic library were grown on 5 µg/ml tetracycline.

5.1 Introduction

5.1.1 ATP Binding Cassette Transporters

ATP Binding Cassette (ABC) transporters are a large superfamily of conserved transporters that play a key role in cell metabolism in all kingdoms of life. As primary transporters, they utilise the hydrolysis of ATP to power the movement of a wide range of molecules including proteins, drugs, metals and ions across cell membranes (Davidson *et al.*, 2008; Wilkens, 2015). The importance of these proteins is illustrated by the fact that mutations in them can lead to disease in humans; adrenoleukodystrophy is caused by mutations in the ABCD1 gene, and that cell death in bacteria can result from their deletion; the loss of MsbA results in a lethal build-up of lipid A in *E. coli* (Kemp *et al.*, 2001; Wiesinger *et al.*, 2015). ABC transporters are so important to *E. coli* that 5 % of its genome encodes for ABC transporter related components (Karow and Georgopoulos, 1993; Linton and Higgins, 1998). Examples of the various functions attributed to different ABC transporters is provided in Table 5.1.

Table 5.1 ABC Transporter Functions.

Function	ABC Transporter	Reference
Lipid Export (Flippase)	MsbA (<i>E. coli</i> ; Lipid A)	(Oram, 2003; Doshi and van Veen, 2013)
Ion Export	ABCA1 (Human; cholesterol)	(Hwang and Kirk, 2013)
Vitamin Import	CFTR (Human; Chloride ions)	(Borths <i>et al.</i> , 2002)
Nucleoside/Nucleotide Export	BtuCD (<i>E. coli</i> ; Vitamin B ₁₂)	(Adema <i>et al.</i> , 2014)
Sugar Transport	MRP4 and MRP5 (Human)	(Kemner <i>et al.</i> , 1997; Ehrmann <i>et al.</i> , 1998)
Amino acid Import	MalEFGK ₂ (<i>E. coli</i> ; Maltose Import) ChvE-GguAB (<i>A. tumefaciens</i> ; Glucose and Galactose import)	(Obis <i>et al.</i> , 1999; Caldara <i>et al.</i> , 2007)
Metal Export	HisQMP ₂ (<i>E. coli</i> ; Histidine and Arginine Import) BusA (<i>L. lactis</i> ; Proline and Glycine Import)	(Achard-Joris <i>et al.</i> , 2005; Ichikawa <i>et al.</i> , 2012)
Iron (Siderophore and Haem) Uptake	OmrA (<i>Oenococcus oeni</i> ; Cadmium Export) ABCB8(Mouse Mitochondria; Iron Export)	(Cheung <i>et al.</i> , 2009; Grigg <i>et al.</i> , 2010)
Bacteriocin Export	SirABC (<i>S. aureus</i> ; staphyloferrin B import) IsdEF (<i>S. aureus</i> ; Haem Transfer)	(Collins <i>et al.</i> , 2010; Biswas and Biswas, 2013)
Drug Export	SmbFT (<i>Streptococcus</i> spp.; Smb lantibiotic export) AnaRB (<i>L. monocytogenes</i> ; nisin export)	(Loo <i>et al.</i> , 2003)

5.1.2 Structure of ABC Transporters

ABC transporters have two nucleotide binding domains (NBDs) and two transmembrane domain (TMDs) with each TMD being linked to one of the NBDs by a coupling helix, Figure 5.1 (Rees *et al.*, 2009; Wilkens 2015). The side of the membrane where the NBD resides is called the cis-side and the side of membrane where the TMD emerges is termed the trans-side. ABC exporters move ligands from the cis-side of the membrane to the trans-side (Ter Beek *et al.*, 2014).

Figure 5.1

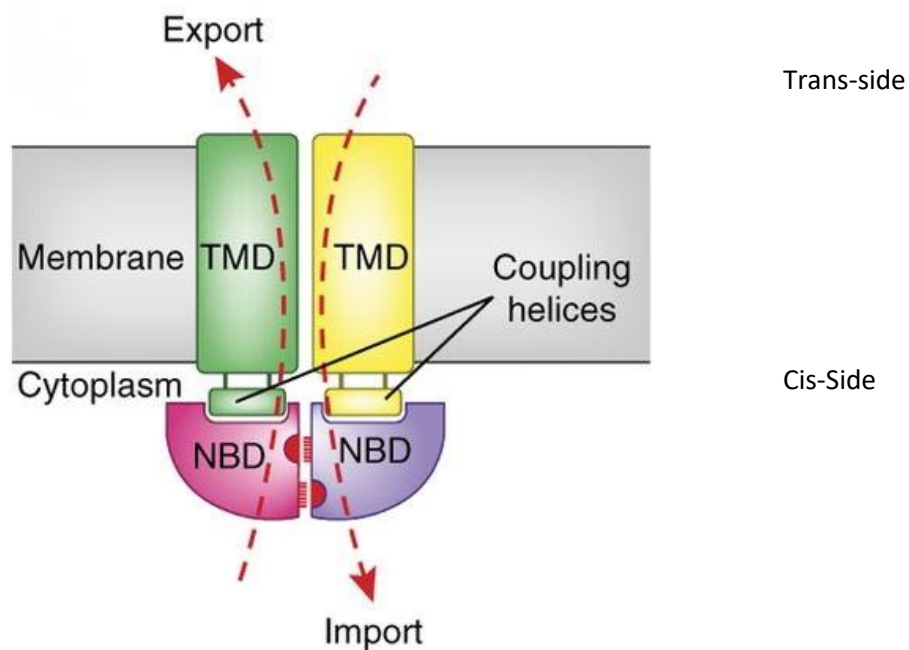


Figure 5.1 Diagram of an ABC Transporter. An illustration of the structure of an ATP Binding Cassette (ABC) transporter spanning the cytoplasmic membrane with the Trans- and Cis-sides of the cytoplasmic membrane highlighted. The transmembrane domains (TMDs) are highlighted in yellow and green with their coupling helices interacting with the nucleotide binding domains (NBDs) of the transporter, highlighted in pink and purple. Reproduced from Locher *et al.* (Locher, 2016).

ABC transporters can be composed of a single peptide, two half transporters (identical or non-identical) or four peptides. Half transporter peptides may contain two TMDs, two NBDs or a single TMD and NBD. Homodimeric transporters are formed when the two identical half transporters interact. Heterodimeric transporters are composed of two non-identical half transporters (Linton and Higgins, 1998).

5.1.2.1 Nucleotide Binding Domains

Among ABC transporters the NBDs are highly conserved and their amino acid sequences can be used as identifying features (Linton, 2007; Davidson *et al.*, 2008; Ter Beek *et al.*, 2014).

The NBD contains two domains; the RecA and helical domains, and seven conserved motifs, Figure 5.2.

Figure 5.2

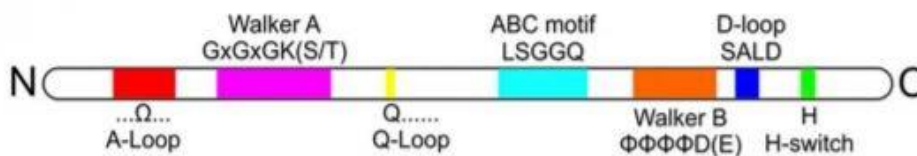


Figure 5.2 The Motifs of the ABC Transporter NBD. An illustration of the seven motifs and their relative positions in the NBD of ABC transporters. The Walker A and Walker B motifs and the H-switch (H-loop) that make up the RecA fold are highlighted in purple, orange and green respectively. The ABC motif present in the helical domain of ABC transporter NBDs is highlighted in blue. This figure was reproduced from Ter Beek *et al.* (Ter Beek *et al.*, 2014).

RecA folds are ATP binding domains found in a number of proteins that utilise the hydrolysis of ATP to function, including ABC transporters (Smith *et al.*, 2002). The RecA domain contains the Walker A and B motifs and the H-loop, Figure 5.3.

Walker A motifs are ATP binding motifs and have the following sequence: GXXGXGKT/S (where X is any residue) (Ramakrishnan *et al.*, 2002). X-ray crystallography studies have

shown that the backbone amino groups of the conserved lysine (K) residue positions ATP via hydrogen bonds with the oxygen atoms of the β and γ phosphates of ATP (Verdon *et al.*, 2003; Hohl *et al.*, 2012), Figure 5.3.

The Walker B motif has the amino acid sequence $\phi\phi\phi\phi$ DE, where ϕ is a hydrophobic residue. It is involved in the coordination of a divalent magnesium cation via a conserved aspartate residue (Schneider and Hunke, 1998; Ter Beek *et al.*, 2014). Magnesium coordinates the phosphate groups of ATP within the catalytic site (Frick *et al.*, 2007). A conserved acidic residue, which is usually a glutamate, acts as a general base to polarise the water molecule that will take part in the ATP hydrolysis. Indeed, the crystal structure of the *E. coli* MalGFK transporter bound to 5'-(β , γ -imido) triphosphate (AMP-PNP), an ATP analogue, shows the Walker B glutamate forming a hydrogen bond with the catalytic water molecule (Sauna *et al.*, 2002; Oldham and Chen, 2011), Figure 5.3.

The H-loop is a conserved histidine residue that interacts with the acidic residue of the Walker B motif, the polarised water molecule, the magnesium cation and the γ -phosphate, contributing to their positioning within the catalytic site of the NBD (Zaitseva *et al.*, 2005).

Outside the RecA fold lies the A-, D- and Q-loops. The A-loop is an aromatic residue, often a tyrosine. The aromatic rings of this residue interact with the adenine ring of ATP via pi stacking and aid in the placement of it in the NBD (Kim *et al.*, 2006), Figure 5.3.

Figure 5.3

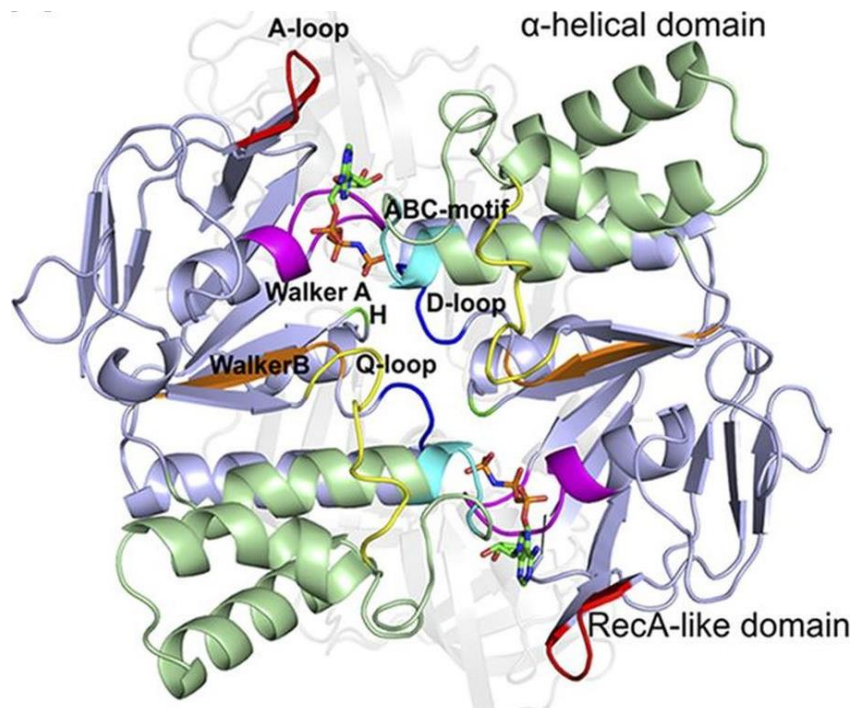


Figure 5.3 Structure of the MalK NBD Dimer of the MalEFGK₂ ABC Transporter. The RecA domains are highlighted in light blue and the Signature motifs in green. The bound 5'-(β, γ-imido) triphosphate (AMP-PNP) molecules are indicated as sticks. The interactions of the A-loop (red), Walker A motif (purple), Walker B motif (orange), D-loop (Blue), H-loop (green), the ABC motif (cyan) and the Q-loop (yellow) of each NBD with AMP-PNP are indicated. This figure was reproduced from Ter Beek *et al.* (Ter Beek *et al.*, 2014).

The D-loop, typically has a conserved SALD amino acid sequence, and when the NBDs are in a dimer formation run alongside each other contributing to the formation of the catalytic sites (Grossmann *et al.*, 2014).

The Q-loop is composed of 8 residues: an N-terminal glutamine residue acts as link between the RecA and helical domains of the NBD. Upon binding of Mg-ATP conformational changes within the NBD allow the Q-loop to contribute to the formation of the catalytic site. Crystal

structures of Sav1886 shows the Q-loop to interact with regions of the TMD indicating the motif's role in transmitting conformational changes from the NBD to the TMD

(Jones and George, 2002; Dawson and Locher, 2006; Ter Beek *et al.*, 2014).

The second domain, the helical domain, contains the signature motif (or C-loop) that is unique to ABC transporters. The signature motif has a sequence of LSGGQ and functions to direct the positively charged N-terminal helix of the domain toward the γ -phosphate of ATP, as has been observed from the crystal structures of a number of ABC transporters (Chen *et al.*, 2003; Dawson and Locher, 2006).

Crystal structures have shown that NBD dimers form in an asymmetrical head-to-tail manner, in which the signature motif of one monomer is opposed to the Walker A and B motifs and the H-loop of the other. These interactions form two composite ATP-binding sites, with two ATP molecules 'sandwiched' at the interface, Figure 5.4 (Jones and George, 1999; Locher *et al.*, 2002; Dawson and Locher, 2006).

Figure 5.4

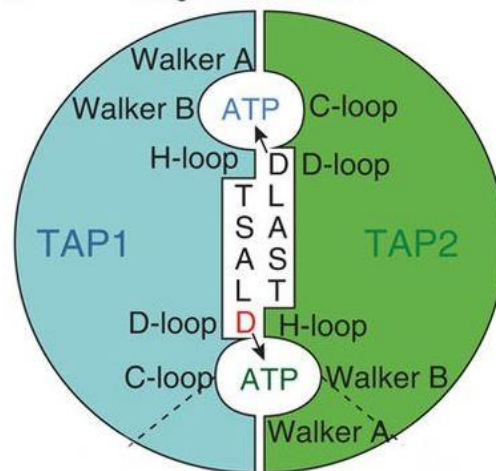


Figure 5.4 The ATP Binding Motif of TAP. An illustration of the two ATP binding sites of the human TAP ABC transporter with the RecA domain of one NBD subunit opposed to the signature motif (C-loop) of the other (Grossmann *et al.*, 2014).

5.1.2.2 Transmembrane Domains

Unlike the NBDs of ABC transporters, there is little amino acid homology between the TMDs of ABC transporters, although they do exhibit tertiary structure homology. This is likely a consequence of the wide range of substrates ABC transporters can recognise and that some transporters act as importers and others as exporters (Rees *et al.*, 2009). The TMD is composed of a number of hydrophobic α -helices that span the lipid bilayer. The α -helices from both TMD subunits align in such a way that they form a channel across the membrane that is open to either the cis or trans face of the cytoplasmic membrane to facilitate substrate transport, Figure 5.5(a-b) (Davidson *et al.*, 2008; Ter Beek *et al.*, 2014). The number of α -helices in ABC exporters is conserved at 12 with each subunit contributing 6 as

has been observed from the crystal structures of numerous transporters including the homodimeric ABC transporter Sav1886 of *S. aureus* and the P-glycoprotein ABC transporter, Figure 5.5(a-b) (Dawson *et al.*, 2007; Jin *et al.*, 2012).

Figure 5.5

(a)



(b)

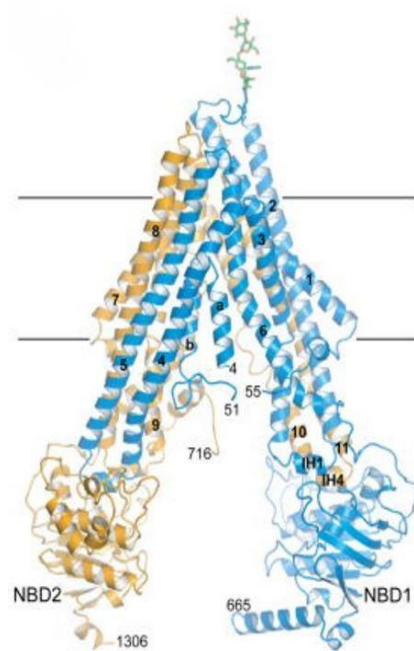


Figure 5.5 Structure of Sav1866 and P-glycoprotein. (a) Structure of the heterodimeric ABC transporter Sav1866 from *S. aureus* open to the trans-side of the cytoplasmic membrane. The six transmembrane helices of each subunit are highlighted (yellow and green) crossing the cytoplasmic membrane (shaded grey), reproduced from Hollenstein *et al.* (Hollenstein *et al.*, 2007). The two nucleotide-bound NBDs are illustrated also. (b) Structure of the *Caenorhabditis elegans* ABC transporter P-glycoprotein, open to the cis-side of the cytoplasmic membrane. The cytoplasmic membrane is indicated as two horizontal lines (Jin *et al.*, 2012).

Crystal structures of bacterial ABC transporters have shown the coupling helices to extend from the TMD to interact with the Q-loops of the NBDs (Dawson *et al.*, 2007). These coupling helices function to transmit conformational changes in the NBD, that occur during ATP binding and hydrolysis, to the TMD and dictate whether the channel is open to the cis or trans side of the membrane. In the TAP ABC transporter a single substitution in the coupling region R659N did not alter substrate binding or ATP hydrolysis, but did result in a loss of transport highlighting its importance in coupling (Chen *et al.*, 1996; Saveanu *et al.*, 2001).

5.1.3 Models for ABC Transporter Activity

How ATP hydrolysis is coupled to substrate transport is still mechanistically unclear although a number of models have been proposed.

The alternating access model, first drafted in the 1960s by Jardetzky *et al.*, describes a substrate binding site within the channel formed by the TMD that has an altered substrate affinity depending on the transporters conformation (Jardetzky, 1966; Dawson *et al.*, 2007). For instance, an exporter will have a substrate bind site that has a higher affinity when in the cis conformation and a lower affinity when in the trans conformation, thus dictating the direction of movement of the substrate. This model is supported by X-ray crystallography structures obtained for a number of ABC transporters which show ABC transporters in either cis or trans facing conformations. The crystal structure of purified Sav1886 bound to AMP-PNP shows it facing the trans-side of the membrane, Figure 5.5(a) (Dawson and Locher,

2006; Dawson *et al.*, 2007). The P-glycoprotein of *C. elegans* and the human ABCB10 ABC transporter crystal structures have only been crystallised in their cis-facing conformation, Figure 5.5(b) (Jin *et al.*, 2012; Shintre *et al.*, 2013).

The ATP switch model for transport posits that substrate binding within the transmembrane channel initiates binding of ATP by the NBD which powers the transport of the substrate. Hydrolysis of the bound ATP followed by the release of ADP and Pi (inorganic phosphate) destabilised the dimer to reset the ABC transporter, Figure 5.6 (Higgins and Linton, 2004; Linton and Higgins, 2007).

Figure 5.6

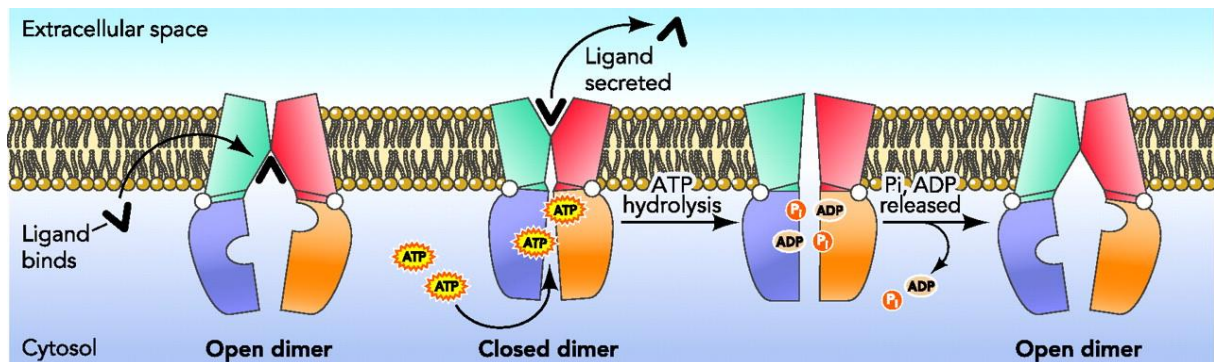


Figure 5.6 The ATP Switch Model. The above illustration shows the transport cycle of an ABC transporter as explained by the ATP switch model. The ABC transporter is cis-facing with its substrate binding site in a high affinity conformation. Following substrate binding the two NBD bind sites bind ATP, forming a closed dimer which acts as the ‘powerstroke’ to change the transporter into a trans-facing conformation to release the substrate. Hydrolysis of the two ATP molecules followed by the release of ADP and inorganic phosphate (Pi) brings the transporter back to a cis-facing conformation. Figure reproduced from Linton *et al.* (Linton, 2007).

Early evidence for substrate binding initiating transport was provided by maltose and histidine permeases with mutations in their TMDs. These mutant transporters no longer required substrate binding and continued to hydrolyse ATP without substrate transport (Petronilli and Ames, 1991; Davidson *et al.*, 1992). Further evidence for the switch model is provided by the crystal structures of various ABC transporters in nucleotide-free states revealing them to have separated NBDs (Jin *et al.*, 2012). The relevance of such nucleotide-free crystal structures is debated; as the physiological concentration of ATP is 10-times greater than the K_m (where K_m is the substrate concentration required to reach half of the maximum rate of reaction) for ATP hydrolysis indicating the resolved structures may not exist in non-laboratory conditions (Gottesman *et al.*, 2009).

Somewhat at odds with the ATP switch model is the constant contact model. This model posits that after substrate transport, hydrolysis at a single site is enough to induce a conformational change that returns the ABC transporter to a cis conformation and that the NBDs are never fully disassociated. Evidence for such a mechanism of transport comes from observations of molecular dynamics simulations using Sav1886 and the MJ0796 transporter from *Methanococcus jannaschii*. These simulations show that when ATP is bound at one site and ADP at the other, the ADP bound site can open, releasing ADP and exchanging it for ATP (Jones and George, 2009; Oliveira *et al.* 2011). Additionally, the crystal structure of the TM287/288 ABC transporter of *Thermotoga maritime* shows it to have a dimeric ABC with its coupling helices separated enough to open the substrate bind site, supporting the constant contact model (Hohl *et al.*, 2012).

Further evidence that hydrolysis at a single site may facilitate substrate transport comes from the functional and sequence asymmetry of the NBDs of heterodimeric ABC

transporters. Heterodimeric ABC transporters have asymmetry in their NBDs, as they have a consensus and a so-called degenerate ATP binding site. The ATP binding and hydrolysis sequence motifs described above differ at these degenerate sites (Lubelski *et al.*, 2006; Zutz *et al.*, 2011; Hohl *et al.*, 2012).

TmrAB is a heterodimeric ABC transporter produced by *T. thermophilus* that has a consensus and a degenerate ATP bind site due to the presence of an aspartate residue in place of a canonical glutamate in the TmrB Walker B motif (Zutz *et al.*, 2011). *In vitro* ATPase and vanadate trapping assays using [γ^{32} -P]ATP and [α^{32} -P]ATP showed that hydrolysis only occurred at the consensus ATP binding site and that substitutions to the consensus glutamate residue diminished ATP hydrolysis. Substitution of the degenerate aspartate residue for an asparagine did not impact the K_m of TmrAB hydrolysis (Zutz *et al.*, 2011).

Similar results were obtained for LmrCD, a heterodimeric transporter produced by *L. lactis*, following substitution of its degenerate aspartate residue for an asparagine. Additionally, whole cell assays demonstrated that *L. lactis* cells expressing this mutant LmrCD could transport ethidium bromide as effectively as those producing the wild type transporter. Substitution at the consensus glutamate residue however resulted in a loss of ethidium efflux (Lubelski *et al.*, 2006).

5.1.4 ABC Transporters in Antibiotic Resistance

Drug resistance in cells can result from the expression of efflux systems that work to decrease the cellular concentration of the toxic compound. In Eukaryotic cells, ABC

transporters are the main export proteins associated with drug resistance. The resistance of cancerous cells to chemotherapy has been attributed to the over-expression of ABC transporters including the P-glycoprotein, multidrug resistance protein 1 (MRP1) and the breast cancer resistance protein (ABCG2) (Cole, 2014; Callaghan *et al.*, 2014; Mao and Unadkat, 2015).

Although less reported than secondary efflux systems such as MFS and RND transporters, ABC transporters are also involved in drug resistance in bacteria (Li and Nikaido, 2004). In fact, mutations and increased expression of ABC transporter genes have been associated with extensive drug-resistance (XDR) in clinical isolates of *Mycobacterium tuberculosis* (Kuan *et al.*, 2015). Due to the multifaceted nature of antibiotic resistance and the diversity of exporters produced by bacteria, it may be that ABC transporters contribute more to antibiotic resistance in bacteria than the literature would suggest. Table 5.2 is a list of antimicrobial ABC transporters.

Table 5.2 Bacterial Antimicrobial ABC Transporters.

Host	ABC Transporter	Antibiotic Substrate	Reference
<i>B. subtilis</i>	BmrCD	Hoechst 33342, doxorubicin, mitoxantrone	(Torres <i>et al.</i> , 2009)
<i>Bifidobacterium breve</i>	AbcAB	Nisin, Polymyxin B	(Margolles <i>et al.</i> , 2006)
<i>E. faecalis</i>	EfrAB	Norfloxacin, Ciprofloxacin, Doxycycline, Novobiocin, Arbekacin	(Davis <i>et al.</i> , 2001; Lee <i>et al.</i> , 2003)
	EfrCD	Tetracycline, Rifampin, Ethidium Bromide, Hoechst 33342	(Hürlimann <i>et al.</i> , 2016)
	EfrEF	Fluoroquinolones, Ethidium Bromide, Hoechst 33342	(Hürlimann <i>et al.</i> , 2016)
<i>E. faecium</i>	MsrC	Quinupristin, Macrolide	(Singh <i>et al.</i> , 2001; Reynolds and Cove, 2005)
<i>L. lactis</i>	LmrCD	Ethidium Bromide, Hoechst 33342	(Lubelski <i>et al.</i> , 2006)
<i>Mycobacterium bovis</i>	Bcg0231	Vancomycin, Ampicillin, Chloramphenicol, Streptomycin	(Danilchanka <i>et al.</i> , 2008)
<i>M. tuberculosis</i>	Rv0194	Ampicillin, Erythromycin, Vancomycin, Novobiocin	(Danilchanka <i>et al.</i> , 2008)
	Rv1258C	Fluoroquinolones, Rifampin, Tetracyclines	(Siddiqi <i>et al.</i> , 2004)
	Rv2686c- Rv2687c- Rv2688c	Fluoroquinolones	(Pasca <i>et al.</i> , 2004)
<i>S. enterica</i> serovar Typhimurium	MacAB	Erythromycin	(Nishino <i>et al.</i> , 2006)
<i>S. marcescens</i>	SmdAB	Norfloxacin, Tetracyclines	(Matsuo <i>et al.</i> , 2008)
<i>S. aureus</i>	AbcA	Penicillin G, Methicillin, Cefotaxime, Moenomycin, Ethidium Bromide	(Truong-Bolduc and Hooper, 2007; Villet <i>et al.</i> , 2014)
	Sav1866	Ethidium Bromide, Hoechst 33342	(Velamakanni <i>et al.</i> , 2008)
<i>S. australis</i>	TetAB(46)	Tetracycline	(Warburton <i>et al.</i> , 2013)
<i>S. pneumoniae</i>	PatAB	Fluoroquinolones	(Baylay and Piddock, 2015)
	SP2073/SP2075	Ethidium Bromide, Novobiocin, Fluoroquinolone	(Robertson <i>et al.</i> , 2005)
	Spr0812/Sp r0813	Bacitracin	(Becker <i>et al.</i> , 2009)
<i>V. cholerae</i>	VcaM	Tetracycline, Ciprofloxacin, Norfloxacin, Ofloxacin	(Huda <i>et al.</i> , 2003)

5.2 Chapter Aims

The aim of the work presented in this chapter was to determine the genes cloned in PS9 required for the observed tetracycline resistance. Additionally, the level and spectrum of resistance conferred to *E. coli* EPI300 T1^R::pCC1BAC by the gene(s) was also determined as well as if the tetracycline resistance genes had an associated fitness cost. We aimed to clone the tetracycline resistance gene(s) into *Streptococcus mutans* UA159. Finally, RT-PCRs were conducted to determine if the tetracycline resistance gene(s) were transcribed in the *E. coli* hosts.

5.3 Methods and Materials

5.3.1 Determining the Size of PS9

The pCC1BAC insert from PS9 was extracted, digested using *HindIII* and analysed by agarose gel electrophoresis as described in 2.3.1. The 1 Kb extended ladder from NEB was used as a DNA fragment size reference.

5.3.2 Sequencing of PS9

The PS9 insert was sequenced using 454 sequencing and primer extension Sanger sequencing. The primers used to sequence PS9 using Sanger sequencing are included in Table 5.3.

Table 5.3 Primers for Subcloning, Mutagenesis and RT-PCR.

Name	Sequence (5'-3')	Information	Source
pCC1-F	GGATGTGCTGCAAGGCGATTAAGTTGG	End sequencing of pCC1BAC	Epicentre®
pCC1-R	CTCGTATGTTGTGTGGAATTGTGAGC	End Sequencing of pCC1BAC	Epicentre®
M13-F	GTTTTCCAGTCACGAC	End sequencing of inserts in pHSG396	Beckman Coulter Genomics
M13-R	CAGGAAACAGCTATGAC	End sequencing if inserts in pHSG396	Beckman Coulter Genomics
TetRseq1R	CTTTTCTACGGTGGGTATC	Sequencing of ABC transporter region of PS9	This Study

TetRseq2R	GATGCGAAACGGAAAGGG	Sequencing of ABC transporter region of PS9	This Study
TetRseq3R	GGCAGAGAACCGTTGGTACG	Sequencing of ABC transporter region of PS9	This Study
TetRseq4R	CTCAACAATGAATCGATTC	Sequencing of ABC transporter region of PS9	This Study
TetRseq5R	CAACTGTCCGATAATACG	Sequencing of ABC transporter region of PS9	This Study
TetRseq6R	CCACAATTGCTCATGCTG	Sequencing of ABC transporter region of PS9	This Study
ABC1FH	GGTGGT <u>AAGCTT</u> GGTGAATGAAGTATAGC	Amplification of ABC transporter genes introduced <i>Hind</i> III site underlined	This Study
ABC1FB	GGTGGT <u>GGATCC</u> GGTGAATGAAGTATAGC	Amplification of ABC transporter genes introduced <i>Bam</i> HI site underlined	This Study
ABC1RB	GGTGGT <u>GGATCC</u> CCTGCTTTGAAATCATGCG	Amplification of ABC transporter genes introduced <i>Bam</i> HI site underlined	This Study
ABC2FH	GGTGGT <u>AAGCTT</u> GCAATTCGTCATGCGGATG	Amplification of ABC transporter genes introduced <i>Hind</i> III site underlined	This Study
ABC2RB	GGTGGT <u>GGATCC</u> CCTCAATCAGTTTCCTAC	Amplification of ABC transporter genes introduced <i>Bam</i> HI site underlined	This Study
ABC2FB	GGTGGT <u>GGATCC</u> GGTGAATGAAGTATAGC	Amplification of ABC transporter genes introduced <i>Bam</i> HI site underlined	This Study
ABC1delF	CCGTACCGAGGAGAGAAGCTG	Deletion of Walker A motif from <i>tetA</i> (60)	This Study
ABC1delR	CTCGCCTTACGTAACG	Deletion of Walker A motif from <i>tetA</i> (60)	This Study
ABC2delF	CGTTCTACGACCCAACG	Deletion of Walker A motif from <i>tetB</i> (60)	This Study
ABC2delR	CGTCTGCTCTGGTTCTAC	Deletion of Walker A motif from <i>tetB</i> (60)	This Study
ABC1delseqF	CAGCTTCTCTCCTCGGTACGG	Amplifying <i>tetA</i> (60) Walker A motif	This Study

ABC1delseqR	CGTTACGTAAAGGCGAG	Amplifying <i>tetA</i> (60) Walker A motif	This Study
ABC2delseqF	CGTTGGGTCGTAGAAGCG	Amplifying <i>tetB</i> (60) Walker A motif	This Study
ABC2delseqR	GTAGAACCAGGACAGACG	Amplifying <i>tetB</i> (60) Walker A motif	This Study
27f	AGAGTTTGATCMTGGCTCAG (M= A or C)	16S gene Amplification	(DJ, 1991)
1492r	TACCTTGTTACGACTT	16S gene Amplification	(Levy <i>et al.</i> , 1999)

5.3.3 Sequence Analysis of PS9

PS9 sequences were assembled using DNASTAR and analysed using the *in silico* tools described in 2.4.1 and 2.4.2. Two ORFs encoding hypothetical ABC half transporter genes were named *tetA*(60) and *tetB*(60) by the Stuart B. Levy lab according to tetracycline resistance gene nomenclature guidelines as their putative protein products had less than 80 % amino acid similarity to any other known tetracycline resistance protein (Levy *et al.*, 1999).

5.3.4 ABC Transporter Amino Acid Alignments

The putative amino acid sequences of TetA(60) and TetB(60) were compared with other phenotypically validated tetracycline and multidrug ABC transporter protein sequences from Gram-positive bacteria [TetAB(46), BmrCD, LmrCD, PatAB and EfrAB] by alignment using Clustal Omega at <http://www.ebi.ac.uk/Tools/msa/clustalo/>.

5.3.5 Subcloning into *E. coli* strains

Regions of the PS9 metagenomic clone were amplified using primer pairs that introduced *Hind*III and *Bam*HI sites that flanked the promoter regions and translation stop sites of genes of interest. *tetA*(60) was amplified using ABC1FH and ABC1RB primers, *tetB*(60) was amplified using ABC2FH and ABC2RB primers and *tetAB*(60) were amplified using ABC1FH and ABC2RB primers. The primers used for subcloning are listed in Table 5.3. The relative positions of these primer pairs are included in Figure 5.8.

The amplicons and pHSG396 were double digested by *Hind*III and *Bam*HI and dephosphorylated using CIAP as described in 2.3.2 and 2.3.3. The digestion reactions were cleaned using the QIAquick PCR Purification Kit. Following clean up, the digested amplicons were ligated into pHG396 using T4 DNA ligase (NEB) as described in 2.3.5.

Amplicons were ligated into pGEM[®]-T Easy using the 2X Rapid Ligase Buffer (Promega) in a 3:1 ratio of insert to plasmid as described in 2.3.5. Ligation reactions were inactivated by incubating at 70 °C for 15 min.

Ligation reactions were transformed into *E. coli* Alpha-Select Silver Efficiency (Bioline). The cells were thawed on ice and transferred to a sterile centrifuge tube containing 5 µl of ligation reaction. The cells were kept on ice for 30 min before being heat shocked at 42 °C for 30 s and cooled on ice for 2 min. 950 µl of SOC media (NEB) was then added to the cells and they were incubated at 37 °C for 1 h with shaking at 200 RPM.

Ligation reactions were desalted before electroporating into *E. coli* EPI300 T1^R, as described in chapter 2. Cells were plated onto LB agar containing ampicillin (100 µg/ml; for pGEM[®]-T Easy) or chloramphenicol (12.5 µg/ml; for pHSG396). The constructs and strains created are detailed in Table 5.4.

Sequencing of the *tetAB*(60) *E. coli* subclones was conducted using the M13 primers and primers TetRseq1R - TetRseq4R, Table 5.3.

Table 5.4 Vectors, Constructs and Strains.

	Name	Information	Source
Vectors	pCC1BAC	8.1 Kb cloning vector that can maintain large inserts (>250 Kb). Low copy number inducible to high copy number. CmR.	Epicentre [®] CopyControl™
	pHSG396	2.2 Kb cloning vector. High copy number. CmR	Takara Bio©
	pVA838	9.2 Kb <i>E. coli</i> – <i>Streptococcus</i> spp. shuttle vector. ErmR and CmR.	ATCC [®]
	pGEM [®] -T Easy	3 Kb cloning vector capable of ligating to polyadenylated PCR products. High copy number. AmpR.	Promega
Constructs	pCC1BAC::PS9	pCC1BAC containing 7,765 bp metagenomic DNA insert.	This Study
	pHSG396::tetA(60)	pHSG396::tetA(60)	This Study
	pHSG396::tetB(60)	pHSG396::tetB(60)	This Study
	pHSG396::tetAB(60)	pHSG396::tetAB(60)	This Study
	pHSG396::tetB(60)ΔtetA(60)	pHSG396::tetB(60)ΔtetA(60)	This Study
	pHSG396::tetA(60)ΔtetB(60)	pHSG396::tetA(60)ΔtetB(60)	This Study
	pGEM::tetAB(60)	pGEM [®] -T Easy::tetAB(60)	This Study
	pVA838::tetAB(60)	pVA838::tetAB(60)	This Study
Bacterial Strains	<i>E. coli</i> EPI300 T1 ^R	Electrocompetent, inducible <i>trfA</i> gene for pCC1BAC copy number control.	Epicentre [®] CopyControl™
	<i>E. coli</i> Alpha-Select Silver Efficiency	Chemically competent <i>E. coli</i> K-12.	Bioline Ltd
	<i>S. mutans</i> UA159	Cariogenic oral isolate	(Ajdić <i>et al.</i> , 2002)

<i>E. coli</i> ::pCC1BAC	<i>E. coli</i> EPI300 T1 ^R ::pCC1BAC	P. Warburton, Eastman Dental Institute
PS9	<i>E. coli</i> EPI300 T1 ^R ::[pCC1BAC::PS9]	This Study
<i>E. coli</i> ::pHSG396	<i>E. coli</i> EPI300 T1 ^R ::pHSG396	This Study
<i>E. coli</i> ::pHSG396tetA(60)	<i>E. coli</i> EPI300 T1 ^R ::[pHSG396::tetA(60)]	This Study
<i>E. coli</i> ::pHSG396tetB(60)	<i>E. coli</i> EPI300 T1 ^R ::[pHSG396::tetB(60)]	This Study
<i>E. coli</i> ::pHSG396tetAB(60)	<i>E. coli</i> EPI300 T1 ^R ::[pHSG396::tetAB(60)]	This Study
<i>E. coli</i> ::pHSG396tetB(60) Δ tetA(60)	<i>E. coli</i> EPI300 T1 ^R ::[pHSG396::tetB(60) Δ tetA(60)]	This Study
<i>E. coli</i> ::pHSG396tetA(60) Δ tetB(60)	<i>E. coli</i> EPI300 T1 ^R ::[pHSG396::tetA(60) Δ tetB(60)]	This Study
<i>S. mutans</i> ::pVA838tetAB(60)	<i>S. mutans</i> UA159::[pVA838::tetAB(60)]	This Study
<i>E. coli</i> ::pGEM	<i>E. coli</i> EPI300 T1 ^R ::pGEM [®] -T Easy	This study
<i>E. coli</i> ::pGEMtetAB(60)	<i>E. coli</i> EPI300 T1 ^R ::[pGEM [®] -T Easy::tetAB(60)]	This Study
DH5 α ::pHSG396	<i>E. coli</i> Alpha-Select Silver Efficiency::pHSG396	
DH5 α ::pHSG396tetAB(60)	<i>E. coli</i> Alpha-Select Silver Efficiency::[pHSG396::tetAB(60)]	This Study
DH5 α ::pGEM	<i>E. coli</i> Alpha-Select Silver Efficiency:: pGEM [®] -T Easy	This Study
DH5 α ::pGEMtetAB(60)	<i>E. coli</i> Alpha-Select Silver Efficiency::[pGem [®] -T Easy::tetAB(60)]	This Study

5.3.6 Subcloning into *S. mutans* UA159 Competent Cells

5.3.6.1 *S. mutans* UA159 Competent Cell Preparation

To make electrocompetent *S. mutans* cells, 800 μ l of a 16 h culture, grown aerobically at 37 °C with shaking at 200 RPM, was inoculated into 40 ml of fresh BHI. The suspension was incubated at 37 °C at 200 RPM until an OD₆₀₀ of 0.4 was reached (approximately 4 h). The cell suspension was then centrifuged at 4200 g for 10 min at 4 °C. The resulting cell pellet was washed three times with 4 ml of 0.5 M sucrose containing 10 % glycerol by centrifuging

at 4200 g for 10 min at 4 °C. After the final wash the cell pellet was suspended in 0.5 M sucrose containing 10 % glycerol and stored at -80 °C.

5.3.6.2 Transformation of *S. mutans* UA159 Competent cells

A region of PS9 that contained *tetAB*(60) was amplified using the primers, ABC1FB and ABC2RB, that introduced *Bam*HI restriction sites that flanked the genes, Table 5.3. The resulting amplicon and pVA838 were digested with *Bam*HI (NEB). The free ends of pVA838 were then dephosphorylated with CIAP and the digestion reactions cleaned with the QIAquick PCR Purification Kit. Following clean-up of the PCR, the product was ligated into pVA838 using T4 DNA ligase (NEB). The ligation reaction was desalted in an agarose cone and 5 µl of the ligation reaction was added to a 0.1 cm electroporation containing 50 µl of electrocompetent *S. mutans* UA 159. Electroporation was conducted at 1.25 kV, 200 Ω and 25 µF. 950 µl of SOC was immediately added to the cells and they were incubated at 37 °C with shaking at 200 rpm for 1 h. The cell suspensions were then plated onto BHI agar plates containing erythromycin (5 µg/ml) with or with tetracycline (5 µg/ml) and they were incubated for 16 h at 37 °C in 5% CO₂. Construct and strain information from this subcloning are included in Table 5.4. Sequencing of the *tetAB*(60) *S. mutans* UA159 subclones was conducted using primers TetRseq1R – TetRseq6R, Table 5.3.

5.3.7 Site Directed Mutagenesis

To make in-frame deletions of the regions of the ABC transporter genes encoding the Walker-A motif, the Q5® Site Directed Mutagenesis Kit (NEB) was used. Two pairs of non-

overlapping primers were designed to amplify the pHG396 plasmid containing the ABC transporter using the Q5[®] polymerase, Table 5.3. The first primer pair, ABC1delF and ABC1delR, amplified pHSG396::*tetAB*(60) without a 69 bp region containing the *tetA*(60) Walker-A motif, keeping *tetB*(60) full length. The second primer pair, ABC2delF and ABC2delR and ABC amplified pHSG396*tetAB*(60) without a 57 bp region containing the Walker-A motif of *tetB*(60) keeping *tetA*(60) intact. The resulting linear PCR products were phosphorylated by a kinase at their ends, circularised by a DNA ligase, and the plasmid that was the template for the PCR was digested by DpnI as follows: 5 µl of PCR reaction was incubated with 1 µl of 10X kinase, ligase, DpnI (KLD) enzyme mix (NEB) in a 10 µl for 15 min at RT. The reactions were then desalted using an agarose cone and electroporated into *E. coli* EPI300 T1^R. Mutant constructs and strain information are detailed in Table 5.4. Mutant clones were sequenced and the Walker A motifs of *tetA*(60) and *tetB*(60) and their respective mutants were amplified to check the mutations; the primers used are detailed in Table 5.3.

5.3.8 MIC of Tetracyclines

The susceptibilities of *E. coli* EPI300 T1^R, *E. coli*::pHSG396, *E. coli*::pHSG396*tetAB*(60), *E. coli*::pHSG396*tetB*(60) Δ *tetA*(60) and *E. coli*::pHSG396*tetA*(60) Δ *tetB*(60) to tetracycline, minocycline and tigecycline (Sigma-Aldrich Company Ltd) were determined using the broth microdilution method described in 2.5.1. The concentrations of tetracycline minocycline and tigecycline tested were 0.25 – 32 µg/ml, 0.25 – 10 µg/ml and 0.25 – 10 µg/ml respectively.

5.3.9 Antibiotic Disk Susceptibility Assay

The susceptibilities of *E. coli* EPI300 T1^R, *E. coli*::pHSG396 and *E. coli*::pHSG396tetAB(60) to various antibiotics were evaluated using the disk diffusion assay, Table 5.5. 16 h cultures of each clone were diluted 1 in 100 using sterile 0.1M PBS (pH 7.0) and the diluted suspensions were then swabbed onto the surface of MH agar. Antibiotic containing disks (Oxoid, Ltd) were then placed on the lawn, and the plates were incubated for 16 h at 37 °C. The zones of inhibition were measured in cm.

Table 5.5 Antibiotic Disks.

Antibiotic (Concentration)	Concentration
Cefotaxime	30 µg
Metronidazole	50 µg
Neomycin	10 µg
Ciprofloxacin	1 µg
Nalidixic Acid	30 µg
Gentamicin	10 µg
Amoxicillin/Clavulanate	20 µg/10 µg
Trimetoprim/Sulfametoxazole	23.75 µg/1.25 µg
Amikacin	30 µg
Tetracycline	10 µg
Ceftazidime	30 µg
Erythromycin	5 µg

5.3.10 Growth Curves

16 h cultures of *E. coli*::pHSG396, *E. coli*::pHSG396tetAB(60), *E. coli*::pHSG396tetB(60)ΔtetA(60) and *E. coli*::pHSG396tetA(60)ΔtetB(60) grown in LB with chloramphenicol (12.5µg/ml) and tetracycline (5µg/ml; when required) were adjusted to an OD₆₀₀ of 0.05 in LB and chloramphenicol (12.5µg/ml). Cell suspensions were grown at 37 °C

with shaking at 200 rpm for 7 h, and their cell density was measured every 30 min using spectrophotometry (OD₆₀₀). *E. coli*::pHSG396tetAB(60) was also grown in LB and chloramphenicol (12.5 µg/ml) with tetracycline (5 µg/ml). Growth rates were measured for each clone as the slope of the line between two time points on the growth curve. The equation, $N_t = N_0 \cdot (1 + r)^t$ was used to calculate the maximum growth rate between 60 and 240 min. Technical and biological triplicates were conducted for all growth curves and growth rate calculations.

5.3.11 Determining Cellular ATP Concentration

The concentration of ATP in *E. coli*::pHSG396, *E. coli*::pHSG396tetAB(60), *E. coli*::pHSG396tetB(60)ΔtetA(60) and *E. coli*::tetA(60)ΔtetB(60) was determined using the Abcam luciferase Luminescent ATP Detection Assay (Abcam plc, Cambridge, UK). This kit measures ATP concentrations using an ATP dependent luciferase and its substrate (luciferin). If ATP is present in a sample, the luciferase oxidises luciferine to produce oxyluciferin and light. ATP concentrations can then be determined by comparing sample luminescence to a standard ATP concentration/luminescence curve. 16 h cultures of these strains grown in LB and chloramphenicol (12.5 µg/ml) were subcultured in fresh LB and chloramphenicol (12.5 µg/ml) at 37 °C for 4 h.

A 16 h culture of *E. coli*::pHSG396tetAB(60) in LB containing chloramphenicol (12.5 µg/ml) and tetracycline (5 µg/ml) was also subcultured in fresh LB and chloramphenicol (12.5 µg/ml) and tetracycline (5 µg/ml) as above.

The subcultures were adjusted to an OD₆₀₀ 0.05. In a 96-well plate 100 µl of each adjusted culture and 50 µl of detergent solution (sodium hydroxide 0.1-1 % w/v; Abcam) were

incubated for 5 mins at RT to lyse cells and inhibit ATPase activity. To each well 50 μ l of assay substrate (contains luciferase) was added and the samples were incubated for 5 min at RT in the dark. This assay was also conducted with fresh LB containing no antibiotic and known concentrations of ATP. The samples were left in the dark for 10 mins prior to measuring luminescence. To estimate the ATP concentration, the luminescence of each clone was compared with a (100 pM to 1 μ M) log₁₀ standard ATP curve (equation: $y = 118625\ln(x) + 635449$). Technical and biological triplicates were conducted for all ATP assays.

5.3.12 Statistical Analysis

Standard deviations were calculated for each clone using the data obtained from the growth curve and ATP assays, which included nine data points encompassing biological and technical replicates. Standard deviations were used as error bars in Figure 5.14 and Figure 5.15 for comparison of the mean OD₆₀₀ and ATP concentrations for each clone. Two tailed t-tests with 95 % confidence intervals were used to determine the significance of differences between clones and the control (*E. coli*::pHSG396) in terms of OD₆₀₀ at 420 min, growth kinetics and OD₆₀₀ 0.05 culture ATP concentrations.

5.3.13 RNA Extraction

RNA was extracted from pure cultures using the RNeasy Plus Mini Kit (Qiagen). 1 ml of 16 h cultures of DH5 α ::pGEM, DH5 α ::pGEM $tetAB(60)$, *E. coli*::pGEM and *E. coli*::pGEM:: $tetAB(60)$ grown in LB supplemented with ampicillin (100 μ g/ml) and DH5 α ::pHSG396,

DH5 α ::pHSG396*tetAB*(60), *E. coli*::pHSG396 and *E. coli*::pHSG396*tetAB*(60) grown in LB supplemented with chloramphenicol (12.5 μ g/ml) were centrifuged at 15700 g for 1 min. The supernatants were removed and 400 μ l RLT buffer containing β -mercaptoethanol (10 μ l/10 ml) was added and the cells were lysed by pipetting up and down and by vortexing for 30 s. To remove genomic DNA from the samples, the suspensions were passed through a genomic DNA Eliminator column by centrifuging at 15700 g for 30 s. The flow-throughs were transferred to clean centrifuge tubes and 1 ml of 70% EtOH was added. The suspensions were then passed through RNeasy columns, 700 μ l at a time by centrifuging at 15700 g for 15 s. This was repeated twice, discarding the flow-throughs between washes. The columns were then washed with 700 μ l of RW1 buffer by centrifuging at 15700 g for 15 s. The flow-throughs were discarded and the columns washed twice with 500 μ l of RPE buffer by centrifuging at 15700 g. The columns were then transferred to clean centrifuge tubes and RNA was eluted with 30 μ l of molecular grade water by centrifuging at 15700 g for 1 min. The RNA preparations were then incubated with DNase I (Thermo Fisher Scientific, Loughborough). 25 μ l of RNA, 3 μ l of 10X reaction buffer, 1 μ l DNase I and 1 μ l of molecular grade water were incubated for 30 min at RT. The reaction was stopped by adding 3 μ l of 25mM EDTA (pH 8.0) followed by heating to 65 $^{\circ}$ C for 10 min. RNA samples were stored at -20 $^{\circ}$ C.

5.3.14 Reverse Transcription PCR

RT-PCRs were conducted using the ABC1FH and ABC2RB primers, Table 5.3. For RT-PCRs the OneTaq[®] One-Step RT-PCR kit (NEB) was used. Reactions contained 25 μ l of OneTaq[®] One-Step Reaction Mix (2X), 2 μ l OneTaq[®] One-Step Enzyme Mix (25X), 0.4 μ M primers and 40 ng

of RNA. RT-PCR programmes began with a complimentary DNA (cDNA) synthesis step; a 15 min incubation at 48 °C. An initial denaturing step at 94 °C was then followed by 40 cycles of 15 s denaturing at 94 °C, 30 s of annealing at a temperature specific for the primer pair and extension at 68 °C for 1 min per Kb. A final extension step at 68 °C for 1 min per Kb plus 1 min was then conducted. The reactions were then cooled at 4 °C. As a control to ensure RNA samples were free of genomic DNA RT-PCR, reactions were set up but the cDNA synthesis step was omitted from the programme. RT-PCRs using the 16S primers, 27f and 1492r, were also conducted as a positive control to show RNA was present in the RNA extractions.

5.4 Results

5.4.1 Analysis of PS9 Insert

The pCC1BAC::insert plasmid was extracted from PS9 and digested with *Hind*III revealing an insert size of approximately 8.25 Kb, Figure 5.7 (a).

Figure 5.7

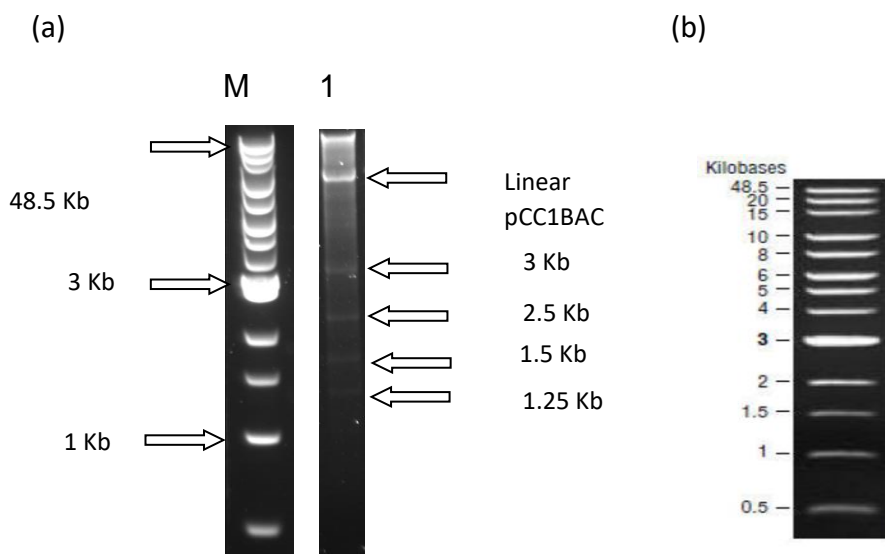


Figure 5.7 Agarose Gel Analysis of *Hind*III Digest PS9 Insert. The lane marked M contains the NEB 1Kb extended ladder and the lane Marked 1 contains the *Hind*III digested insert. (a) A 0.9 % agarose gel stained with gel red showing the *Hind*III digestion fragments of the PS9 insert. The gap in the gel is due to the removal of lanes not pertinent to the results being discussed. (b) An image depicting the NEB 1Kb extended ladder with size labels.

Sequencing of the PS9 plasmid was conducted using 454 and Sanger sequencing which revealed it to contain a 7,765 bp fragment (accession number KX887332). PS9 sequencing primers are listed in Table 5.3 and the full sequence is included in Appendix III. BLASTN analysis revealed the insert to have similarity along its entire length to *Streptococcus* sp. 263_SSPC (accession: GCA_001071995.1, 98 % cover and 90 % identity) and *Granulicatella adiacens* ATCC 49175 (accession: NZ_ACKZ00000000, 94 % cover 92 % identity), Appendices

IV and V. The alignments between the PS9 insert and these two genomes also identified an inversion in PS9 between 1,600 bp and 1,789 bp relative to these *Streptococcus* spp. and *Granulicatella* spp. genomes (Appendix IV and Appendix V). BLASTX analysis of the sequence predicted the sequence to have 5 putative ORFs, three of which encoded a putative sulfurtransferase, UDP-galactopyranose and an amidohydrolase, Figure 5.8 and Table 5.6. The two remaining ORFs were predicted to encode half ABC transporters. These transporters were named TetA(60) and TetB(60), as their putative amino acid sequences had less than 80% similarity to other tetracycline resistance protein (Levy *et al.*, 1999). The *tetAB*(60) nucleotide sequence has been submitted to Genbank (accession numbers KX000272.1 and KX000273.1) and is included in Appendix VI.

Figure 5.8

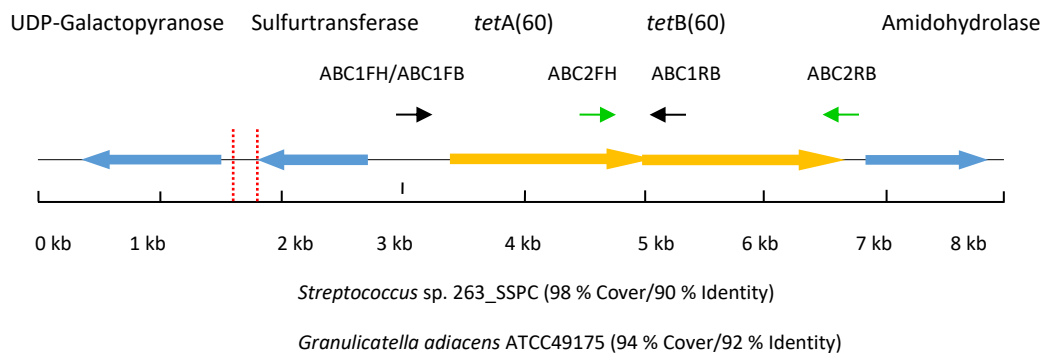


Figure 5.8 PS9 Schematic Illustrating Subcloned *tetAB*(60) Region. The 5 ORFs identified in the PS9 clone are presented. The vertical dashed red lines indicate the sequence inversion. The primers used to amplify *tetA*(60) are represented by two black arrows. The primers used to amplify *tetB*(60) [highlighted in orange] are represented by two green arrows. The black arrow labelled ABC1FH/ABC1FB and the green arrow labelled ABC2RB indicate the primers used to amplify *tetAB*(60), Table 5.3. The closest nucleotide relatives are detailed as are the 5 ORFs present on the insert, according to BLASTX.

Table 5.6 Putative Open Reading Frames Identified in PS9

Open Reading Frame	Streptococcus sp.263_SSPC	Granulicatella adiacens ATCC 49175
	Accession Numbers	Accession Numbers
Sulfurtransferase	WP_048781756.1	WP_005607217.1
UDP-galactopyranose	WP_048781754.1	WP_005607219.1
Amidohydrolase	WP_048781763.1	WP_005607209.1
TetA(60) Homolog	WP_048781758.1	WP_005607214.1
TetB(60) Homolog	WP_048781760.1	WP_005607213.1

5.4.2 Analysis of TetAB(60) Amino Acid Sequence

Both TetA(60) and TetB(60) were predicted to be 579 amino acids and contain a predicted NBD and TMD. Additionally, there was a 4 bp overlap in the genes as the start codon of *tetB(60)* was present in *tetA(60)*, although the genes were in different reading frames.

ClustalΩ alignments of the putative amino acid sequences of TetA(60) and TetB(60) to characterised antimicrobial resistance heterodimeric ABC transporters showed that they were more closely related to TetA(46) and TetB(46) (39.27 % and 42.28 % identity respectively) and BmrC and BmrD (40.93 % and 46.61 % respectively) than they were to the MDR ABC transporters EfrAB, PatAB and LmrCD of *E. faecalis*, *S. pneumonia* and *L. lactis*, respectively (≤ 34.46 %), Table 5.7.

Table 5.7 Alignment of TetA(60) to other Antibiotic Resistance ABC Transporters.

ABC Half Transporter	Percentage Similarity		Accession Numbers	References
	TetA(60)	TetB(60)		
TetA(60)	100 %	24.69 %	KX000272.1	This Study
TetB(60)	24.69 %	100 %	KX000273.1	This Study
BmrC	40.93 %	27.11 %	BG13040	(Torres <i>et al.</i> , 2009)
BmrD	24 %	46.61 %	BG13041	(Torres <i>et al.</i> , 2009)
TetA(46)	39.27 %	24.16 %	AET10444.1	(Warburton <i>et al.</i> , 2013)
TetB(46)	24.69 %	42.28 %	AET10445.1	(Warburton <i>et al.</i> , 2013)
EfrA	28.62 %	27.37 %	CDO61511	(Lee <i>et al.</i> , 2003)
EfrB	28.96 %	34.46 %	CDO61515	(Lee <i>et al.</i> , 2003)
PatA	25.9 %	26.69 %	ABJ55477	(Baylay and Piddock, 2015)
PatB	23.77 %	29.68 %	ABJ53826	(Baylay and Piddock, 2015)
LmrC	27.69 %	26.5 %	ABF66011	(Lubelski <i>et al.</i> , 2004)
LmrD	25.14 %	30.07 %	ABF66027	(Lubelski <i>et al.</i> , 2004)

The NBDs of TetA(60) and TetB(60) were also non-identical. The Walker B motif of TetB(60) had the conserved glutamate residue needed for polarising the γ phosphate; however, TetA(60) had an aspartate residue instead. The TetA(60) walker B motif also contained a polar cysteine residue where a hydrophobic residue would ideally be. The Walker A motif of TetA(60) and TetB(60) are non-identical, although both had consensus sequences. Additionally, there are amino acid substitutions between the signature motifs of TetA(60) and TetB(60), with the former following the consensus, Figure 5.9. This suggests that the Walker A and Walker B motifs of TetB(60) interact with the TetA(60) signature motif to form a consensus NBD. Following on from this the non-consensus TetB(60) Walker B motif and TetA(60) signature motif most likely form a degenerate ATP binding and hydrolysis site.

Figure 5.9

	A-Loop	Walker A	Q-Loop	Signature Motif	Walker B	D-Loop	H-Loop
TetA(60)	Y	GKTGSGKT	QEHTLFSR	LSGGQ	CLILDD	SAVD	H
TetB(60)	Y	GHTGSGKS	QDPYLFTG	FSSGE	ILILDE	SHID	H
TetA(46)	Y	GRTGAGKT	QEHLFSK	VSGGQ	LLLLDD	SAVD	H
TetB(46)	Y	GHTGSGKS	QDPYLFTG	FSSGE	ILILDE	SHID	H
BmrC	Y	GRTGAGKT	QDPYLFSG	LSGGQ	ILILDD	SAVD	H
BmrD	Y	GHTGSGKS	QHDLLFSR	LSSGE	ILILDE	AHID	H
Consensus	Y	GXXGXGKT/S	QXXXXXXXX	LSSGQ	ϕ ϕ ϕ ϕ DE	SALD	H

Figure 5.9 TetAB(60) NBD Motif Alignments. An alignment of the conserved motifs and loops of the NBDs of the ABC half transporters compared with the consensus; consensus amino acid residues are highlighted in red. The alignment shows that TetA(60), TetA(46) and BmrC have Walker B motifs that deviate from the consensus as they have an aspartate (D) residue in place of the consensus glutamate (E) residue that is needed for ATP hydrolysis. Their counterpart half transporters contain consensus Walker B motifs. Additionally, the signature motif of TetB(60) contains a number of mutations resulting in it differing from the consensus while TetA(60) does not. This is observed for TetB(46) and BmrD also. This suggests that the full TetAB(60) transporter contains both a consensus and degenerate NBD. The remaining motifs and loops appear to follow the consensus though there is some variation between their D-loops.

5.4.3 Subcloning of *tetAB(60)*

Having shown that *tetAB(60)* encoded a putative heterodimeric ABC transporter, *tetA(60)* and *tetB(60)* were amplified individually and together by PCR and cloned into *E. coli* EPI300 T1^R and *E. coli* Alpha-Select Silver Efficiency using the pHSG396 and pGEM[®]-T Easy vectors to determine if both were required for tetracycline resistance. Restriction enzyme digests of pHSG::*tetAB60*, pGEM::*tetAB(60)* are shown in Figure 5.10 (a-b). A schematic diagram of the subcloned *tetAB(60)* genes is included in Figure 5.11 (a).

Figure 5.10

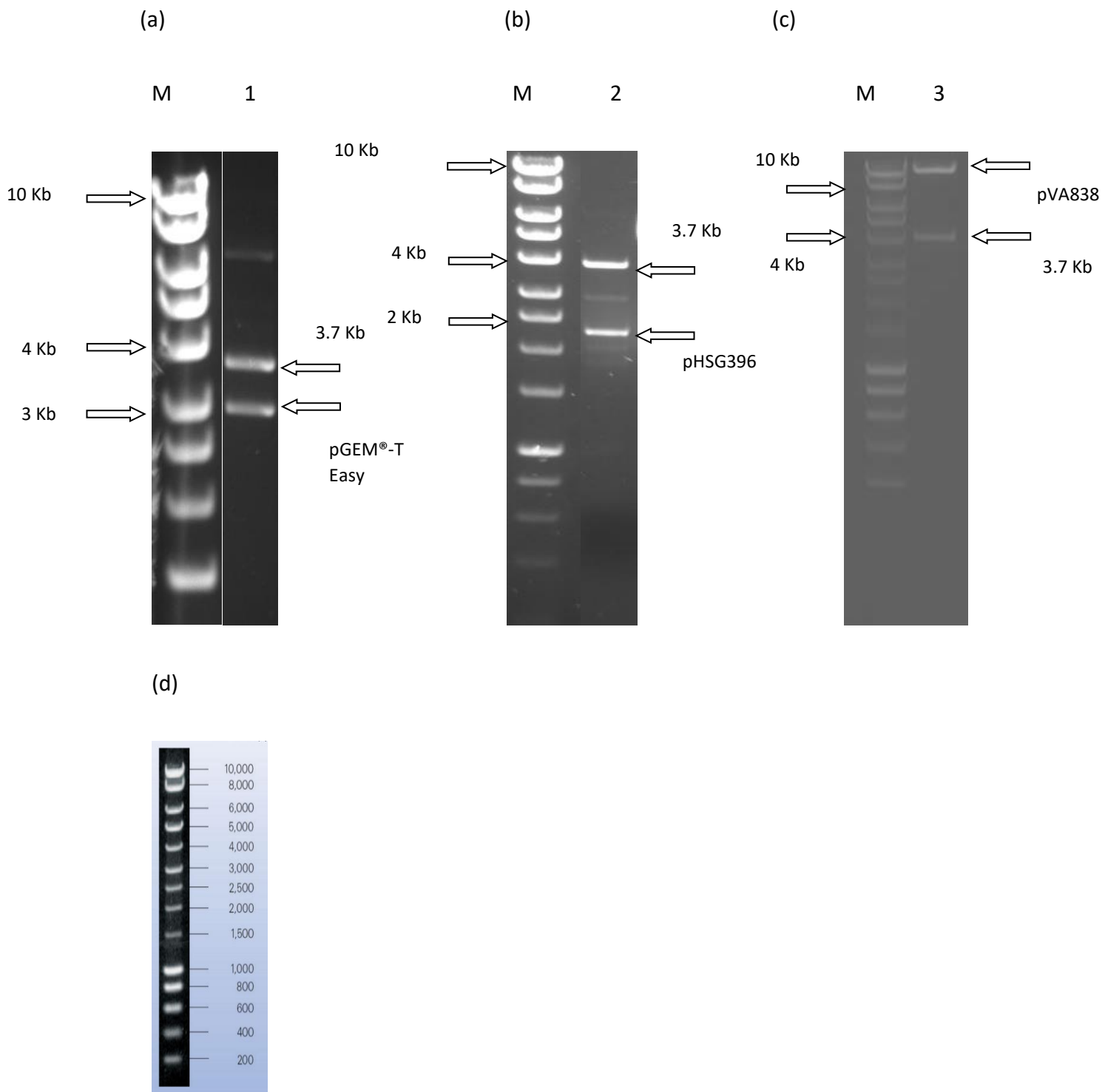
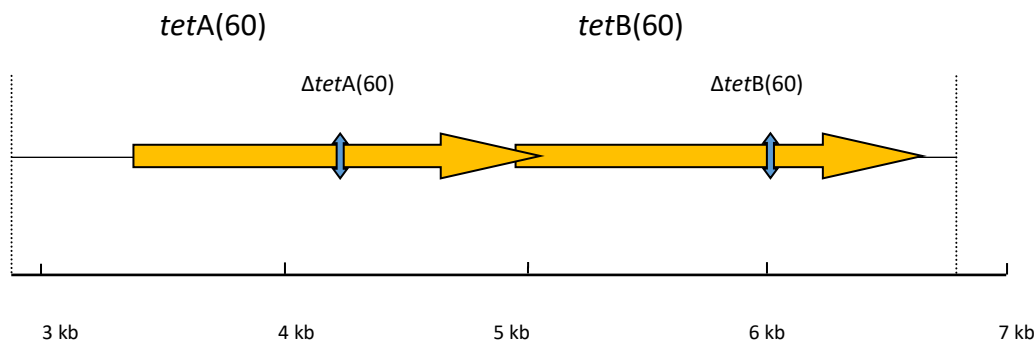


Figure 5.10 *tetAB(60)* Subclones. (a) Lane 1: *Hind*III/*Bam*HI double digest of pGEM::*tetAB(60)*. (b) Lane: *Hind*III/*Bam*HI double digest of pHSG396::*tetAB(60)*. (c) Lane 3: *Bam*HI digest of pVA838::*tetAB(60)*. Lanes marked M contain Hyper Ladder I. (d) Hyper Ladder I (Bioline)

Figure 5.11

(a)



(b)

*tetB*Δ*tetA*(60)

Amino Acid N-T L G I V **G K T G S G K T** T L L M Q L L H Q F -C
 Nucleotide 5'-ACGCTTGAATCGTT**GGAAAAACGGTTCTGGGAAGACG**ACGCTCTTGATGCAATTATTACATCAATTT-3' (4220-4288)

*tetA*Δ*tetB*(60)

Amino Acid N-V A F V **G H T G S G K S** S I M N L L F -C
 Nucleotide 5'-GTTGCTTTTGT**CGACATACTGGTTCAGGGAAATCA**TCCATTATGAACTTACTGTTT-3' (5974-6030)

Figure 5.11 *tetAB*(60) Deletion Mutants. (a) Diagram depicting the positions and orientations of *tetAB*(60) in the 3, 703 bp subclones as marked by the vertical dashed lines. The positions of the Walker A motifs that were deleted to make pHSG396::*tetB*(60)Δ*tetA*(60) and pHSG396::*tetA*(60)Δ*tetB*(60) are marked by vertical double headed blue arrows. (b) The nucleotide sequences of the deleted regions are given above with the Walker A motif of each gene highlighted in yellow and translated. The scale indicates the nucleotide positions relative to the full length PS9 clone.

E. coli::pHSG396*tetA*(60), *E. coli*::pHSG396*tetB*(60), *E. coli*::pGEM*tetAB*(60),

DH5α::pHSG396*tetAB*(60) and DH5α::pGEM*tetAB*(60) were unable to grow on LB

supplemented with 5 μg/ml tetracycline. Only *E. coli*::pHSG396*tetAB*(60) was able to grow

on LB supplemented with 5 μg/ml tetracycline which indicates that both *tetA*(60) and

tetB(60) are required for the tetracycline resistance.

The *tetAB(60)* genes were also cloned into *S. mutans* UA159 using pVA838. The resulting clone, *S. mutans*::pVA838*tetAB(60)*, showed no increase in tetracycline resistance compared with *S. mutans* UA159::pVA838, Figure 5.10 (c).

5.4.4 Mutagenesis of the *tetAB(60)* Walker A Motif

Having shown that both *tetA(60)* and *tetB(60)* were required for tetracycline resistance in *E. coli* EPI300 T1^R we next set out to ascertain whether the gene products functioned as an ABC transporter that conferred resistance to tetracycline. In order to do this a 69 and 57 base pair deletion was made to remove the Walker A motif of the nucleotide binding domain from either *tetA(60)* or *tetB(60)*, respectively, Figure 5.11(a, b). PCR amplicons of the Walker A motifs from the wild type and mutant *tetA(60)* and *tetB(60)* are shown in Figure 5.12(a). Nucleotide alignments of the wild type and mutant Walker A motifs from *tetA(60)* and *tetB(60)* are shown in Figure 5.13(a,b). Both mutants, *E. coli*::pHSG396 *tetB(60)*Δ*tetA(60)* and *E. coli*::pHSG396*tetA(60)*Δ*tetB(60)* were unable to grow on LB supplemented with tetracycline (5 µg/ml). Taken together these subcloning and mutagenesis results confirmed that the ABC transporter activity of these gene products is responsible for the tetracycline resistance in PS9 and *E. coli*::pHSG396*tetAB(60)*.

Figure 5.12

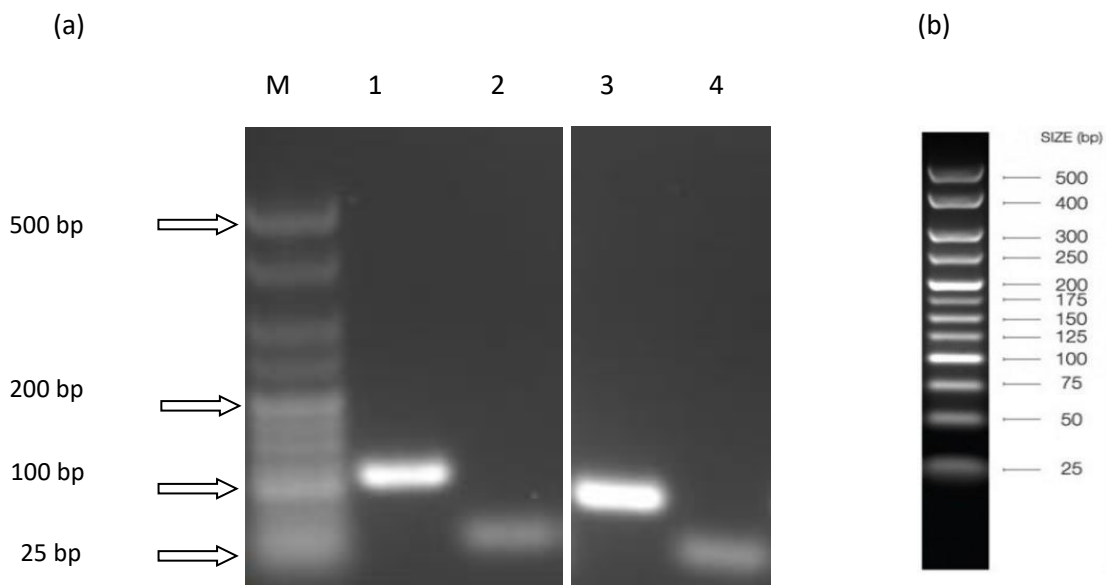


Figure 5.12 Agarose Gel Showing Walker A Motif PCR Products. (a) The amplicons obtained from the amplification of the Walker A motif containing regions from *tetA(60)*, Δ *tetA(60)*, *tetB(60)* and Δ *tetB(60)*. The lane marked M contains HyperLadder V (Bioline). These PCR products were amplified using the ABC1delseq and ABC2delseq primers, Table 5.3. The *tetA(60)* Walker A motif region is approximately 100 bp in size (Lane 1) whereas the Walker A motif region of Δ *tetA(60)* is approximately 40 bp in size (Lane 2), indicating that the Walker A motif has been deleted. The Walker A motif of *tetB(60)* is approximately 75 bp (Lane 3), while that of Δ *tetB(60)* is approximately <25 bp (Lane 4), suggesting that the Walker A motif of *tetB(60)* has been removed. (b) A diagram showing the size fragments of the HyperLadder V.

Figure 5.13

(a)

```

tetA(60)  TATCCAGGCGAAGAGGCACCATTATTTAAAAGAGATTAACCTTACGTTACGTAAAGGCG
           .....
ΔtetA(60) TATCCAGGCGAAGAGGCACCATTATTTAAAAGAGATTAACCTTACGTTACGTAAAGGCG

tetA(60)  AGACGCTTGAATCGTTTAAATACCTTCCGCAATAACGCTCTTGATGCAATTAT
           ::
ΔtetA(60)  AG-----

tetA(60)  TACATCAATTTCCGTACCGAGGAGAGAAGCTGCTCATTACGGAGAGCCATTGATTGATT
           .....
ΔtetA(60)  -----CCGTACCGAGGAGAGAAGCTGCTCATTACGGAGAGCCATTGATTGATT

tetA(60)  ACGACACTCAATCGGTGGCAGGGCATCTAGCCTATGTGCCACAA GAACACACCCTTTTCT
           .....
ΔtetA(60)  ACGACACTCAATCGGTGGCAGGGCATCTAGCCTATGTGCCACAA GAACACACCCTTTTCT

tetA(60)  CACGCACGATTCGCGAGAATATGTTATTCGAAAAGAGGATGCAACGGATGATGAAATTT
           .....
ΔtetA(60)  CACGCACGATTCGCGAGAATATGTTATTCGAAAAGAGGATGCAACGGATGATGAAATTT

tetA(60)  GGAAGCGTTGACGCTAGCCTCTTTTGAAGGAGACGTGAAACGAATGCCAGACGAGCTCG
           .....
ΔtetA(60)  GGAAGCGTTGACGCTAGCCTCTTTTGAAGGAGACGTGAAACGAATGCCAGACGAGCTCG

tetA(60)  ATACGATGGTCGGAGAAAAAGGGGTATCGTCACTGGAGGTCMAAACAACGCTTATCGA
           .....
ΔtetA(60)  ATACGATGGTCGGAGAAAAAGGGGTATCGTCACTGGAGGTCMAAACAACGCTTATCGA

tetA(60)  TTGCTCGTGCTTTCTTACGCAACCGTGAA TGCTTAATTTGGATGATGCGTTA TCTGCAC
           .....
ΔtetA(60)  TTGCTCGTGCTTTCTTACGCAACCGTGAA TGCTTAATTTGGATGATGCGTTA TCTGCAC

tetA(60)  TTGATGCGAAAACGGAAAGGGAAATTATCTCGCACTTGCAACAAGAACGCGGAGGTTGTA
           .....
ΔtetA(60)  TTGATGCGAAAACGGAAAGGGAAATTATCTCGCACTTGCAACAAGAACGCGGAGGTTGTA

tetA(60)  TGAATATCATTTCTGCGCACAGACTTTCTGCAATTCGT CAT
           .....
ΔtetA(60)  TGAATATCATTTCTGCGCACAGACTTTCTGCAATTCGT CAT

```

(b)

```
tetB (60)  TACACAGCGGGAGTTCCTGTGTTGAAACACTTGAATTTACGGTAGAACCAGGACAGACG
           .....
ΔtetB (60)  TACACAGCGGGAGTTCCTGTGTTGAAACACTTGAATTTACGGTAGAACCAGGACAGACG

tetB (60)  GTTGCTTTTGTC GACATATGAACTTACTGTTTCGC TCCATTATGAACTTACTGTTTCGC
           .....
ΔtetB (60)  -----CGC

tetB (60)  TTCTACGACCCAACGAGTGGTGCTATCTTCATCGATGGCAAAAACACACGCGACTTTAAC
           .....
ΔtetB (60)  TTCTACGACCCAACGAGTGGTGCTATCTTCATCGATGGCAAAAACACACGCGACTTTAAC

tetB (60)  AGACGTAGTGTTCTGAAGCGAGATGGGAATCGTTCTT CAAGATCCGTACCTCTTTACAGGA
           .....
ΔtetB (60)  AGACGTAGTGTTCTGAAGCGAGATGGGAATCGTTCTT CAAGATCCGTACCTCTTTACAGGA

tetB (60)  ACGATTGCTTCAAACGTAGGGCTCAACAATGAATCGATTGAGCCTGAGACGATAAAAAGAG
           .....
ΔtetB (60)  ACGATTGCTTCAAACGTAGGGCTCAACAATGAATCGATTGAGCCTGAGACGATAAAAAGAG

tetB (60)  GCGATTATTAAGTGGGTGGAGGACATCTACTTACGAAGAGTGACAAGGGCTTGACTAC
           .....
ΔtetB (60)  GCGATTATTAAGTGGGTGGAGGACATCTACTTACGAAGAGTGACAAGGGCTTGACTAC

tetB (60)  GAGGTGAAAGAAAAGGAATGGAT TTCTCTTCAGGAGAACGCCAAGTATTTCATTTGCT
           .....
ΔtetB (60)  GAGGTGAAAGAAAAGGAATGGAT TTCTCTTCAGGAGAACGCCAAGTATTTCATTTGCT

tetB (60)  CGTGCGATCGTCTTTGACCCGAAAATCTTAATTTTAGACGAGGCAACT TCGCATATCGA
           .....
ΔtetB (60)  CGTGCGATCGTCTTTGACCCGAAAATCTTAATTTTAGACGAGGCAACT TCGCATATCGA

tetB (60)  ACCGAGACAGAAGAGATTATTCAGAATGCAATTAATGTCGTCAAAGAAGGGCGTACAACC
           .....
ΔtetB (60)  ACCGAGACAGAAGAGATTATTCAGAATGCAATTAATGTCGTCAAAGAAGGGCGTACAACC

tetB (60)  TTTATGATTGCT CAC
           .....
ΔtetB (60)  TTTATGATTGCT CAC
```

Figure 5.13 Alignments of the NBDs of *tetA*(60) and *tetB*(60). A-loops are highlighted in yellow, Walker A motifs in green, Q-loops in purple, signature motifs in red, Walker B motifs in grey, D-loops in dark blue and H-loops in light blue. (a) A nucleotide alignment between the nucleotide binding domains of *tetA*(60) and Δ *tetA*(60). (b) A nucleotide alignment between the nucleotide binding domains of *tetB*(60) and Δ *tetB*(60).

5.4.5 Tigecycline and Minocycline as Substrates for TetAB(60)

Using the broth dilution method, the MIC of tetracycline for *E. coli* EPI300 T1^R, *E. coli*::pHSG396, *E. coli*::pHSG396*tetAB*(60), *E. coli*::pHSG396*tetB*(60) Δ *tetA*(60) and *E. coli*::pHSG396*tetA*(60) Δ *tetB*(60) was determined, Table 5.8.

The MIC of tetracycline for *E. coli*::pHSG396*tetAB*(60) was found to be 32 μ g/ml. The MICs for the mutants and the control strains were 16-fold lower at 2 μ g/ml. To determine if *tetAB*(60) was able to confer resistance to later-generation tetracycline derivatives, MIC assays were conducted using minocycline and tigecycline. The MIC of minocycline for all strains and clones was 1 μ g/ml. The MIC of tigecycline for *E. coli*::pHSG396*tetAB*(60) was 16-fold higher than the control and mutant strains at 8 μ g/ml, which was above the clinical break point for *Enterobacteriaceae* (0.5 μ g/ml).

Table 5.8 MICs of Tetracycline Antibiotics for *E. coli*::pHSG396*tetAB*(60) and Mutant Strains.

Strain	Tetracycline (μ g/ml)	Minocycline (μ g/ml)	Tigecycline (μ g/ml)
<i>E. coli</i> ::pHSG396	2	1	0.5
<i>E. coli</i> ::pHSG396 <i>tetAB</i> (60)	32	1	8
<i>E. coli</i> ::pHSG396 <i>tetB</i> (60) Δ <i>tetA</i> (60)	2	1	0.5
<i>E. coli</i> ::pHSG396 <i>tetA</i> (60) Δ <i>tetB</i> (60)	2	1	0.5

5.4.6 TetAB(60) Substrate Spectrum

Disk diffusion assays were used to discern the spectrum of resistance for this transporter. *E. coli*::pHSG396tetAB(60) was less sensitive to tetracycline but equally sensitive to cefotaxime, ceftazidime, metronidazole, neomycin, ciprofloxacin, nalidixic acid, gentamicin, amikacin, amoxicillin/clavulanate and trimetoprim/sulfamethoxazole as *E. coli* EPI300 T1^R and *E. coli*::pHSG396, indicating that TetAB(60) is a tetracycline and tigecycline-specific ABC transporter. *E. coli* EPI300 T1^R was intrinsically resistant to erythromycin, which has been described previously, and attributed to AcrAB-TolC mediated efflux and membrane impermeability (Chollet *et al.*, 2004; Vaara, 1993).

5.4.7 Impact of TetAB(60) on *E. coli* Growth

When PS9 was first isolated, we noticed that it grew more slowly and formed smaller colonies than *E. coli*::pCC1BAC, even in the absence of tetracycline in the growth media. This phenotype was also observed for the *E. coli*::pHSG396tetAB(60) subclone. Furthermore, the *E. coli*::pHSG396tetB(60)ΔtetA(60) and *E. coli*::pHSG396tetA(60)ΔtetB(60) mutants did not have such a large growth defect.

In order to determine the extent of this growth defect, growth curves were conducted. These growth curves revealed that although there were significant differences between *E. coli*::pHSG396 and *E. coli*::pHSG396tetA(60)ΔtetB(60) maximum growth rates (0.993 ± 0.05 and 0.917 ± 0.05 , respectively; $p = 0.005$) there was no significant difference in their OD₆₀₀ of

cultures at 7 h (1.65 ± 0.08 and 1.565 ± 0.16 ; $p = 0.1807$) when grown in the absence of tetracycline, Figure 5.14.

Figure 5.14

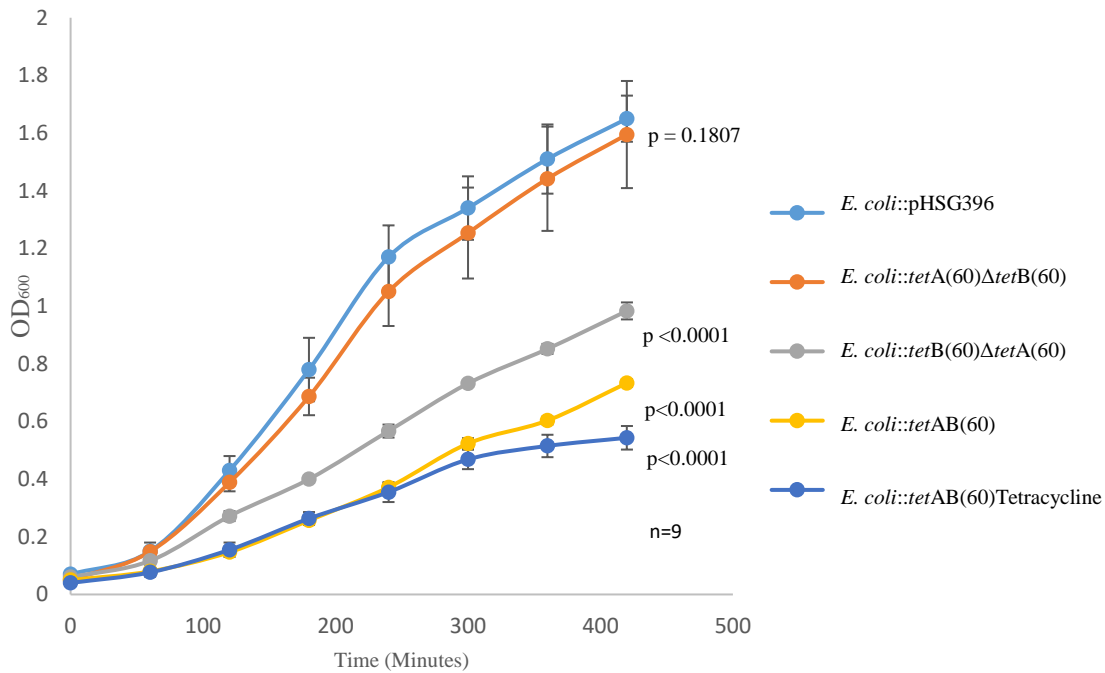


Figure 5.14 Growth Curves of *tetAB*(60) Expressing *E. coli* EPI300 T1^R Clones. *E. coli*::pHSG396, *E. coli*::pHSG396*tetB*(60) Δ *tetA*(60) and *E. coli*::pHSG396*tetA*(60) Δ *tetB*(60) were grown in LB and chloramphenicol for 7 h. This figure also shows the growth curve for *E. coli*::pHSG396*tetAB*(60) grown in LB and chloramphenicol (12.5 μ g/ml) with and without tetracycline (5 μ g/ml). *P*-values for OD₆₀₀ at 7 h were calculated from biological triplicate OD₆₀₀ measurements at 420 min for each clone compared with *E. coli*::pHSG396 are indicated beside each growth curve. *P*-values from biological triplicate ATP assays for each clone compared with *E. coli*::pHSG396 are shown.

Compared with *E. coli*::pHSG396, *E. coli*::pHSG396*tetB*(60) Δ *tetA*(60) and *E. coli*::pHSG396*tetAB*(60) reached lower OD₆₀₀ at 7 h, when they were grown in the absence of tetracycline (0.983 ± 0.03 , 0.733 ± 0.01 , respectively, $p < 0.0001$). Additionally, when grown in the presence of tetracycline, *E. coli*::pHSG396*tetAB*(60) reached an even lower

OD₆₀₀ at 7 h (0.543 ± 0.04 , $p < 0.0001$), Figure 5.14. Whilst the maximum growth rates of *E. coli*::pHSG396*tetB(60)*Δ*tetA(60)* grown without tetracycline and *E. coli*::pHSG396*tetAB(60)* grown without or with tetracycline were not significantly different from each other (0.702 ± 0.06 , 0.68 ± 0.03 and 0.692 ± 0.09 , respectively; $p = 0.435$ to 0.879), they were 1.14-1.46-fold lower than the maximum growth rate of *E. coli*::pHSG396 grown without tetracycline ($p < 0.0001$). This suggested that there was a fitness cost associated with the activity of TetAB(60) rather than the carriage of the plasmid itself, and that TetB(60) contributed more to this fitness cost than TetA(60).

5.4.8 Effect of TetAB(60) on Cellular ATP Concentration

Further to conducting growth curves we set out to determine if harbouring *tetAB(60)* or their mutant variants imposed a fitness cost on *E. coli* EPI300 T1^R by affecting cellular ATP concentrations. ATP levels were estimated for cultures at OD₆₀₀ of 0.05 using a luciferase assay and the results indicated that *E. coli*::pHSG396 and *E. coli*::pHSG396*tetA(60)*Δ*tetB(60)* had the lowest ATP concentrations that were not significantly different from each other ($0.23 \mu\text{M} \pm 0.01$ and $0.23 \mu\text{M} \pm 0.01$ respectively, $p=0.7378$), Figure 5.15. A 1.2-fold increase in ATP concentration was observed for *E. coli*::pHSG396*tetB(60)*Δ*tetA(60)* compared with *E. coli*::pHSG396 ($0.285 \mu\text{M} \pm 0.008$; $p=0.00623$).

E. coli::pHSG396*tetAB(60)* exhibited the highest ATP concentrations at 1.4-, and 1.65- fold higher than the control when grown without ($0.32 \mu\text{M} \pm 0.01$; $p=0.0006$) and with tetracycline ($0.385 \mu\text{M} \pm 0.007$; $p < 0.0001$) respectively. Taken together these results suggested that the activity of TetAB(60) was responsible for the observed fitness cost in *E.*

coli::pHSG396tetAB(60), and that the fitness cost was a result of the transporter altering cell ATP levels.

Figure 5.15

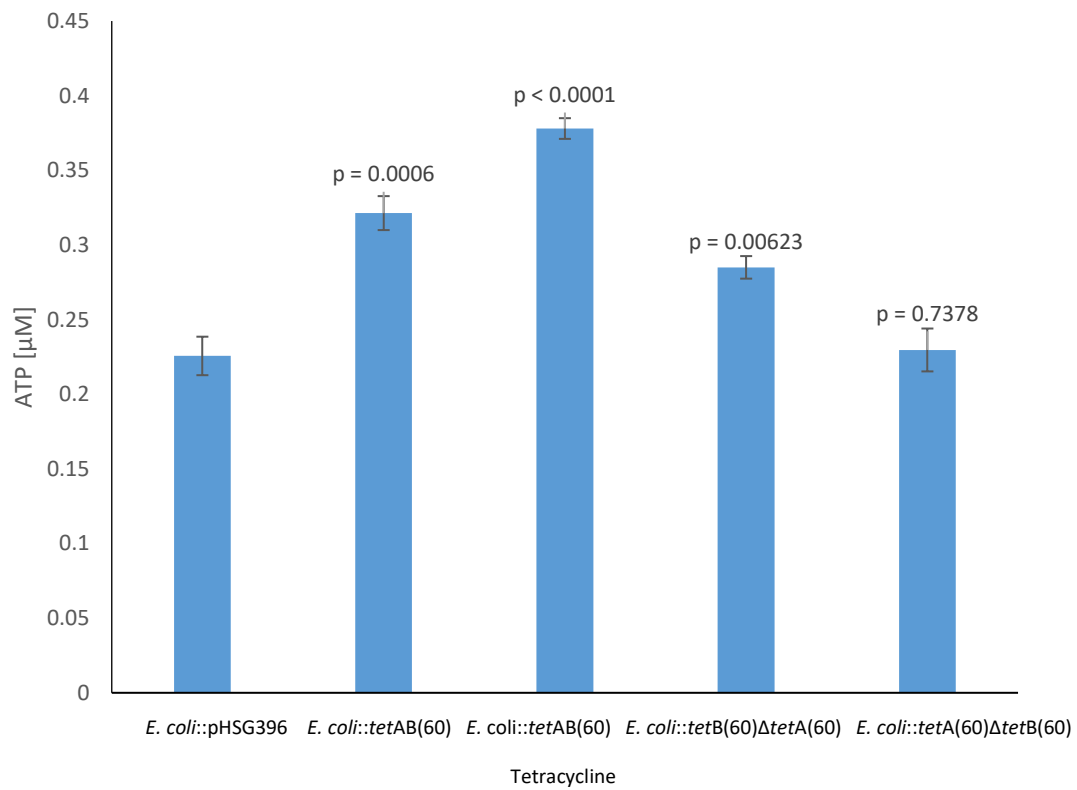


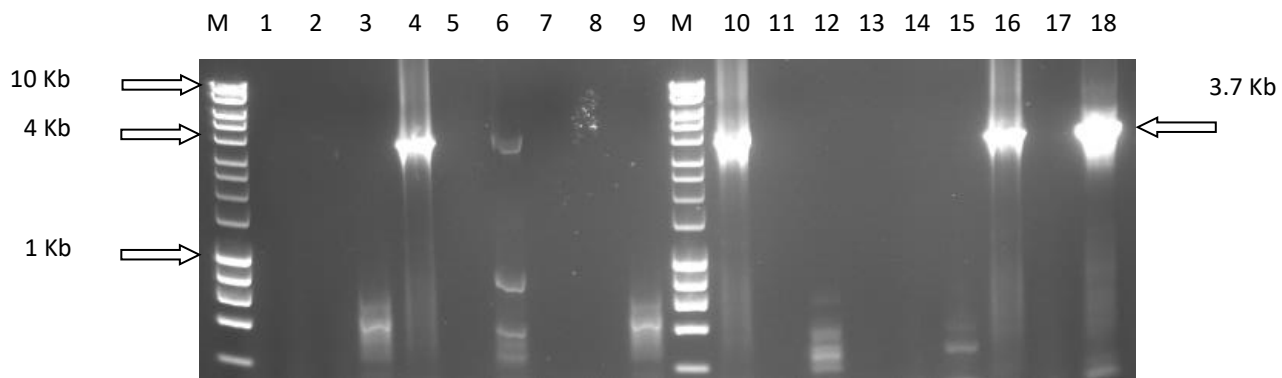
Figure 5.15 ATP Concentration of *tetAB(60)* Expressing *E. coli* EPI300 T1^R. This bar graph displays the ATP concentration of 0.05 OD₆₀₀ cell suspensions of *E. coli::pHSG396*, *E. coli::pHSG396tetAB(60)*, *E. coli::pHSG396tetB(60)ΔtetA(60)* and *E. coli::tetA(60)ΔtetB(60)* in LB containing chloramphenicol (12.5 µg/ml). The ATP concentrations of OD₆₀₀ 0.05 cultures of *E. coli::pHSG396tetAB(60)* grown in LB with chloramphenicol (12.5 µg/ml) and tetracycline (5 µg/ml) is also represented. P-values from biological triplicate ATP assays for each clone compared with *E. coli::pHSG396* are shown.

5.4.9 *tetAB(60)* expression - Reverse Transcription PCR Results

Using the primers ABC1FB and ABC2RB, Table 5.3, RT-PCRs were conducted to determine if the *tetAB(60)* genes were transcribed as a single transcript and if these genes were being transcribed in *E. coli* EPI300 T1^R and *E. coli* Alpha-Select Silver Efficiency. A product of approximately 3.7 kb was produced from the RT-PCR reaction from the RNA preparations of *E. coli*::pHSG396*tetAB(60)*, indicating that two genes were transcribed as a single mRNA. Additionally, a 3.7 Kb amplicon was also obtained from DH5 α ::pGEM*tetAB(60)* and *E. coli*::pGEM*tetAB(60)*; this amplicon was not produced from DH5 α ::pHSG396*tetAB(60)*, Figure 5.16. The 16S rRNA PCR and RT-PCR controls are shown in Figure 5.17. Thus, *tetAB(60)* was transcribed in *E. coli*::pHSG396*tetAB(60)* but not in DH5 α ::pHSG396*tetAB(60)*.

Figure 5.16

(a)



(b)

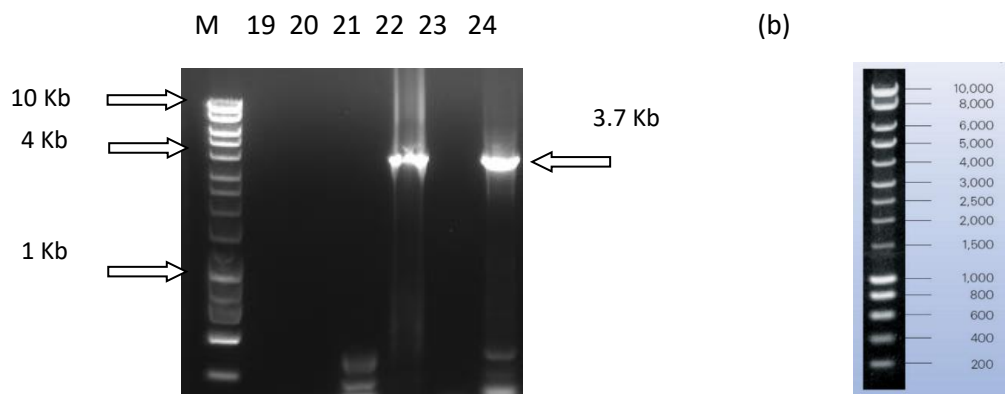
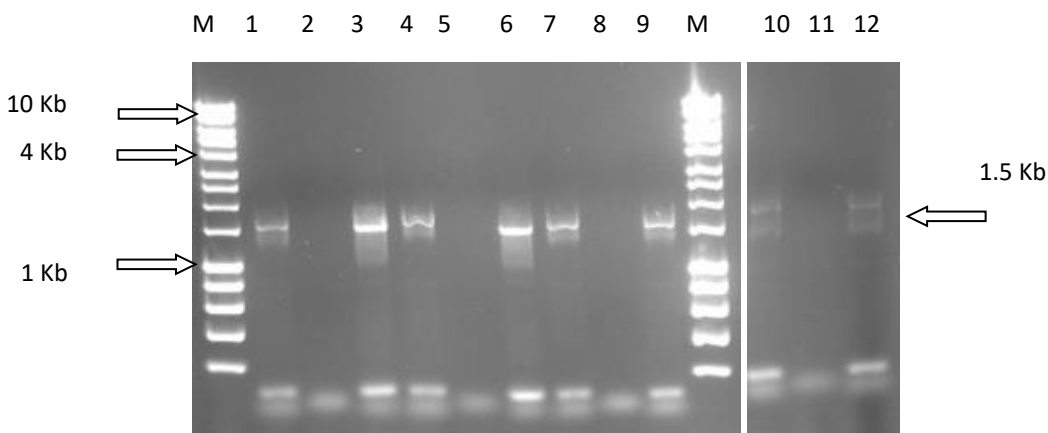


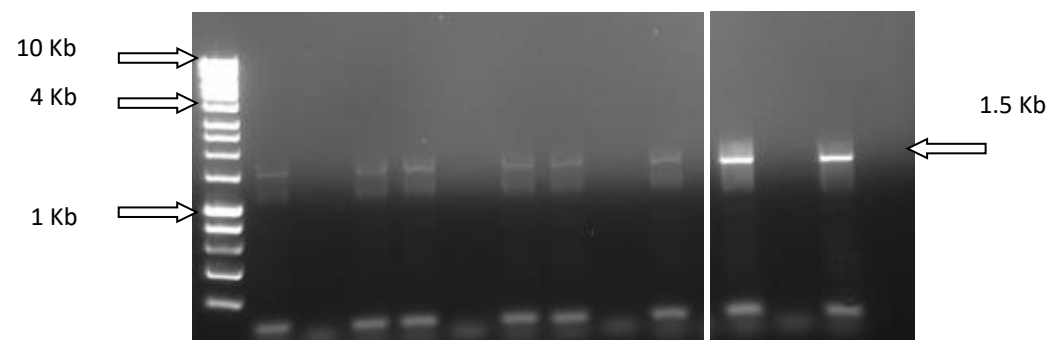
Figure 5.16 RT-PCR Results of *tetAB(60)* Expressing *E. coli* EPI300 T1^R. (a) Agarose gel showing the results of RT-PCRs conducted using ABC1FH and ABC2RB primers. RNA was isolated from clones harbouring empty vectors or vectors containing *tetAB(60)*. PCRs without the RT step, conducted using DNA and RNA isolated from the clones, to show that the primers amplify *tetAB(60)* and that there was no DNA in the RNA preparations respectively are also included. For each clone the reactions are ordered in the gel as PCR, RNA-PCR and RT-PCR. Lanes marked M contain HyperLadder I (Bioline). Lanes 1, 2 and 3 show DH5 α ::pGEM, lanes 7, 8 and 9 show DH5 α ::pHSG396, lanes 13, 14 and 15 show *E. coli*::pGEM and lanes 19, 20 and 21 show *E. coli*::pHSG396. *tetAB(60)* is not present or expressed in this clone. Lanes 4, 5 and 6 show that *tetAB(60)* is transcribed in DH5 α ::pGEM*tetAB(60)* as there is a positive RT-PCR reaction. Lanes 10, 11 and 12 represent DH5 α ::pHSG396*tetAB(60)*; showing *tetAB(60)* to be present but not transcribed as no 3.7 Kb amplicon was observed. Lanes 16, 17 and 18 show *E. coli*::pGEM*tetAB(60)* and show that *tetAB(60)* is present and transcribed. Similarly in lanes 22, 23 and 24 that contain *E. coli*::pHSG396*tetAB(60)* are being transcribed. (b) A diagram showing the size fragments of the HyperLadder I.

Figure 5.17

(a)



M 13 14 15 16 17 18 19 20 21 22 23 24



(b)

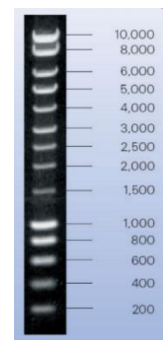


Figure 5.17 16S RT-PCR. Agarose gel showing the results of RT-PCRs conducted using 16S primers (27f and 1492r primers). RNA was isolated from clones harbouring empty vectors or vectors containing *tetAB(60)*. PCRs without the RT step, conducted using DNA and RNA isolated from the clones, to show that the primers amplify *tetAB(60)* and that there was no DNA in the RNA preparations respectively are also included. For each clone the reactions are ordered in the gel as PCR, RNA-PCR and RT-PCR. Lanes marked M contain HyperLadder I (Bioline). Lanes 1, 2 and 3 contain DH5 α ::pGEM. Lanes 4, 5 and 6 contain DH5 α ::pGEM*tetAB(60)*. Lanes 7, 8 and 9 contain DH5 α ::pHSG396. Lanes 10, 11 and 12 contain DH5 α ::pHSG396*tetAB(60)*. Lanes 13, 14 and 15 contain *E. coli*::pGEM. Lanes 16, 17 and 18 contain *E. coli*::pGEM*tetAB(60)*. Lanes 18, 19 and 20 contain *E. coli*::pHSG396. Lanes 22, 23 and 24 contain *E. coli*::pHSG396*tetAB(60)*. In the DNA PCR and the RT-PCR reaction the 1.5 Kb 16S amplicon is observed. No amplicon is observed in the RNA extractions without the RT step. (b) A diagram showing the size fragments of HyperLadder I.

5.5 Discussion

The work described in chapter 4 included the isolation of a clone, PS9, that exhibited decreased susceptibility to tetracycline, which was identified and sequenced. The insert contained in PS9 was 7,765 bp and had 90 % and 92 % nucleotide identity to *Streptococcus* sp. 263_SSPC and *G. adiacens* ATCC 49175, respectively, indicating a Gram-positive origin for the insert. *Streptococcus* spp. are predominant in the oral cavity, although to the best of our knowledge *Streptococcus* sp. 263_SSPC has not been isolated from the oral cavity (Segata *et al.*, 2012). *Granulicatella* spp. including *G. adiacens* are also abundant in the oral cavity, typically inhabiting the mucosa (Aas *et al.*, 2005). Tetracycline resistance has been described in oral *Streptococcus* spp. which has been discussed in chapter 4. Tetracycline resistance has also been observed in *Granulicatella* spp., although minimal characterisation studies have been conducted (Zheng *et al.*, 2004; De Luca *et al.*, 2013).

Analysis of the insert revealed it contained five ORFs, predicted to encode a putative UDP-galactose mutase, a sulfurtransferase, an amidohydrolase and two ABC half transporters [TetAB(60)]. Each predicted protein had amino acid sequences with high similarity (>90% identity) to proteins from *Streptococcus* sp. 263_SSPC and *G. adiacens* ATCC 49175.

Alignment of the putative amino acid sequences of TetAB(60) to other antibiotic resistance heterodimeric ABC transporters showed that they were most closely related to TetAB(46) and BmrCD, and less so to the MDR ABC transporters EfrAB, PatAB and LmrCD. As TetAB(46) has been shown to be most closely related to BmrCD, this suggested that TetAB(60) was also tetracycline specific (Warburton *et al.*, 2013).

The alignments also revealed that some of the motifs in the NBDs of TetA(60), TetA(46) and BmrC did not conform to the consensus, Figure 5.9. The NBDs of TetB(60), TetB(46) and BmrD conformed to consensus, however their signature motifs deviated somewhat with the TetB(60) signature motif containing three amino acid changes relative to the consensus, Figure 5.9. This suggested that a heterodimeric transporter formed by TetAB(60) would contain a degenerate ATP binding site composed of the Walker B and Walker A motifs of TetA(60) and the signature motif of TetB(60) and a consensus site containing the Walker B and Walker A motifs of TetB(60) and the signature of TetA(60). This data suggests that TetAB(46) and BmrCD are also likely to form asymmetric ABC transporters. This is congruent with what has been discussed in the literature where it is understood that bacterial heterodimeric ABC transporters commonly have an asymmetric nature (Lubelski *et al.*, 2006; Zutz *et al.*, 2011; Hohl *et al.*, 2012).

We showed that both *tetA*(60) and *tetB*(60) were required to confer tetracycline resistance in *E. coli* EPI300 T1^R, suggesting that the product of these genes formed a heterodimeric ABC transporter with each gene product containing a TMD and NBD, as revealed by BLASTX (Dawson and Locher, 2006). Interestingly, transformation of *tetAB*(60) genes into *E. coli* Alpha-Select Silver Efficiency or *S. mutans* UA159 did not confer resistance to these strains. This suggests that in these hosts the *tetAB*(60) genes may not be transcribed or translated, or that their products were not properly folded or transported.

Walker A motifs are found in many ATP-utilizing enzymes including ABC transporters and are required for binding and stabilizing ATP (Ramakrishnan *et al.*, 2002; Hirakata *et al.*, 2002; Verdon *et al.*, 2003; Hohl *et al.*, 2012). Deletion of these motifs from ATP transporters has been shown to result in a loss of function (Warburton *et al.*, 2013). Individual in-frame deletions of these motifs from either *tetA*(60) or *tetB*(60) led to a loss of the tetracycline

and tigecycline resistance phenotype providing further evidence that the products of these genes form a heterodimeric ABC transporter.

Compared with *E. coli*::pHSG396, *E. coli*::pHSG396*tetAB*(60) was 16-fold more resistant to tetracycline (MIC of 32 µg/ml) and tigecycline (MIC of 8 µg/ml). Although *E. coli*::pHSG396*tetAB*(60) showed levels of resistance to tetracycline and tigecycline beyond the EUCAST breakpoints, it was as susceptible to minocycline as *E. coli*::pHSG396, indicating that minocycline is not a substrate for this transporter (Olson *et al.*, 2006; Ramos *et al.*, 2009). Efflux-mediated tigecycline resistance has been described previously in *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, being attributed to the activity of an ABC and an RND transporter, respectively (Dean *et al.*, 2003; He *et al.*, 2015; McDaniel *et al.*, 2016).

TetAB(60) appeared to be specific for tetracycline and tigecycline, as disk diffusion assays demonstrated *E. coli*::pHSG396 to be as susceptible as *E. coli*::pHSG396*tetAB*(60) to cefotaxime, ceftazidime, metronidazole, neomycin, ciprofloxacin, nalidixic acid, gentamicin, amikacin, amoxicillin/clavulanate and trimetoprim/sulfamethoxazole and erythromycin.

The observed fitness cost associated with *tetAB*(60) was not observed in either mutant as although *E. coli*::pHSG396*tetA*(60) Δ *tetB*(60) had a lower maximum growth rate than the control, it had a comparable final OD₆₀₀ to *E. coli*::pHSG396, and *E.*

coli::pHSG396*tetB*(60) Δ *tetA*(60) exhibited faster growth than *E. coli*::pHSG396*tetAB*(60).

This indicated that the growth defect was a result of *TetAB*(60) activity rather than from maintenance of the plasmid. Additionally, *E. coli*::pHSG396*tetB*(60) Δ *tetA*(60) grew less well than *E. coli*::pHSG396*tetA*(60) Δ *tetB*(60) suggesting that *TetB*(60) produces a greater cost to the *E. coli* host than *TetA*(60).

ATP assays revealed a correlation between the growth phenotype and culture ATP concentration of the clones as the clones that reached the highest OD₆₀₀ had the lowest ATP concentrations.

For example, *E. coli*::pHSG396 and *E. coli*::pHSG396*tetA*(60) Δ *tetB*(60) reached the highest OD₆₀₀ and both had the lowest ATP concentration (0.23 μ M). *E.*

coli::pHSG396*tetA*(60) Δ *tetB*(60) grew less well than these clones but had a higher ATP concentration (0.285 μ M). This suggested that this clone produced an ABC transporter that could bind ATP at its non-mutated site and prevent it from taking part in cell metabolism.

During the luciferase assay, this ATP was liberated from the transporter resulting in a higher ATP concentration being observed. The comparable ATP concentrations of *E. coli*::pHSG396 and *E. coli*::pHSG396*tetA*(60) Δ *tetB*(60) suggested that the latter produced an ABC transporter that could not sequester ATP, explaining why it had no associated fitness cost. Further to this, *E. coli*::pHSG396*tetAB*(60) cultures grown without tetracycline had a higher ATP concentration (0.32 μ M) than either mutant, likely a result of its two non-mutated ATP binding sites sequestering but not hydrolysing ATP, resulting in a greater fitness cost in this clone.

When grown in tetracycline *E. coli*::pHSG396*tetAB*(60) cultures had higher ATP levels (0.385 μ M) than when grown without. This may be a result of TetAB(60) having a higher affinity for ATP when it binds its tetracycline which is in accordance with the ATP switch model mechanism of efflux. This higher ATP concentration observed in *E. coli*::pHSG396*tetAB*(60) may also be attributed to tetracycline getting into the cell and interfering with protein synthesis. A build-up of charged tRNAs could result in a downregulation of their synthesis by aminoacyl tRNA synthases, a process that is ATP dependent, resulting in an increase in ATP

concentration (Green *et al.*, 2010; Pontes *et al.*, 2015). Thus, it appears that when the TetAB(60) is expressed in *E. coli* EPI300 T1^R it acts as a sink for ATP resulting in a growth defect.

Together, the growth curve and ATP assays indicate that *E. coli*::pHSG396tetA(60) Δ tetB(60) produces an ATP transporter unable to bind and hoard ATP and so it has a similar growth phenotype to *E. coli*::pHSG396 that doesn't express TetAB(60). In contrast, *E. coli*::pHSG396tetB(60) Δ tetA(60) expresses a mutated transporter that can bind some ATP and so it acts as an ATP sink. *E. coli*::pHSG396tetAB(60) even in the absence of tetracycline showed poor growth indicating that TetAB(60) may still bind ATP molecules, acting as an ATP sink in the absence of tetracycline resulting in a reduction of total ATP available for cellular metabolism.

RT-PCRs showed that tetAB(60) was not transcribed in DH5 α ::pHSGtetAB(60) explaining why no tetracycline resistance was observed in this clone. In all tetAB(60) subclones the genes were cloned with their native promoters. It may be possible that secondary structures at the promoter binding site prevent recognition by *E. coli* Alpha-Select Silver σ -factors. It is unlikely to be a lack of promoter sequence recognition as tetAB(60) was transcribed in this host from pGEM[®]-T Easy.

Transcription of tetAB(60) was also observed in *E. coli*::pGEMtetAB(60), although both DH5 α ::pGEMtetAB(60) and *E. coli*::pGEMtetAB(60) were tetracycline-sensitive. The lack of tetracycline resistance in these clones may result from secondary structures in the mRNA preventing ribosomal binding and translation. Additionally, as discussed in chapter 1, improper folding, PTM or transport of the translated TetAB(60) peptides may be responsible

for the lack of tetracycline resistance in these clones. This is especially true for proteins with large hydrophobic regions such as ABC transporters that may aggregate in the more hydrophilic cytoplasm (Vabulas *et al.*, 2010). A failure to traffic proteins correctly can also result in non-functional proteins as a result of incorrect positioning of the protein and also loss of further processing. For example, sulfide bridge formation, which is important for many ABC transporters, takes place mainly in the periplasm by Dsb oxidoreductases (Denoncin and Collet, 2013).

tetAB(60) is transcribed in *E. coli*::pHSG396*tetAB(60)* and the clone is tetracycline resistant indicating that *E. coli* EPI300 T1^R can translate the *tetAB(60)* mRNA from pHSG396 as well as correctly fold and traffic TetAB(60). Further work is necessary to determine why transcription of *tetAB(60)* does not occur in DH5 α ::pHSG396*tetAB(60)* and what post-transcriptional step(s) fail in DH5 α ::pGEM*tetAB(60)* and *E. coli*::pGEM*tetAB(60)*.

To conclude, we have identified two novel genes from the human oral cavity that likely produce a heterodimeric ABC transporter, TetAB(60). TetAB(60) specifically exports tetracycline and tigecycline conferring high levels of resistance to these antibiotics in an *E. coli* host. A limitation of this work is that we do not know the prevalence of these genes in the human oral cavity. Further work should be undertaken to survey its prevalence in various niches, to determine how common these genes are, and their possible clinical relevance for treating bacterial infections with tetracycline derivatives. This work also shows that the human oral cavity harbours unknown tetracycline resistance determinants in the absence of any obvious selection pressure; although *tetAB(60)* has an associated fitness cost in *E. coli* EPI300 T1^R it may not have this fitness cost in its native host. There is potential for these genes to be acquired by mobile genetic elements and transferred to bacterial

pathogens, which is particularly worrying given the recent identification of a carbapenem and colistin resistant strains of *E. coli* some of which could only be inhibited by doxycycline and tigecycline (Liu *et al.*, 2015; Mediavilla *et al.*, 2016; Yao *et al.*, 2016). However, the associated fitness cost of *tetAB(60)* observed in *E. coli* may limit any possible fixation following dissemination of the genes from their native host to *E. coli* strains in the absence of a tetracycline or tigecycline selective pressure.

Chapter 6

Analysis of a Novel Tn916-like Element from the Human Saliva Metagenomic Library

TT31 was identified when the human saliva metagenomic library was cultured on 5 µg/ml tetracycline.

6.1 Introduction

6.1.1 Conjugation

As discussed in chapter 1, conjugation is a mechanism of HGT that involves the transfer of genetic material from a donor to a recipient cell through cell-to-cell contact. Conjugative transposons (CTNs) are DNA that encode for the machinery required for their transfer between cells. The first conjugative element discovered was the F-plasmid (fertility plasmid) from *E. coli* (Lederberg *et al.*, 1952). The first CTN, a mobile genetic element (MGE) that can integrate into the host chromosome through site-specific recombination, was discovered later when tetracycline resistance was observed to transfer to a susceptible strain of *E. faecalis* from a resistant strain in the absence of a plasmid and was called Tn916 (Franke and Clewell, 1981). Further discussion of conjugative elements will focus on CTNs or integrative and conjugative elements (ICEs) of the Tn916 family.

6.1.2 The Modular Nature of Tn916

Tn916 is an 18 Kb CTN that contains 4 functional modules encoding genes involved in conjugation, regulation, recombination (excision and integration) and accessory functions, Figure 6.1 (Flannagan *et al.*, 1994).

Figure 6.1

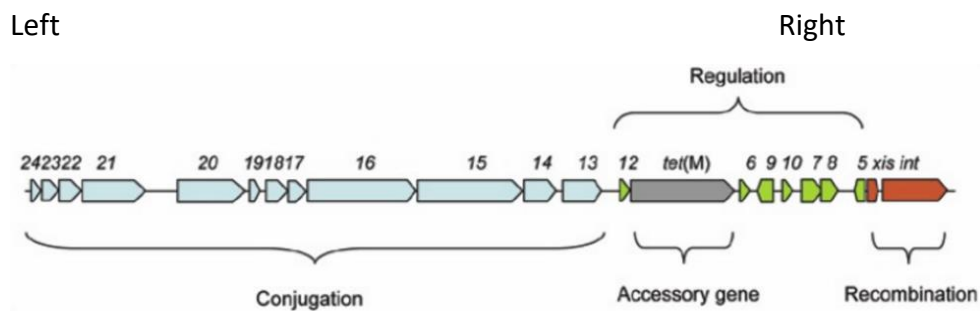


Figure 6.1 Schematic of the Modular Nature of Tn916. The blue arrows on the left side of Tn916 indicate the ORFs involved in conjugation. On the right side of Tn916, the green arrows show genes of the regulation module while the grey arrow and the red arrows show the *tet(M)* and genes required for excision and integration respectively. This figure was reproduced from Roberts and Mullany (Roberts and Mullany, 2009).

6.1.2.1 The Tn916 Recombination Module

In Tn916-like elements the recombination module typically contains two genes encoding a tyrosine recombinase (*int*) and excisionase (*xis*), although numerous CTns encode a single serine recombinase (*tndX*), such as Tn5397 and Tn1116 from *C. difficile* and *Streptococcus pyogenes* respectively (Mullany *et al.*, 1996; Brenciani *et al.*, 2007). The tyrosine recombinase catalyses the integration and excision of Tn916 with the latter reaction being favoured in the presence of the excisionase (Rudy *et al.*, 1997; Hinerfeld and Churchward, 2001). The recombinase cleaves at sites at the end of the CTn producing staggered, non-identical overhangs that form a heteroduplex at the joint of the circular intermediate CTn structure. During integration, the recombinase creates cuts in the circular transposon and the chromosome target site and mediates insertion of the CTn into the host chromosome. The heteroduplexes flanking the integrated CTn are then resolved during host cell

replication and mismatch repair (Scott and Churchward, 1995; Taylor and Churchward, 1997).

6.1.2.2 A Tn916 Module Regulates its Transposition and Conjugation

In the regulation module of Tn916, *orf12*, *orf9*, *orf7*, *orf8* and *tet(M)* play an important role in controlling excision of the CTn from the chromosome, through regulation of *xis* and *int* expression, and a transcription attenuation mechanism of regulation in response to tetracycline has been proposed by Su *et al.* (Su *et al.*, 1992). During transcription, stem-loop terminator structures form in the *orf12* mRNA transcript. As mRNA codons transcribed from this region encode for rare codons, the ribosome lags behind the RNA polymerase (RNAP) allowing the stem loop structure to form, disrupting the RNAP-DNA complex, halting transcription. When tetracycline is present, protein translation is greatly reduced and charged tRNAs build up in the cell. However, a small number of ribosomes are still active due to protection from basal levels of Tet(M). These protected ribosomes can transcribe *orf12* and due to the increased abundance of charged tRNAs, the ribosome can translate the mRNA at a faster rate such that it doesn't lag behind the RNAP. This prevents the stem loop structure from forming as its sequence is bound by the RNAP and ribosome and so the RNAP is able to continue transcribing into *tet(M)* and downstream regulation ORFs.

Orf9 is a putative repressor of *orf7* and *orf8* transcription. It has been hypothesised that when tetracycline is present RNAP produces an antisense *orf9* mRNA transcript, resulting in the down-regulation of this gene and subsequent de-repression of *orf7* and *orf8*. Orf7 and

Orf8 then induce their own expression and the expression of the downstream *xis* and *int* genes and thus act as amplifiers of the tetracycline signal that triggers excision of the CTn (Celli and Trieu-Cuot, 1998; Roberts and Mullany, 2009).

6.1.2.3 Accessory Functions of Tn916

Following on from the initial discovery of Tn916, Tn916-like elements have been identified in more than 36 genera from 6 phyla (Roberts and Mullany, 2009). Identification of these CTNs is based on nucleotide sequence identity as well as the conservation of their modular structure and the genes they encode. While early studies relied on Southern blotting techniques to identify Tn916-like elements in bacteria, including oral *Streptococcus* spp., the identification of conserved Tn916 genes, typically *int* and *tet(M)*, by PCR or array analysis are frequently used to rapidly determine the presence of the element in a strain or metagenomic sample (Hartley *et al.*, 1984; Procino *et al.*, 1988; Bentorcha *et al.*, 1992; Spigaglia *et al.*, 2006; Seville *et al.*, 2009; Guglielmini *et al.*, 2011; Dong *et al.*, 2014).

With the introduction of low-cost genomic sequencing, putative Tn916-like elements have been identified *in silico* in a number of bacteria species including *Listeria monocytogenes* and *Streptococcus* spp. (Kuenne *et al.*, 2013; Puymege *et al.*, 2015). In a study of invasive macrolide resistant *S. pneumoniae* isolates, Chancey *et al.* (2015) identified Tn916-like elements in 53 of the 86 isolates they studied (Chancey *et al.*, 2015). Functional metagenomic studies have also identified novel Tn916-like CTNs, such as Tn6079 identified from a human infant faecal fosmid library (De Vries *et al.*, 2011).

These works have led to the identification of Tn916-like elements encoding accessory genes in addition to or in place of *tet(M)* that alter the host cells phenotype. These include genes conferring resistance to biocides, heavy metals and antibiotics other than tetracycline. Schematics of some of the Tn916-like elements that have been identified are shown in Figure 6.2.

Figure 6.2

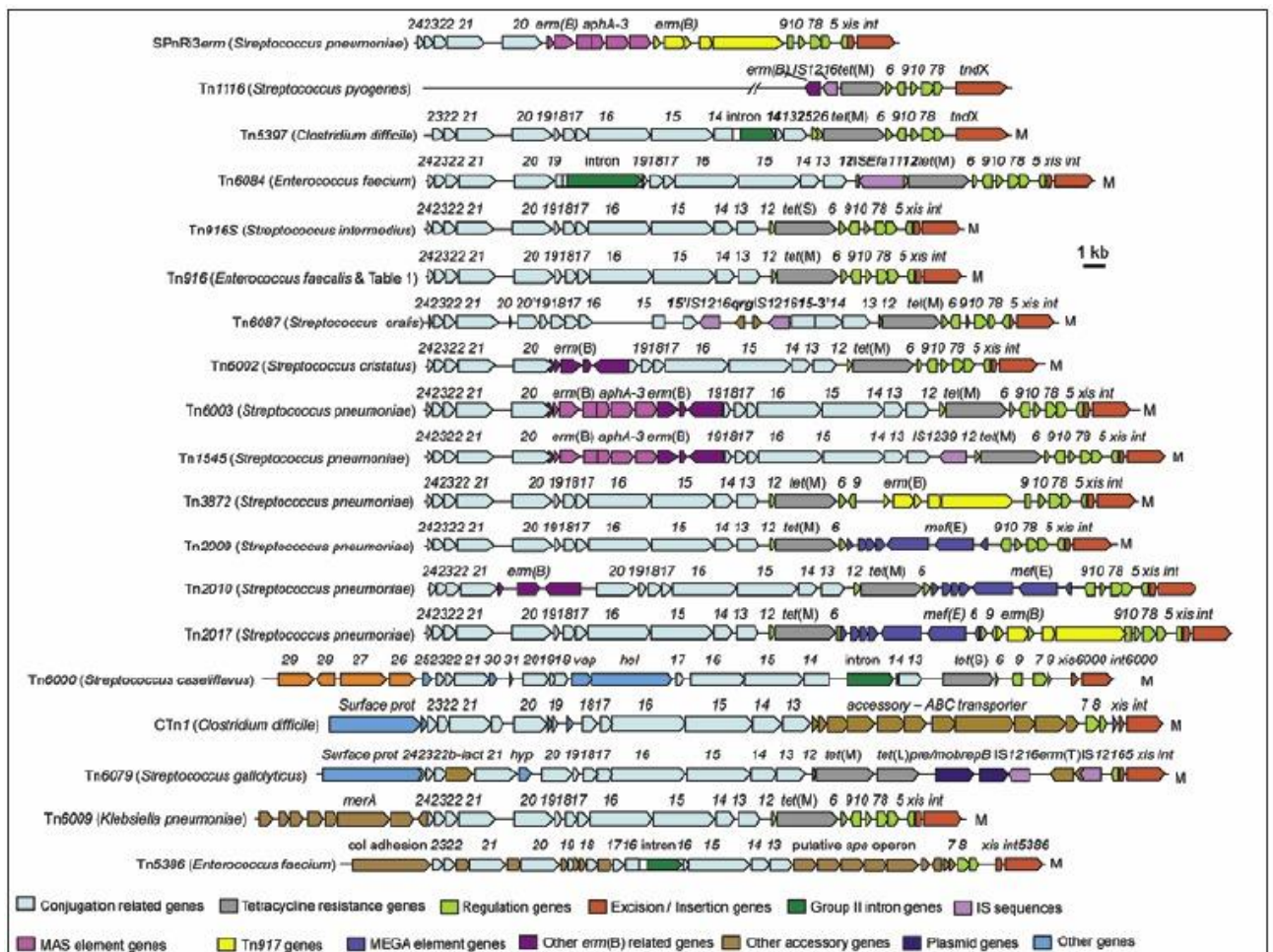


Figure 6.2 Illustration of Tn916-like Elements. A schematic of a selection of characterised Tn916-like elements detailing their conjugation, regulation and recombination modules as well as the different accessory genes and mobile elements they contain. This figure was reproduced from Ciric *et al.* (Ciric *et al.*, 2011a).

6.1.2.4 Tn916 Modifications by MGEs

A number of the Tn916-like elements discussed in this introduction contain other smaller MGEs including plasmids, IS elements and introns, Figure 6.2. These interactions allow Tn916-like elements to acquire new accessory genes and influence the structure of the regulation, conjugation and recombination modules.

Tn916 has itself been identified as part of a larger CTn, Tn5251. It is composed of a Tn5252-like CTn, into which a Tn916-like element has inserted; Tn916 can also independently transpose from Tn5251 (Provvedi *et al.*, 1996; Iannelli *et al.*, 2014).

6.1.3 The Effect of CTNs on the Host Genome

In addition to altering the host's phenotype through expression of accessory genes, CTNs may alter the host's genome in a number of ways. For instance, a CTn may insert into one of the host organism's genes, disrupting its expression. In fact, Tn916 is used as a mutagenesis tool to identify gene function (Lin and Johnson, 1991; Mullany, 2012). Alternatively, the insertion of a CTn into a gene may result in a fusion event giving rise to a novel protein. Full genome sequencing of the *C. difficile* strain 630 identified CTn5 inserted into a gene encoding for a putative surface protein (CD1844) resulting in a potential fusion protein between the CTn5 recombinase and the C-terminal end of CD1844 (Sebahia *et al.*, 2006). Fusion events have also been described within Tn916-like elements as a result of recombination. Croucher *et al.* (2011) analysed the genome sequences of 240 *S.*

pneumoniae isolates and identified such a recombination event that resulted in the loss of *aphA-3* and concomitant creation of a fusion between a repressor gene and *orf20* on a Tn916-like element (Croucher, 2011).

6.2 Chapter Aims

The aim of the work presented in this chapter was to analyse the sequence of the TT31 tetracycline-resistant clone, that was identified from the human saliva metagenomic library, to determine the likely source of the insert and what ORFs present on the insert may be responsible for the tetracycline resistance phenotype.

6.3 Methods

6.3.1 Determining the Size of TT31

The pCC1BAC insert from TT31 was extracted, digested using *HindIII* and analysed by agarose gel electrophoresis as described in 2.3.1.

6.3.2 Sequencing TT31

Primers used to sequence the TT31 insert by primer walking are detailed in Table 6.1.

Table 6.1 Primers used to Sequence the TT31 Insert.

Name	Sequence (5'-3')	Purpose	Source/Reference
pCC1-F	GGATGTGCTGCAAGGCGATTAAGTTGG	End sequencing of pCC1BAC	Epicentre®
pCC1-R	CTCGTATGTTGTGTGGAATTGTGAGC	End Sequencing of pCC1BAC	Epicentre®
TT31_F1	GGTTGACTCAGTGCTTATCG	Sequencing of TT31	This Study
TT31_R1	GAGGTCAGTCTGAACTTTGCG	Sequencing of TT31	This Study
TT31_F2	GAAGATGGAAGTGATGGAG	Sequencing of TT31	This Study
TT31_R2	GCAAACGACTGTTGAACC	Sequencing of TT31	This Study
TT31_F3	CTATCGCGACTAACATGG	Sequencing of TT31	This Study
TT31_R3	CTGCTCGGTGTATTCAAG	Sequencing of TT31	This Study
TT31_F4	CGTAATGGTTGTAGTTGC	Sequencing of TT31	This Study
TT31_R4	CGATTGGTGGAAATGATAGC	Sequencing of TT31	This Study
TT31_F5	GCTAAACCGAATAGAGCTC	Sequencing of TT31	This Study
TT31_R5	GCTTTCGTTACCAAAAACAG	Sequencing of TT31	This Study
TT31_F6	CGAAAGTTATCGGGACTG	Sequencing of TT31	This Study
TT31_R6	CTTTGCTGAGGTGGCAG	Sequencing of TT31	This Study
TT31_F7	GTTAGTATGGCCGCTTCAAG	Sequencing of TT31	This Study
TT31_R7	CCATCTTCTTTTCAGACC	Sequencing of TT31	This Study
TT31_F8	CGTTTAGCGATGAGTCGTG	Sequencing of TT31	This Study
TT31_R8	CTAACGAGCCATTGTTC	Sequencing of TT31	This Study
TT31_F9	GTAGGTTCTGTCGTATCG	Sequencing of TT31	This Study

TT31_R9	GTCGTATCAGGGCTCTTCC	Sequencing of TT31	This Study
TT31_F10	CTTACGATGGTAAACGAG	Sequencing of TT31	This Study
TT31_R10	CCGTAACAGGAGAACAG	Sequencing of TT31	This Study
TT31_R11	CGCTGAACATTACGCACAC	Sequencing of TT31	This Study

6.3.3 Sequence Analysis of TT31

The sequences obtained from primer walking were assembled to obtain the full TT31 sequence, using DNASTAR, 2.4.1, Appendix VII. The full TT31 sequence was analysed and putative ORFs were identified using the *in silico* tools as described in chapter 2.

6.3.4 Alignment of TT31 Sequence

As regions of TT31 had highest identity to Tn916 (accession: U09422), this sequence was obtained from the NCBI database and aligned with TT31. Additionally, as TT31 encoded a putative *tet(L)* gene, the Tn6079 (accession: GU951538), sequence as well as the *Lactobacillus johnsonii* strain BS15 (accession: CP016400) genome sequence [both of which encode *tet(L)*] were also obtained from the NCBI database and aligned with the TT31 sequence. Alignments were made using the ClustalΩ software.

6.4 Results

6.4.1 Size of the TT31 Insert

The pCC1BAC vector from TT31 was extracted and after digestion with *Hind*III and analysis by agarose gel electrophoresis showed it contained a 5 Kb insert, Figure 6.3 (a).

When the insert was sequenced it was revealed to be 14,226 bp in size. Apart from the *Hind*III sites flanking the insert, there were a further four *Hind*III sites within the insert; positioned at 8,532bp, 9,095 bp, 9,793 bp and 14,071 bp from the flanking *Hind*III site adjacent to the pCC1R primer binding site. If these three sites were successfully targeted by *Hind*III it would result in 5 DNA fragments (8,532 bp, 563 bp, 698 bp, 4,278 bp and 155 bp) upon digestion. DNA fragments of these sizes were not observed, Figure 6.3(a). It may be that the expected 8, 532 bp fragment is obscured by the 8 Kb pCC1BAC fragment and that the smaller than 1 Kb fragments are too faint to see on the gel. This would leave only the approximately 5 Kb fragment visible on the gel.

Figure 6.3

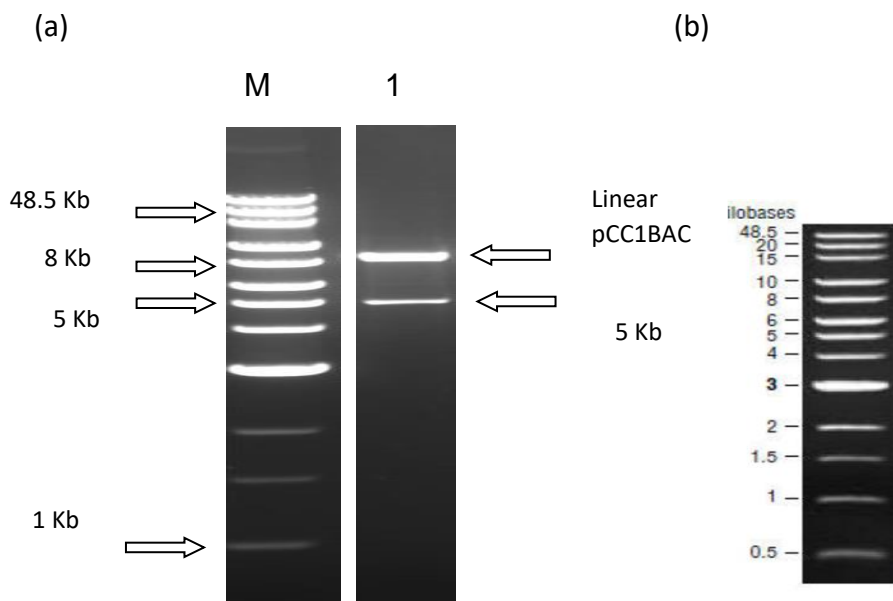


Figure 6.3 Agarose gel containing *HindIII* Digested TT31. The lane marked M contains the NEB 1Kb extended ladder and the lane Marked 1 contains the *HindIII* digested insert. (a) A 0.9 % agarose gel stained with ethidium bromide showing the *HindIII* digestion fragments of the TT31 insert. The gap in the gel is due to the removal of lanes not pertinent to the results being discussed. (b) An image depicting the NEB 1Kb extended ladder with size labels.

6.4.2 TT31 Sequence

BLASTN analysis of the 14,226 bp insert showed that from 1 bp to 7,226 bp (the left side of Figure 6.4) had 97 % nucleotide identity to Tn916 from *Enterococcus faecalis* (74 % cover). A 1,853 bp region of the left side of the TT31 insert did not align with the Tn916 sequence, Figure 6.5. Nine ORFs were identified in this region of the TT31 insert including *orf5*, *orf8*, *orf7* and *orf10* found in Tn916-like elements. A truncated *orf9* gene, *orf9t* was also present on TT31. The putative protein encoded by *orf9t* had 100 % amino acid identity to an *E. faecalis* T8 hypothetical protein (accession: EEU27120) that contains a DNA binding, helix-

turn-helix, motif. An excisionase and tyrosine integrase as well as *tet(M)* (also found on Tn916-like elements) were also encoded in this region of TT31. A *tet(L)* ORF with 99 % nucleotide identity to *tet(L)* from multiple species including *Enterococcus* spp. and *Streptococcus* spp. was also identified, Figure 6.4.

The right side of TT31, from 7,214 bp to 14,226 bp, exhibited 95 % nucleotide identity to *Gemella haemolysans* ATCC 10379 (accession: NZ_ACDZ00000000). Analysis of the right end of the TT31 insert, from 7,227 bp – 14,226 bp, revealed this region of TT31 to encode two half-ABC transporters (ABC2; 112 bp – 1,851 bp and ABC1; 1,838 bp and 3,598 bp), a SIR2 family protein deacetylase (3,599 bp – 4,345 bp) and a SecA translocase (4,428 bp – 6,998 bp) all of which had high amino acid similarity to proteins from *G. haemolysans* ATCC 10379 (>98 %), Figure 6.4. The full TT31 sequence is included in Appendix VII. No *HindIII* site was identified at the boundary between the left side of the TT31 insert (that had homology to Tn916) and the right side of the TT31 insert (that had homology to *G. haemolysans* ATCC 10379).

Figure 6.4

Left

Right

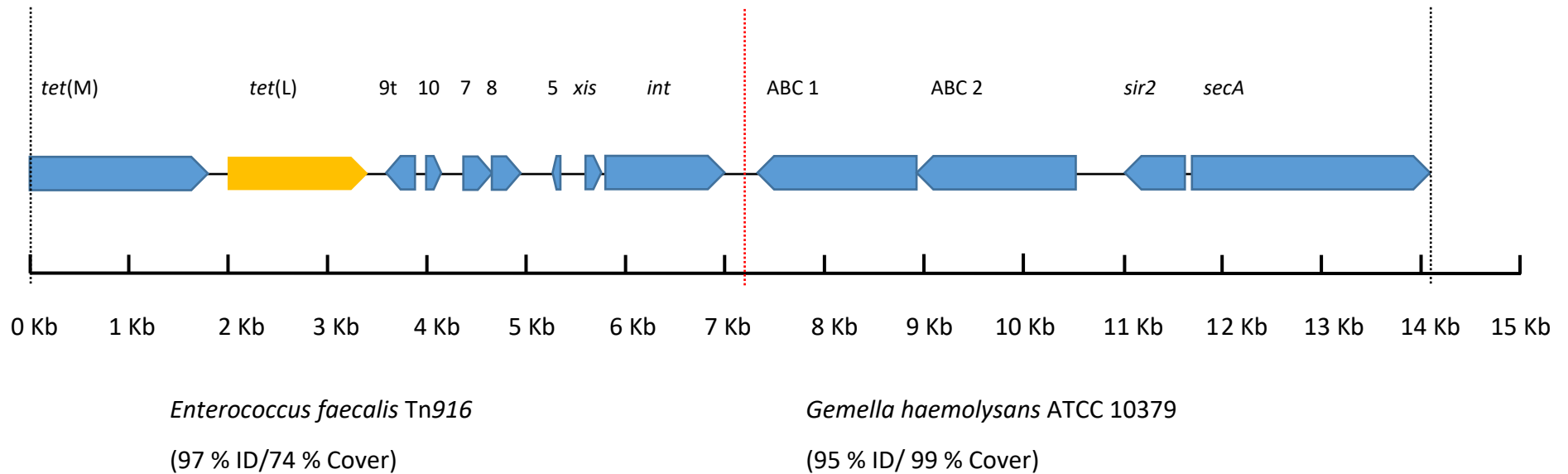


Figure 6.4 Schematic of the ORFs identified from the TT31 Clone. ORFs are represented by blue arrows. The vertical black dashed lines indicate the ends of the sequence.

The vertical dashed red line indicates the boundary between the regions of the sequence with nucleotide identity to Tn916 on the left and *Gemella haemolysans* ATCC 10379 on the right. *orf9t*, *orf10*, *orf7*, *orf8*, and *orf5* are indicated by 9t, 10, 7, 8 and 5 respectively. The excisionase and integrase genes are indicated by *xis* and *int* respectively. The closest nucleotide relatives are indicated. *tet(L)* is highlighted orange as it has no homologous region in Tn916.

6.4.3 TT31 Alignment with Tn916

An alignment between 1 bp - 7,226 bp of TT31 and a 5,844 bp region of *Enterococcus faecalis* Tn916 was conducted due to their high nucleotide identity. This region of *E. faecalis* Tn916 contained the regulatory genes, excision/insertion genes and *tet(M)* (Roberts and Mullany, 2009). The first 1,874 bp of TT31 aligned with the *tet(M)* encoding region of *Enterococcus faecalis* Tn916 (12,185 bp,– 14,058 bp; 96 % identity), although the first 62 bp of *tet(M)*, including the start codon, were not present in TT31, Figure 6.5 and Figure 6.6. The sequence alignment between TT31 (1 – 7,226 bp) and Tn916 is included in Appendix VIII.

Figure 6.5

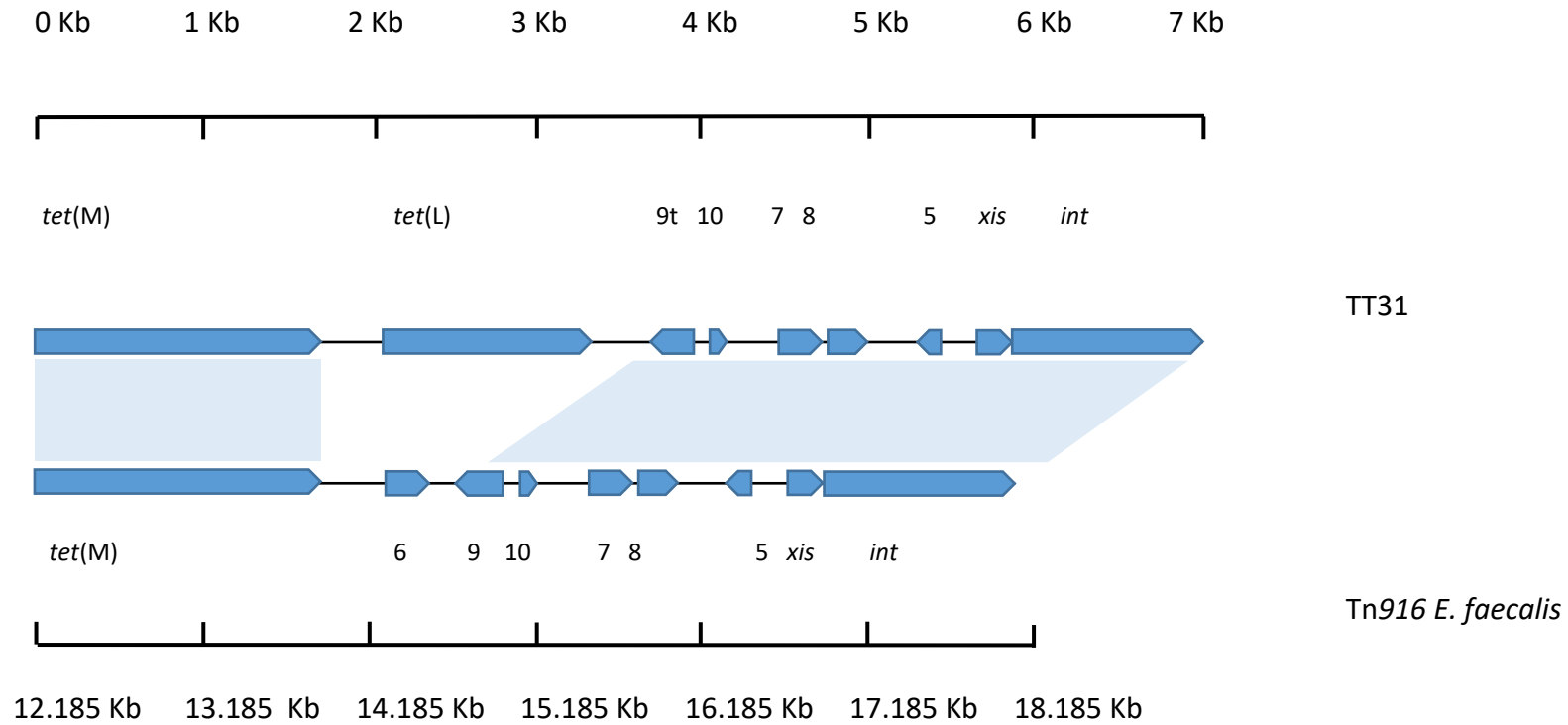


Figure 6.5 Schematic of TT31 Alignment with Tn916. An alignment between the left 7,226 bp of TT31 and the right 5,844 bp of Tn916. Regions of Tn916 and TT31 that aligned are indicated by the blue shaded areas. Each gene schematic is accompanied by its own scale. *tet(M)* is present in both as is the Tn916 regulation and recombination modules. TT31 however does not encode *orf6* and Tn916 does not encode *tet(L)*. Additionally, the TT31 *orf9* is truncated (9t) compared with Tn916 *orf9*.

Figure 6.6

```

TT31_1_7226 -----
Tn916      ATGAAAATTATTAATATTGGAGTTTTAGCTCATGTTGATGCAGGAAAAAC

TT31_1_7226 -----AAGCTTATTATATAACAGTGGAGCGATTACAGAATTAG
Tn916      TACCTTAACAGAAAGCTTATTATATAACAGTGGAGCGATTACAGAATTAG
                *****

TT31_1_7226 GAAGCGTGGACAAAGGTACAACGAGGACGGATAATACGCTTTTAGAACGT
Tn916      GAAGCGTGGACAAAGGTACAACGAGGACGGATAATACGCTTTTAGAACGT
                *****

TT31_1_7226 CAGAGAGGAATTACAATTCAGACAGGAATAACCTCTTTTCAGTGGGAAAA
Tn916      CAGAGAGGAATTACAATTCAGACAGGAATAACCTCTTTTCAGTGGGAAAA
                *****

TT31_1_7226 TACGAAGGTGAACATCATAGACACGCCAGGACATATGGATTTCTTAGCAG
Tn916      TACGAAGGTGAACATCATAGACACGCCAGGACATATGGATTTCTTAGCAG
                *****

TT31_1_7226 AAGTATATCGTTCATTATCAGTTTTAGATGGGGCAATTCTACTGATTTCT
Tn916      AAGTATATCGTTCATTATCAGTTTTAGATGGGGCAATTCTACTGATTTCT
                *****

```

Figure 6.6 Alignment of *tet(M)* from TT31 and Tn916. A ClustalΩ alignment between the first 300 bp of *tet(M)* from Tn916 and nucleotide positions 1 bp to 238 bp of TT31; indicating that the first 62 bp of *tet(M)* were not cloned in TT31. The conserved *Hind*III site between both sequences is highlighted in blue and this represents the start of the TT31 insert. The Tn916 *tet(M)* start codon is highlighted in yellow.

From 3,759 bp to 7,216 bp, TT31 had 97 % nucleotide identity to *Enterococcus faecalis* Tn916 (14,571 bp, – 18,032 bp), Figure 6.5. This region of TT31 encoded *orf10*, *orf7*, *orf8*, *orf5*, the *xis* and *int* genes and part of the *orf9* gene which is found in Tn916, Figure 6.5 (Roberts and Mullany, 2009). 1,875 bp – 3,759 bp of TT31 did not align with Tn916, this region encoded *tet(L)* which is not present in Tn916. Additionally, 14,058 bp – 14,570 bp of Tn916, that encoded *orf6*, did not align with TT31.

Interestingly, TT31 *orf9* appeared to be a truncated form, *orf9t*, of that found in Tn916. The first 172 nucleotides in *orf9* showed >99 % nucleotide identity with TT31 *orf9t*, which was 84 bp shorter at the 3' - end compared with its Tn916 homolog (354 bp), Figure 6.7.

Figure 6.7

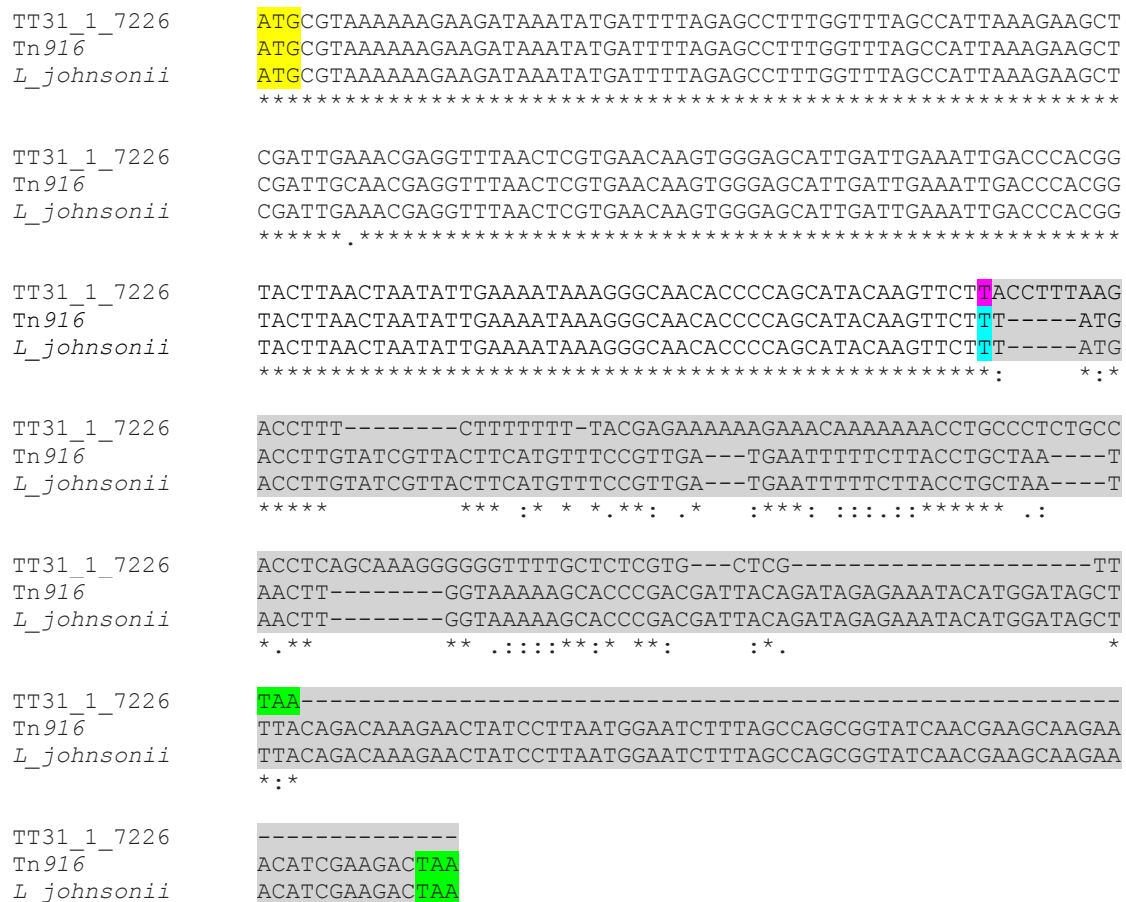


Figure 6.7 Alignment of *orf9* ORFs from TT31, Tn916 and *L. johnsonii*. A ClustalΩ alignment between the 354 bp *orf9* ORFs from Tn916 and *L. johnsonii* and the 270 bp *orf9* from TT31. The start codons are highlighted in yellow and the stop codons in green. TT31 *orf9* had 83.6 % and 84 % to its counterparts in Tn916 and *L. johnsonii* respectively. *orf9* ORFs from Tn916 and *L. johnsonii* were 84 bp longer than that encoded by TT31. The nucleotides following those highlighted in light blue are the 3'-end nucleotides of the full length *orf9* in Tn916 and *L. johnsonii* that are not present in TT31. The nucleotides following the nucleotide highlighted in purple are the 3'-end nucleotides of *orf9t*. These purple and blue highlighted nucleotides mark the point of divergence between *orf9* and *orf9t*. The nucleotide regions following these purple and blue nucleotides are highlighted in grey; they are unrelated sequences that ClustalΩ has attempted to align.

6.4.4 TT31 Alignment with Tn6079

An alignment of the left 7,226 bp of TT31 with the right 11,701 bp of Tn6079 revealed 99 % nucleotide identity (82 % cover) between these sequences. TT31, 1 bp - 3,758 bp, aligned with Tn6079 between 17,176 bp and 20,917 bp (99 % identity); this region contained *tet(M)*, *tet(L)* and the 3'-end of *orf9t* identified in TT31. The sequences also aligned between 4,993 bp and 7,226 bp on TT31 and 26,639 bp and 28,876 bp on Tn6079 (97 %), Figure 6.8. These regions contained *orf5* and the *xis* and *int* genes. The region of TT31 containing *orf8*, *orf7*, *orf10* and the 5' – end of *orf9t* (3,759 bp – 4,993 bp) did not align with Tn6079. Additionally, the region of Tn6079 that encodes a plasmid recombination protein (*pre/mob*), a plasmid replication protein (*rep*) and *erm(T)* that is flanked by *IS1216* sequences (20,918 bp – 26,639 bp) did not align with TT31, Figure 6.8. The sequence alignment between TT31 (1 – 7,226 bp) and Tn6079 is included in Appendix IX.

Figure 6.8

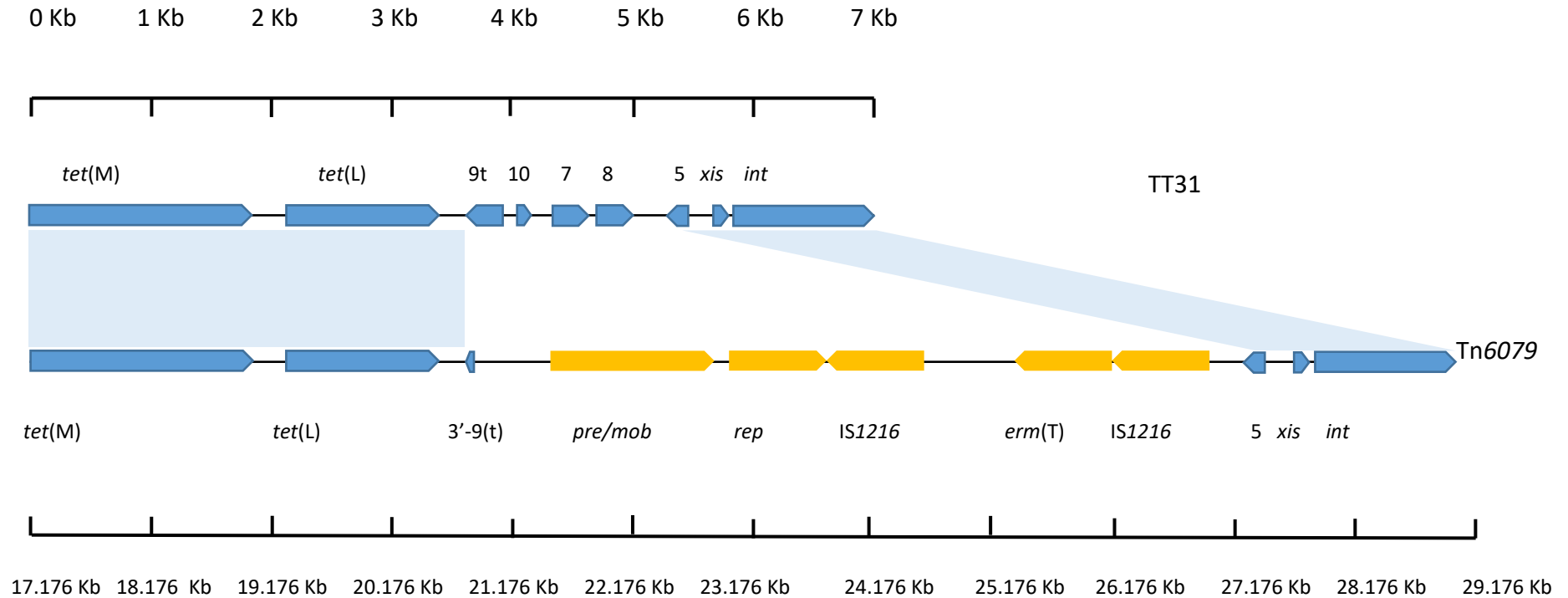


Figure 6.8 Schematic of TT31 Alignment with Tn6079. An alignment between the right 7,226 bp of TT31 and the right 11,701 bp of Tn6079. Regions of Tn6079 and TT31 that aligned are indicated by the blue shaded areas. Each gene schematic is accompanied by its own scale. *tet(M)* and *tet(L)* are present in both as are *orf5*, *xis* and *int*. Tn6079 however does not encode *orf9*, *orf10*, *orf7* or *orf8*. Additionally, Tn6079 encodes *pre/mob*, *rep* and *erm(T)* genes (the latter of which is flanked by IS1216 sequences) that are not present on TT31, highlighted in orange.

6.4.5 TT31 Alignment with *Lactobacillus johnsonii* strain BS15

An alignment of the left 7,226 bp of TT31 with a 12,313 bp region of the *L. johnsonii* strain BS15 genome revealed the sequences to have high nucleotide identity (99 % cover/97 % ID). There appeared to be an insertion in *L. johnsonii* relative to TT31, in *orf9t*, between nucleotides 3, 758 bp and 3, 759 bp as they aligned to positions 41, 612 bp and 46, 612 bp respectively on the *L. johnsonii* genome, Figure 6.9. The intervening 4,999 bp region was analysed by BLASTX and found to contain 4 ORFs. These ORFs encoded *pre/mob* and *rep* genes, a transposase of the IS110 family and an *orf6* homolog. The *pre/mob* and *rep* genes had >99 % nucleotide identity to those encoded by Tn6079.

This insertion also contained 84 bp of the 3' - end of *orf9* that is found in Tn916 but not in TT31, Figure 6.9. This 3' - end was in frame with the start codon containing 5' - end of the *orf9* and so *L. johnsonii* strain BS15 encoded the full length *orf9*, Figure 6.7. The 3' - end of *orf9t* remained in the *L. johnsonii* genome, outside of the insertion, Figure 6.9. Additionally, a small region from 1,875 bp – 2002 bp on the TT31 insert did not align with the *L. johnsonii* strain BS15 genome between 1,975 bp - 2,085 bp. The sequence alignment between TT31 (1 – 7,226 bp) and *L. johnsonii* strain BS15 is included in Appendix X.

Figure 6.9

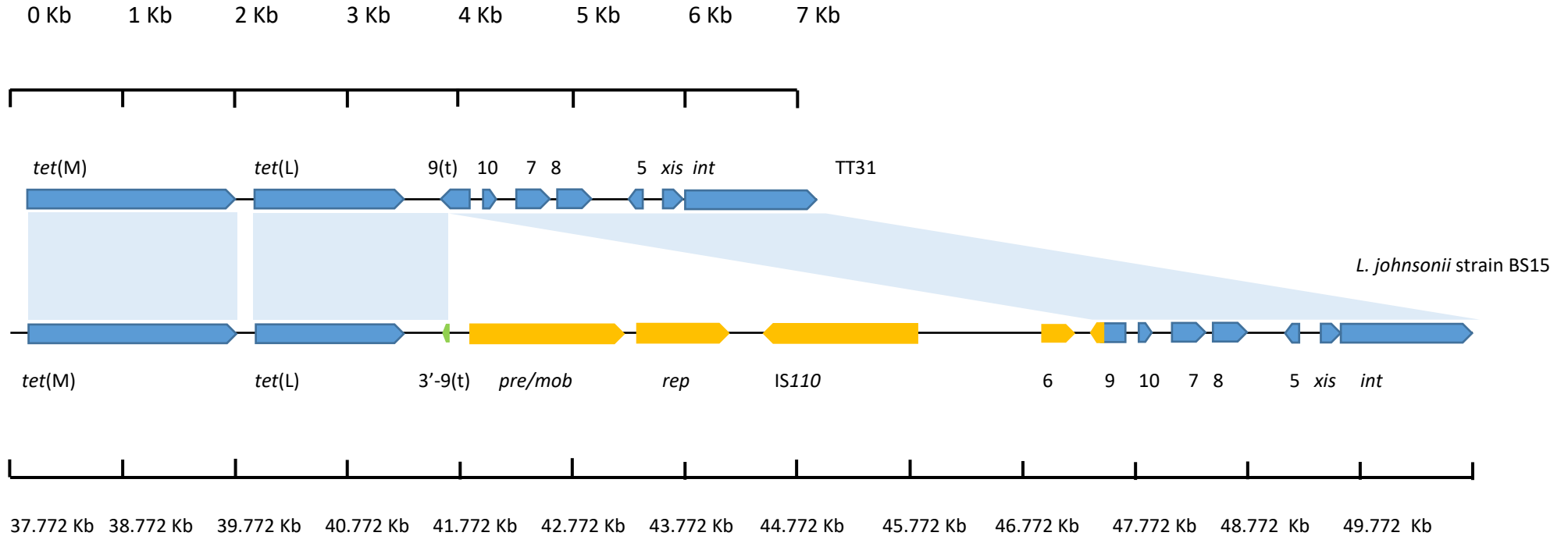


Figure 6.9 Schematic of TT31 Alignment with *L. johnsonii* strain BS15 Genome. An alignment between the right 7,226 bp of TT31 and a 12,313 bp region of the *L. johnsonii* strain BS15 genome. Regions of alignment between these sequences are indicated by the blue shaded areas. Each gene schematic is accompanied by its own scale. *tet(M)* and *tet(L)* are present in both as are *orf10*, *orf7*, *orf8*, *orf5*, *xis* and *int*. TT31 encodes *orf9t*. The *L. johnsonii* strain BS15 genome contains an insertion in its *orf9t* relative to TT31. This insertion encodes *pre/mob*, *rep*, IS110 transposase and *orf6* genes; highlighted in orange. The insertion also contains the 84 bp 3' - end of the full length *orf9* (highlighted in orange) in frame with the start codon of the 5' end of *orf9* that is found in all the sequences (highlighted in blue); thus *L. johnsonii* encodes the full length *orf9*. The 3' - end of *orf9t* is still present in the *L. johnsonii* genome and is highlighted in green.

The *E. faecalis* Tn916 sequence was aligned to the *L. johnsonii* strain BS15 genome. The left 14,132 bp of Tn916 aligned with the *L. johnsonii* genome at positions 25,590 bp – 39,719 bp (99% identity). An alignment with the *L. johnsonii* genome at positions 46,167 bp – 50,073 bp was observed (99% identity), from 14,126 bp – 18,032 bp on Tn916. The region of the *L. johnsonii* strain BS15 genome that did not align with *E. faecalis* Tn916 contained *tet(L)* that was also identified in TT31, and the plasmid recombination and replication and transposase ORFs. Interestingly, although *orf6* and the full length *orf9* are not present on the TT31 insert they are present in both *L. johnsonii* strain BS15 and *E. faecalis* Tn916, Figure 6.10. This comparison indicated that *L. johnsonii* strain BS15 encoded a Tn916-like element. The sequence alignment between Tn916 and the *L. johnsonii* strain BS15 genome is included in Appendix XI.

Figure 6.10

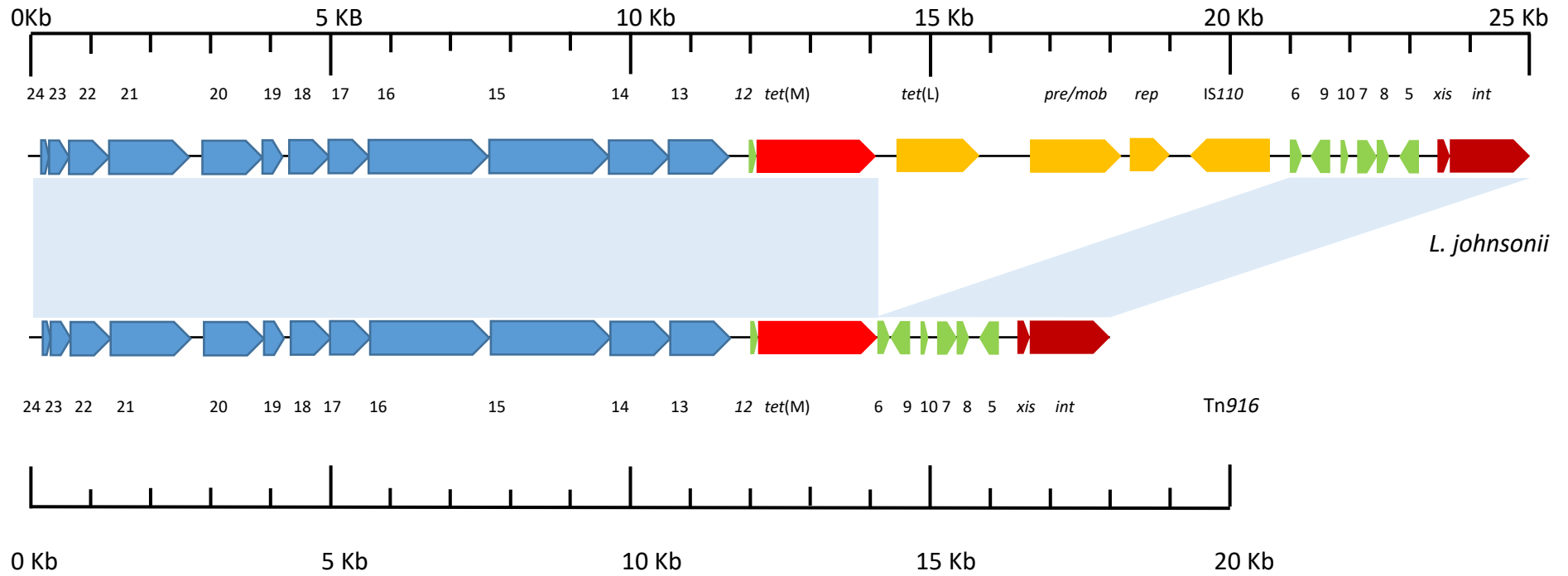


Figure 6.10 Schematic of the Alignment of Tn916 and *L. johnsonii* strain BS15 Genome. Regions of alignment between these sequences are indicated by the blue shaded areas. Each gene schematic is accompanied by its own scale. This region of the *L. johnsonii* strain BS15 genome aligns with the conjugation module (blue arrows), regulation module (green arrows), recombination module (dark red arrows) and the *tet(M)* gene of Tn916. The *tet(L)*, *pre/mob*, *rep* and *IS110* transposase genes encoded by the *L. johnsonii* strain BS15 genome are not present on the Tn916-like element. These results indicate that *L. johnsonii* strain BS15 encodes a Tn916-like element.

6.4.6 Potential Tetracycline Resistance Genes in TT31

The partial *tet(M)* ORF in TT31 showed 96 % nucleotide identity across its length to the *tet(M)* gene in *E. faecalis* Tn916. However, TT31 *tet(M)* appeared to be lacking the first 62 bp compared with Tn916 *tet(M)* as a result of cleavage at a *Hind*III site [conserved in Tn916 *tet(M)*] leading to a loss of its start codon. Thus it is likely that *tet(M)* is not expressed in TT31, Figure 6.6. The putative products of the half-ABC transporter ORFs (ABC1 and ABC2) in TT31 showed high amino acid identity to two multidrug half-ABC transporters from the *G. haemolysans* ATCC 10379 (99 % cover, 99 % and 98 % identity respectively).

TT31 also contained a MFS ORF with high nucleotide similarity to *tet(L)* from *Enterococcus* spp. and *Staphylococcus* spp. (100 % cover/99 % ID) as previously mentioned.

6.5 Discussion

TT31 was identified when the human saliva metagenomic library was screened for tetracycline resistance. *Hind*III digestion of the TT31 insert revealed an apparent 5 Kb insert, although sequencing determined the insert to have a 14,226 bp insert. The insert sequence had 4 *Hind*III sites other than the flanking *Hind*III sites at positions 8, 532 bp, 9,095 bp, 9,793 bp and 14,071 bp. Digestion of the insert at these sites would produce DNA fragments of 8,532 bp, 563 bp, 698 bp, 4, 278 bp and 155 bp. Only a single fragment of approximately 5 Kb was observed which may be represented by the approximately 5 Kb fragment identified by agarose gel electrophoresis. Additionally, the expected 8, 532 bp fragment may have been obscured by the pCC1BAC vector which is 8,128 bp in size. The smaller fragments may have been too faint to be seen on the gel.

Sequencing of the 14,226 bp insert revealed it to be made up of a 7,226 bp region with $\geq 97\%$ nucleotide sequence identity to Tn916 and a region (7,000 bp) with 95% nucleotide identity with *G. haemolysans* ATCC 10379. As no *Hind*III site was found to divide the insert into these Tn916-like and *G. haemolysans* regions, the insert was not a concatemer.

Four ORFs that potentially contributed to the observed tetracycline resistance phenotype were identified on the insert: *tet*(M), *tet*(L) and two genes encoding a putative MDR heterodimeric ABC transporter. Alignment of the TT31 *tet*(M) with its counterpart in Tn916 revealed them to have $>90\%$ nucleotide identity. However, the first 62 bp (including the start codon) of the *tet*(M) gene present in Tn916 was not cloned into TT31 as a result of restriction at a conserved *Hind*III site at this location in the gene. Although there is an ATG codon on the pCC1BAC backbone, between the T7 promoter and the insert, it is not in frame

with the *tet(M)* ORF. Additionally, the T7 polymerase is not encoded by pCC1BAC or *E. coli* EPI300 T1^R. As such, although this *tet(M)* gene may play role in conferring resistance in its host cell, it is unlikely that it is expressed in TT31. Thus, the tetracycline resistance phenotype is likely a result of the expression of *tet(L)* and/or the heterodimeric ABC transporter, though further characterisation of these genes is required to confirm if this is the case.

As TT31 had 95 % nucleotide identity (between 7,214 and 14,226 bp) with a region of the *G. haemolysans* ATCC 10379 genome, it indicated that the cloned insert contained a partial Tn916-like element in a *Gemella* spp. host. *Gemella* spp. are Firmicutes and are normal lactic acid-producing commensals of the oral cavity (Stackebrandt *et al.*, 1982; Dewhirst *et al.*, 2010). *tet(M)* mediated tetracycline resistance has been described in *Gemella* spp. before; evidence for its association with the Tn916 *int* in this genus was generated when Cerdá Zolezzi *et al.* (2004) used PCR to identify these markers in nasopharyngeal *Gemella* spp. isolates (Olsvik *et al.*, 1995; Cerda Zolezzi *et al.*, 2004; Zolezzi *et al.*, 2007). Apart from this study little evidence for the acquisition of *tet(M)* or other antibiotic resistance genes from Tn916-like elements by *Gemella* spp. has been generated, even though they are likely to interact with *Streptococcus* spp. in the oral cavity which are well studied as carriers of these CTns (Ciric *et al.*, 2012).

As a region of TT31 showed $\geq 97\%$ nucleotide identity to the *tet(M)*, regulation and conjugation modules of Tn916, it indicated that part of a Tn916-like CTn was present in the clone. Interestingly, although *orf10*, *orf7*, *orf8*, *orf5*, *xis* and *int* were conserved between TT31 and Tn916, there were substantial differences between TT31 and the Tn916 sequences. For example, TT31 did not encode an *orf6* homolog but did encode a truncated *orf9* that was 84 bp shorter than its Tn916 counterpart. As the *orf9* product is thought to be

responsible for repressing *orf7* and *orf8* expression and consequently excision of Tn916, it would be of interest to determine if the truncated *orf9* product can function in regulating the transposition of a Tn916-like element. Indeed, the fact that the putative *orf9t* product has 100 % amino acid identity to a putative DNA binding protein encoded by *E. faecalis* T8 provides further evidence for such a function of Orf9t. It is also thought that transcription through *tet(M)* results in the transcription of antisense *orf9* mRNA. If *orf9t* is functional it would provide further evidence that the antisense *orf9* transcript inhibits translation of the sense *orf9* transcript, as the *orf9t* antisense transcript would still be able to inhibit translation (Roberts and Mullany, 2009).

The most noticeable difference between TT31 and Tn916 is the presence of a 1,853 bp insert that contains *tet(L)*. If this region of TT31 does represent the right end of a Tn916-like element it is unknown what selection advantage carrying two tetracycline resistance genes of different classes would give to the CTn or the host cell. As *tet(L)* is an antiporter it is possible that under certain environmental conditions, such as low pH, it confers a selection advantage in the presence of tetracycline. Alternatively, the transporter encoded by this *tet(L)* homolog may have different substrates that may result in its selection in the absence of tetracycline.

To the best of our knowledge no other Tn916-like element has this genetic organisation. However, the initial BLAST analysis of the left 7,226 bp of TT31 revealed it to have 97% nucleotide identity to a 12,313 bp region of the *L. johnsonii* strain BS15 genome. Relative to TT31 however, the *L. johnsonii* strain BS15 genome contains a 4,999 bp insert in *orf9t*. This insert contains *pre/mob*, *rep*, transposase and *orf6* ORFs. Interestingly, the insert also contains the 3' - 84 bp section of the full length *orf9* that is missing in TT31. This 84 bp

sequence is in frame with the start codon-containing 5'-end of *orf9t* such that *L. johnsonii* strain BS15 encodes the full length *orf9* found in Tn916. Subsequent analysis of the *L. johnsonii* strain BS15 genome shows that a Tn916-like conjugation module is present upstream of the region that aligns with the TT31 insert, and that the genome contains a Tn916-like element.

This indicated that TT31 contains a region of an 'ancestral' Tn916 element and that a hypothetical series of events of acquisition and subsequent loss of genes by this 'ancestral' element resulted in the creation of Tn916 and that the *L. johnsonii* strain BS15 genome hosts a Tn916-like element that is an intermediate between the two. The series of events may have occurred as follows. A plasmid containing the *pre/mob*, *rep*, transposase and *orf6* ORFs as well as the 3' - 84 bp of the full length *orf9* gene may have inserted into *orf9t* of the TT31 Tn916-like element, resulting in the creation of the Tn916-like element identified in *L. johnsonii* strain BS15. Following this, an imprecise excision or recombination event mediated by the transposase or the plasmid's recombination protein, encoded by *pre/mob*, may have occurred whereby sites outside of the plasmid were recognised resulting in the excision of a region of the CTn containing *tet(L)* and the *pre/mob*, *rep* and transposase genes but leaving *orf6* and the full length *orf9* fusion behind, thus creating Tn916, Figure 6.11. Imprecise excision events have been observed previously. For example, Laverde Gomez *et al.* (2011) observed fragments of the pathogenicity island of *E. faecalis* strain UW3114 to remain in the chromosome following excision. The authors concluded this imprecise excision occurred due to the recognition of sequences flanking the element that had homology to internal sequences required for excision (Laverde Gomez *et al.*, 2011).

Figure 6.11

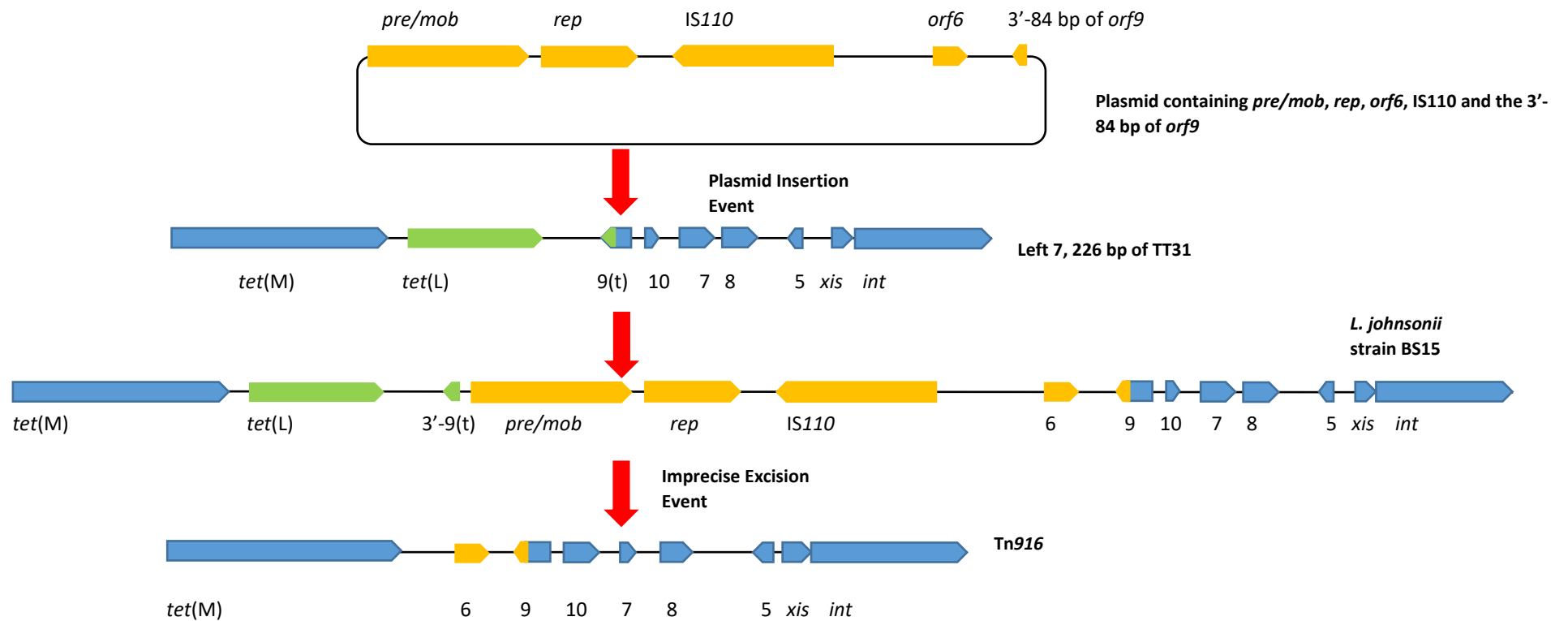


Figure 6.11 Illustration of the Relationship Between TT31 and Tn916. An illustration of the hypothetical events that may have led to the creation of Tn916. This hypothetical event assumes that the left 7,226 bp of TT31 are a part of a larger Tn916-like element. Gene schematics are not to scale. A plasmid containing *pre/mob* and *rep* genes, *orf6*, IS110 and the 3' - end of *orf9* (orange) inserts into the TT31 Tn916-like element between. This creates the Tn916-like that we described above as being encoded by the *Lactobacillus johnsonii* strain BS15 genome. This event disrupts the *orf9t* reading frame (green and blue) and in doing so creates the larger *orf9* (orange and blue). This is followed by an imprecise excision event that results in the loss of the *pre/mob*, *rep* and IS110 as well as *tet(L)* and the 3' - end of *orf9t*; creating Tn916.

Although Tn6079 encodes a *tet(L)* gene there are large differences in the structure of their respective regulation modules. The regulation module is absent from Tn6079 and instead, plasmid genes (*pre/mob* and *rep*) and *erm(T)* flanked by IS1216 elements are encoded between *tet(L)* and its recombination module. TT31 and Tn6079 shared 99% nucleotide identity outside of these regions and the latter's *pre/mob* and *rep* genes were >99 % identical to those of found in *L. johnsonii*; additionally Tn6079 contained the 3' – end of *orf9t*. Thus, it is possible to hypothesise a relationship between TT31 and Tn6079 whereby TT31 is a parent Tn916-like element to Tn6079 and the *L. johnsonii* Tn916 is an 'intermediate'. As described previously for the hypothetical evolution of Tn916, a plasmid encoding *pre/mob*, *rep*, transposase and *orf6* ORFs as well as the 3' - 84 bp of the full length *orf9* inserts into *orf9t* of TT31, creating the *L. johnsonii* CTn element. A recombination event then occurs between two regions of this CTn which results in the deletion of *orf10*, *orf7*, *orf8*, *orf6* and *orf9*. This is similar to the event hypothesised by Croucher *et al.* (2011) to have deleted *aphA-3* in an *S. pneumoniae* Tn916-like element (Croucher, 2011). Following this, *erm(T)* flanked by two IS1216 elements inserts upstream of *orf5* to give rise to Tn6079, Figure 6.12. It is also possible that a lone IS1216 element inserted up stream of *orf5* followed by the insertion of a transposable unit containing a second IS1216 element and *erm(T)*.

Figure 6.12

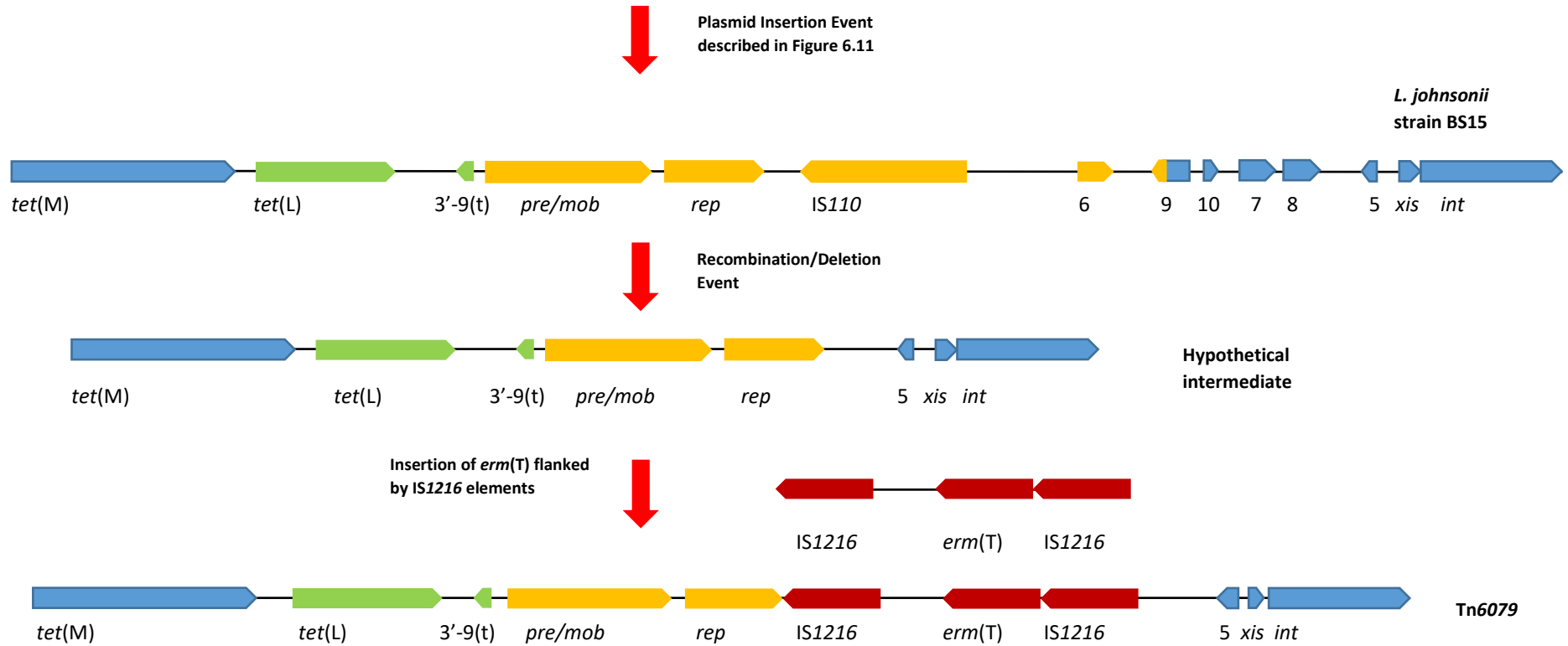


Figure 6.12 Illustration of the Relationship Between TT31 and Tn6079. An illustration of the hypothetical events that may have led to the creation of Tn6079. This hypothetical event assumes that the left 7,226 bp of TT31 are a part of a larger Tn916-like element. Following on from the generation of the *Lactobacillus johnsonii* strain BS15 Tn916-like element described in Figure 6.11, a recombination/deletion event results in the loss of *orf6*, *orf9*, *orf10*, *orf7* and *orf8*; forming a hypothetical intermediate CTn. Following this deletion event, the insertion of *erm(T)* flanked by IS1216 elements (dark red) occurs creating Tn6079. For continuity with Figure 6.11, *tet(M)*, *orf5*, *xis* and *int* are coloured blue, *tet(L)* and the 3' – end of *orf9(t)* are green and the plasmid associated genes are orange.

Similar events resulted in the acquisition of galactose metabolism genes by lambda-specialised transduction phages and have been described in prokaryotes also (Lundblad *et al.*, 1984; Campbell, 2007; Rice *et al.*, 2005). For example, Tn916 has been shown to mediate the excision of itself and an adjacent Tn5386, as well as the genomic material between them from its host cell, resulting in a deletion of 178 Kb from the genome (Rice *et al.*, 2005).

These hypotheses, if correct, point to a Tn916-like element with a gene organisation as described in TT31 as an ancestor of both Tn916 and Tn6079, and suggest that the Tn916-like element encoded by *L. johnsonii* strain BS15 may act as a point of evolutionary divergence between these two CTns. Much of this discussion relies on the assumption that the TT31 sequence is a fragment of a larger Tn916-like element in a *Gemella* spp. host as determined from the sequence of this metagenomic clone and although this seems a reasonable assumption to make, it is just that. Further work is required to discern if a full length Tn916-like CTn with a regulation, recombination and *tet(M)* module structure like the one identified in the TT31 clone exists in the human saliva metagenome. One way of accomplishing this would be to use a DNA hybridization capture protocol. This would involve the design of a biotinylated DNA probe that would hybridize only to the DNA fragments that are complementary to the sequences of the TT31 Tn916-like element. These DNA-probe duplexes can then be removed from the more complicated metagenomic sample using magnetic streptavidin beads and subsequently sequenced. It would also be of interest to determine whether this Tn916-like element is present in oral bacteria other than *Gemella* spp. and how common it is in the human oral cavity.

Chapter 7

Analysis of the CTAB-Resistant Clone: A10(F2)

A10(F2) was identified when a subset of the clones in the human saliva metagenomic library were cultured in 3.7 µg/ml CTAB.

7.1 Introduction

7.1.1 GalE

7.1.1.1 Molecular Mechanism of GalE Activity

GalE is a UDP-glucose 4-epimerase encoded for by *galE*. GalE functions as a homodimeric epimerase that catalyzes the reversible reaction UDP-galactose to UDP-glucose, two nucleotide sugars that differ in the orientation of their 4-hydroxyl group on the sugar, Figure 7.1 (Bengoechea *et al.*, 2002; Pena *et al.*, 2015; Thoden *et al.*, 2001). GalE favours the production of UDP-glucose as the enzyme has a higher affinity for binding UDP-galactose. The K_m of UDP-glucose (1000 μM) has been calculated at almost 10-fold greater than that of UDP-galactose (160 μM) for GalE (Rodríguez-Díaz and Yebra, 2011; Vorgias *et al.*, 1991). X-ray crystallographic analysis of GalE from *E. coli* has shown each GalE subunit to have an independent active site with the N-terminal mediating nicotinamide adenine dinucleotide (NAD) binding and the nucleotide sugar binding in the C-terminal (Bauer *et al.*, 1992; Nayar and Bhattacharyya, 1997).

In a series of publications Thoden *et al.* utilised functional assays in conjunction with mutagenesis and X-ray crystallography to characterise how GalE catalyzes this reaction. NAD is a cofactor in the interconversion of these nucleotide sugars. During the epimerase reaction NAD^+ acts as an oxidising agent, receiving an electron from the 4'-hydroxyl group of either UDP-galactose or UDP-glucose. The resulting 4-ketopyranose intermediate rotates 180° about this 4'-ketone group before NADH, acting as a reducing agent, transfers an electron to it to restore the 4'-hydroxyl group. The transfer of the electron between the

NAD cofactor and the nucleotide sugar is mediated by a conserved tyrosine residue in GalE (Liu *et al.*, 1997; Thoden *et al.*, 1997, 2000, 2002). GalE from *S. sanguinis* and *B. subtilis* have also been shown to be able to catalyse the interconversion of UDP-N-acetylgalactosamine (UDP-GalNAc) and UDP-N-acetylglucosamine (UDP-GlcNAc) (Soldo *et al.*, 2003; Yang *et al.*, 2014a).

Figure 7.1

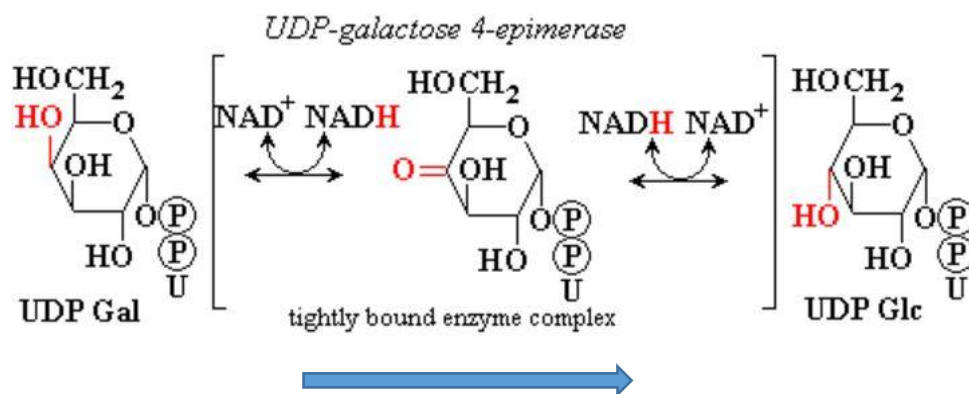


Figure 7.1 The Reaction Catalysed by GalE. A depiction of the reversible conversion of UDP-galactose to UDP-glucose that is catalysed by GalE. The 4-hydroxyl group on both nucleotide sugars is highlighted in red as is the hydrogen that is transferred as NAD^+ is reduced to NADH during the reaction. The blue arrow indicates the direction of the reaction that is favoured by GalE.

This Figure was amended from <http://www.chemistry.uoguelph.ca/educmat/chm452/lecture7.htm>

(Chem*4520 Metabolic Processes).

7.1.1.2 Genetic Locations of *galE*

galE has been identified in numerous organisms including bacteria, plants and mammals (Dormann and Benning, 1998; Krispin and Allmansberger, 1998; Schulz *et al.*, 2004). In *E. coli*, *galE* is found in the *gal* operon along with *galT*, *galK* and *galM* that encode a uridylyltransferase, kinase and mutarotase respectively and are required for D-galactose metabolism (Wang *et al.*, 2014). *gal* operon expression is regulated by the availability of glucose; when glucose levels are high, the GalR repressor binds to operator sequences flanking the *galE* promoter creating a loop structure that is stabilised by the HU protein which results in only basal level transcription of *galE*. When galactose is present and glucose is not available the former can bind GalR and disrupt the nucleoprotein complex, de-repressing *galE* expression. Semsey *et al.* (2006) demonstrated this complex dissociation by showing that increasing concentrations of D-galactose resulted in a reduction in GalR crosslinking and an increase in *in vitro* transcription from these operators. (Lee *et al.*, 2008; Semsey *et al.*, 2006). The limited availability of glucose in such circumstances results in increased levels of cyclic adenosine monophosphate (cAMP) which triggers operon transcription when bound to the cAMP receptor protein (Busby and Ebright, 1999; Narang, 2009).

Interestingly, a second *galE* in an O-antigen biosynthesis locus of *E. coli* strain O113 has been identified. The O-antigen repeat region of this strain is galactose-rich and so the second *galE* may provide a source of UDP-galactose that is not catabolically repressed (Parolis and Parolis, 1995; Paton and Paton, 1999). In *Streptococcus* spp., *galE* may be located in a *gal* operon, though *galE* is typically the third gene in the operon rather than the

first and in *S. pneumoniae* *galE* is separate from the *gal* operon (Vaillancourt *et al.*, 2002). As with *E. coli* O113, some oral *Streptococcus* spp. encode two *galE* genes. The 'second' gene is typically found in receptor polysaccharide synthesis operons important for coaggregation (Yang *et al.*, 2014a).

galE has been identified in loci outside of the typical *gal* operon. In *Pasteurella haemolytica* A1 for instance *galE* was not found with the *galT* and *galK* genes (Potter and Lo, 1996). Similarly, *galE* in *Haemophilus influenzae* is not located in an operon with the other *gal* genes (Maskell *et al.*, 1991, 1992). In *Acidithiobacillus ferrooxidans*, *galE* has been identified in an iron regulated operon with *luxA*, *galM*, *galK* and *pgM* (Barreto *et al.*, 2005). In *Neisseria meningitidis* Group B and *Erwinia stewartii*, *galE* is found in capsule biosynthesis loci (Dolph *et al.*, 1988; Hammerschmidt *et al.*, 1994). The different loci of *galE* in different organisms likely reflects the different functions UDP-galactose plays in these cells.

That *galE* has been found in different loci, and that some genomes encode multiple copies of the gene, suggests that they may be mobilised. For example, two near identical copies of *galE* have been identified in *N. meningitidis* Group B (Jennings *et al.*, 1993). Additionally, *galE* from *P. haemolytica* was found to be bound by 11-bp direct repeats which may be indicative of a transposition event and that may explain its separation from the other *gal* genes (Potter and Lo, 1996).

7.1.1.3 Functions of GalE

GalE along with the previously mentioned GalT, GalK and GalM enzymes are involved in the Leloir pathway. In the Leloir pathway, UDP-galactose is formed when GalT transfers the UDP group of UDP-glucose to galactose-1-phosphate. During this reaction glucose-1-phosphate (G-1-P) is also generated which can be converted to glucose-6-phosphate and used in glycolysis. The epimerase activity of GalE can then regenerate UDP-glucose from UDP-galactose (Holden *et al.*, 2003), Figure 7.2.

Figure 7.2

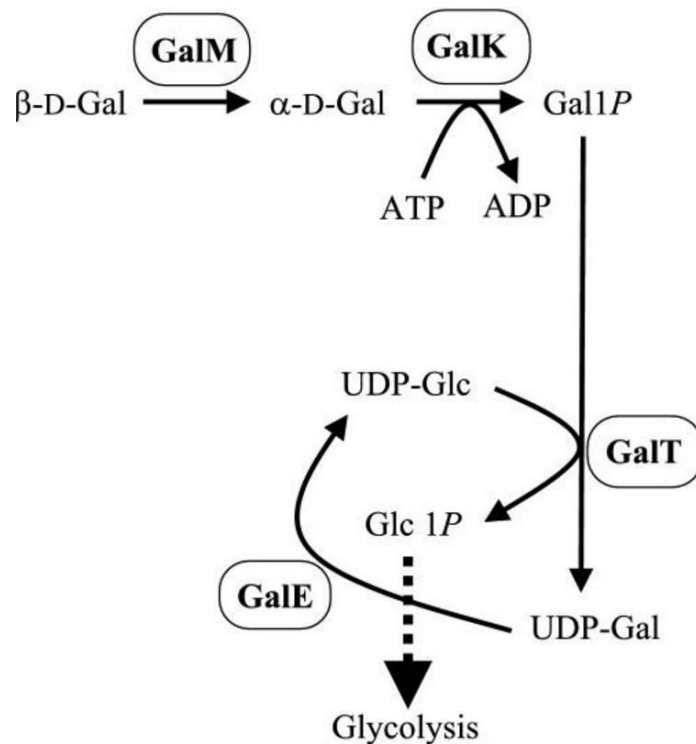


Figure 7.2 The Leloir Pathway. An Illustration of the roles the GalM, GalK, GalE and GalT enzymes play in the Leloir pathway, reproduced from Nishimoto and Kitaoka (Nishimoto and Kitaoka, 2007).

UDP-galactose and UDP-glucose, the final products of the Leloir pathway, can act as donor molecules for the glycosylation of proteins, EPS components and LPS, as such GalE has been shown to be required for the synthesis of these biologically important structures, particularly when exogenous galactose is unavailable.

UDP-Galactose and UDP-GalNAc that are produced by GalE are required for the synthesis of *B. subtilis* EPS (Chai *et al.*, 2012). They are also important components of receptor polysaccharides (RPS) produced by *Streptococcus* spp that are involved in coaggregation. In

fact, Xu *et al.* (2003) showed that insertional inactivation of GalE2 in *S. sanguinis* resulted in a loss of RPS synthesis and coaggregation with *Actinomyces naeslundii* (Xu *et al.*, 2003).

In *E. coli* and *Salmonella enterica* serovar Typhimurium, GalE is required for synthesis of the galactose-containing EPS component colanic acid (Costa *et al.*, 2003; Stevenson *et al.*, 1996). As colanic acid plays a role in *E. coli* biofilm formation, GalE may play a role in the formation of *E. coli* biofilms (Danese *et al.*, 2000). Furthermore, Chai *et al.* (2012) demonstrated that a loss of GalE activity in *B. subtilis* resulted in a loss of biofilm formation in the absence of exogenous galactose (Chai *et al.*, 2012). Somewhat conversely to these previous observations, Nakao *et al.* (2006) demonstrated that *P. gingivalis* containing a *galE* interruption produced denser biofilms than their wild-type counterparts, to which the authors attributed the formation of truncated LPS due to a loss of galactose from this biopolymer (Nakao *et al.*, 2006).

GalE is also involved in capsule biosynthesis in some species including *N. meningitidis* serogroup B, *E. stewartii* and *Erwinia amylovora* (Dolph *et al.*, 1988; Hammerschmidt *et al.*, 1994; Metzger *et al.*, 1994). Thus, GalE can be viewed as a virulence factor. Additionally, in *P. gingivalis*, *B. subtilis* and *E. coli*, GalE has been shown to be important for UDP-galactose detoxification as this molecule is bacteriolytic at high concentrations (Chai *et al.*, 2012; Csiszovszki *et al.*, 2011; Krispin and Allmansberger, 1998).

The above studies indicate the importance of GalE as a source of UDP-galactose in biopolymer synthesis and ultimately cell function and survival.

7.1.2 LPS and GalE

7.1.2.1 LPS Composition

LPS is a major constituent of the Gram-negative bacteria cell envelope. It is a polymer composed of lipid A, an inner- and outer-core polysaccharide and a saccharide O-antigen, Figure 7.3(a). The highly conserved lipid A is a hexa-acylated disaccharide molecule synthesised from the acylation, dimerization and subsequent glycosylation (by two 3-deoxy-D-manno-octulosonic acid [Kdo] molecules) of UDP-GlcNAc and is the minimal LPS component required for *E. coli* viability, Figure 7.3(a) (Klein *et al.*, 2013; Meredith *et al.*, 2006). The hydrophobic nature of the lipid A portion of LPS anchors the polymer to the outer membrane (OM) of the cell.

The inner core is more conserved than the outer core and is composed of phosphorylated Kdo and L-glycero- α -D-manno-heptopyranose (hep) residues. It is directly linked to the lipid A moiety via Kdo (Klein *et al.*, 2013; Yethon *et al.*, 1998).

Figure 7.3

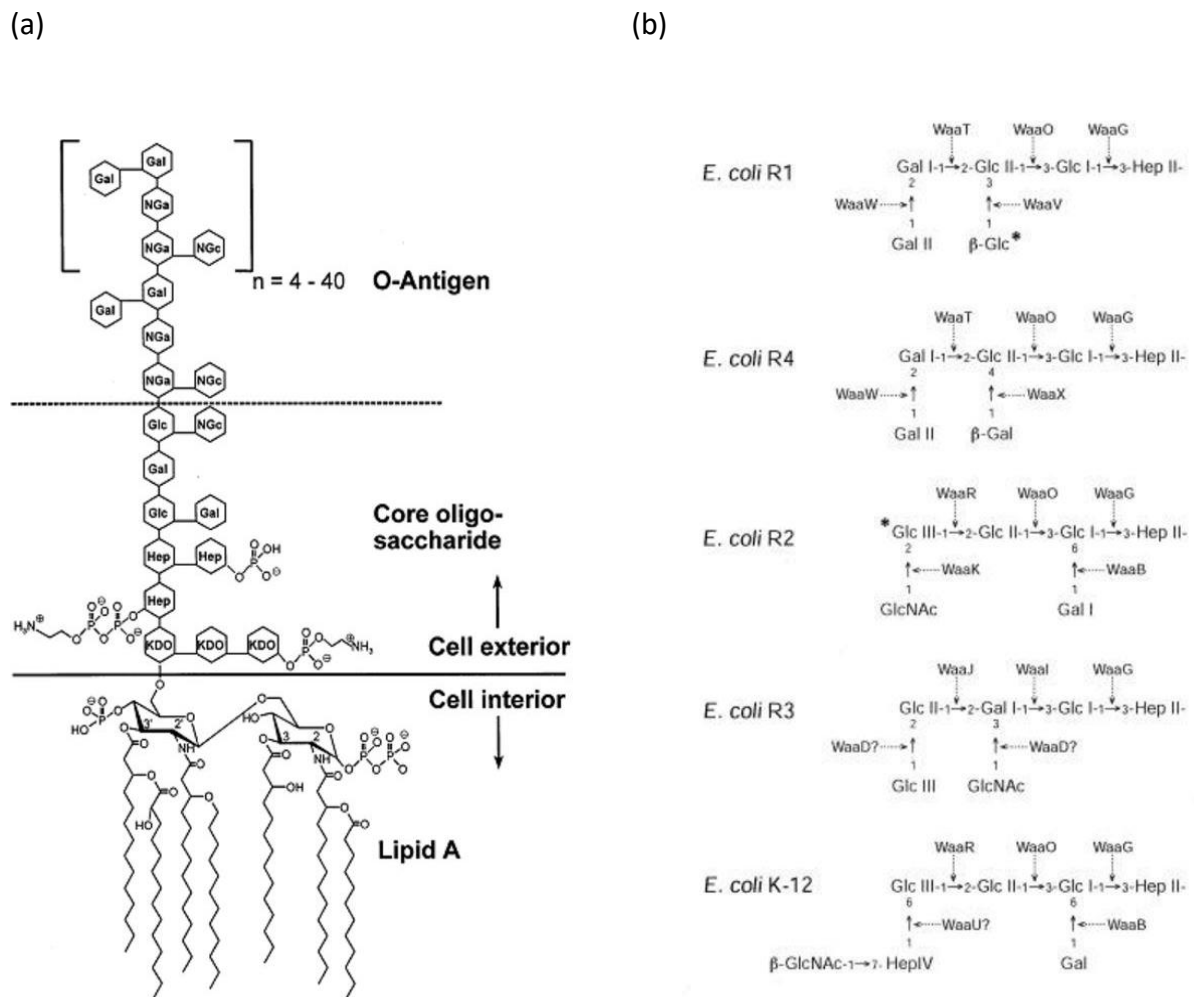


Figure 7.3 LPS Structure and *E. coli* Glycosyltransferases. (a) An illustration the structure of an LPS molecules containing an O-antigen. Lipid A is embedded in the OM. The inner core oligosaccharide is composed of Kdo and Hep residues to which the outer core oligosaccharides are linked. The O-antigen is a polysaccharide chain that is linked to the LPS structure via the inner core oligosaccharide. This Figure was reproduced from Magalhães *et al.* (Magalhães *et al.*, 2007) (b) A depiction of the glycosyltransferases involved in the synthesis of the 5 *E. coli* outer core oligosaccharide types. This diagram was reproduced from Amor *et al.* (Amor *et al.*, 2000).

The outer core polysaccharide is attached to the inner core via the hep residue and is more variable than the inner core; 5 structural forms have been identified in *E. coli*. All of the

forms contain glucose and galactose residues, two contain GlcNAc (*E. coli* R3 and K-12) and one contains a heptose residue (*E. coli* K-12) (Amor *et al.*, 2000). The construction of the core polysaccharide of LPS is achieved a series of glycosyltransferases that act at the cytoplasmic face of the inner membrane. These glycosyltransferases catalyse the transfer of a sugar molecule from a nucleotide sugar to an acceptor sugar molecule in the growing core polysaccharide through the formation of a glycosidic bond (Heinrichs *et al.*, 1998; Wang and Quinn, 2010). In *E. coli*, *waaT*, *waaX*, *waaB*, *waaI* and *waaW* encode galactosyltransferases involved in LPS synthesis, Figure 7.3 (b) (Amor *et al.*, 2000; Leipold *et al.*, 2007; Qian *et al.*, 2014). For example, Qian *et al.* (2014) used an *in vitro* assay to show that WaaB could catalyse the addition of galactose but not glucose to a phosphate radiolabelled lipid A-core oligosaccharide acceptor molecule (measured using electrospray ionization mass spectrometry) (Qian *et al.*, 2014).

Rough LPS is composed of lipid A and the inner and outer core polysaccharides. Smooth LPS also contains the O-antigen polysaccharide that is linked to the outer core and represents the most outer region of the LPS. The O-antigen is extremely variable and more than 180 *E. coli* serogroups have been described (Orskov *et al.*, 1977; Stenutz *et al.*, 2006). The O-antigen is synthesised on the membrane bound undecaprenyl carrier by a series of glycosyltransferases that are specific to the O-antigen (Raetz and Whitfield, 2002; Samuel and Reeves, 2003). The variability within the O-antigen is a result of differences in the sugar residues present and the level of branching within the structure that is mediated in part by differences in glycosyltransferases (Lerouge and Vanderleyden, 2002). Not all organisms produce an O-antigen and *E. coli* K-12 is known to produce rough LPS (Stevenson *et al.*, 1994).

7.1.2.2 GalE and its Effect on LPS Structure

GalE has been shown to play a role in proper LPS and Lipooligosaccharide (LOS; lacking the O-antigen) synthesis in Gram-negative bacteria as it produces nucleotide sugars that act as donors in glycosyl transferase reactions. For example, a loss of *galE* or a reduction in GalE enzyme activity in *Neisseria* spp. can result in a truncated LOS that lacks galactose and lactose residues in the core polysaccharide (Jennings *et al.*, 1993; Lee *et al.*, 1995, 1999; Robertson *et al.*, 1993). GalE is also important for the synthesis of the O-antigen in some species. Nakao *et al.* (2006) showed that a loss of *galE* in *P. gingivalis* led to the production of a truncated O-antigen structure and a rough phenotype (Nakao *et al.*, 2006). An earlier study demonstrated that *P. haemolytica galE* could complement a rough phenotype in a $\Delta galE$ *S. enterica* serovar Typhimurium mutant, reinstating a smooth phenotype (Potter and Lo, 1996). A *galE* mutant *E. coli* K-12 strain (that could produce O-antigen) was shown to be able to produce galactose containing core oligosaccharide and O-antigen structures when grown on LB. Subsequent loss of the *galK* and *galT* genes resulted in a loss of galactose incorporation, however, suggesting that in the absence of GalE activity, *E. coli* may still be able to use exogenous galactose to produce LPS (Pierson and Carlson, 1996; Schnaitman and Austin, 1990).

7.1.2.3 Functions of LPS

LPS is negatively charged as a result of phosphate groups on the lipid A and inner core structures and the presence of anionic Kdo sugar residues (Raetz and Whitfield, 2002). The negative charges are vital for OM integrity as they bind Mg^{2+} and Ca^{2+} causing a tight packing of the structure. LPS may play a role in biofilm formation in some species and the composition of the inner polysaccharide core has been shown to effect biofilm formation in *E. coli*. Nakao *et al.* (2012) showed that mutant *E. coli* strains expressing deep rough LPS (lacking much of the polysaccharide core) exhibited increased biofilm formation, while expression of an O-antigen had the opposite effect (Nakao *et al.*, 2012). O-antigen interference in biofilm formation in other bacteria, including *P. gingivalis* and *Vibrio vulnificus*, has been described also (Lee *et al.*, 2016b; Nakao *et al.*, 2006).

LPS is the target for a number of antimicrobial compounds including QACs such as CTAB and CPC that interact with the structure's negative charges inducing cell lysis (Ding *et al.*, 2003; Domingues *et al.*, 2014; Gilbert *et al.*, 2002; Maillard, 2002). The charged nature of the LPS outer structures and the hydrophobic nature of lipid A also prevent penetration of the OM by charged and hydrophobic molecules (Clifton *et al.*, 2015; Walsh *et al.*, 2000). Mutations resulting in modifications to the LPS have also been described in resistant bacterial strains, including a CTAB resistant *E. coli* mutant strain that exhibited reduced cell surface hydrophobicity (Ishikawa *et al.*, 2002).

The lipid A of *E. coli* growing in biofilms has been shown to undergo palmitoylation resulting in increased antimicrobial peptide resistance. Chalabaev *et al.* (2014) demonstrated that lipid A palmitoylation in *E. coli* biofilms conferred a 3-fold increase in survival against the

antimicrobial peptide protegrin-1 compared with biofilms formed by a *pagP* (palmitoyl transferase) mutant strain that could not modify its lipid A (Chalabaev *et al.*, 2014). Resistance to cationic antimicrobial peptides and polymyxin (which binds to LPS) has been demonstrated to result from an offset or loss of the negative charge on LPS. For example, *Vibrio cholerae* O1 El Tor strain achieves this through the presence of glycine residues in its lipid A that increase its positive charge, and mutants lacking the genes required for this modification have been found to be 100-fold more sensitive to polymyxin (Hankins *et al.*, 2012). Other bacteria such as *P. gingivalis* have been shown to reduce the level of phosphorylation at their lipid A to reduce the negative charge of their LPS (Kumada *et al.*, 1995).

7.2 Chapter Aims

The aim of the work presented in this chapter was to determine the gene(s) required for reduced CTAB susceptibility in the A10(F2) clone and to determine what level of resistance the gene(s) conferred to the *E. coli* EPI300 T1^R host and if they altered the host LPS.

7.3 Methods and Materials

7.3.1 Determining the Size of A10(F2)

The pCC1BAC insert from A10(F2) was extracted, digested using *Hind*III and analysed by agarose gel electrophoresis as described 2.3.1. The 1 Kb extended ladder from NEB was used as a DNA fragment size reference.

7.3.2 Sequencing of A10(F2)

Inserts were sequenced using primer extension Sanger sequencing. The primers used to sequence A10(F2) are included in Table 7.1.

Table 7.1 Primers for Subcloning and ‘Entranceposon’ Sequencing

Name	Sequence (5’-3’)	Information
pCC1-F	GGATGTGCTGCAAGGCGATTAAGTTGG	End sequencing of pCC1BAC (Epicentre®)
pCC1-R	CTCGTATGTTGTGTGGAATTGTGAGC	End sequencing of pCC1BAC (Epicentre®)
CTAB_F2	GGATCTTGTTGAGAATAAGG	Sequencing of A10F2 (This Study)
CTAB_R2	CTGTTATTACCCCTAGTGC	Sequencing of A10F2 (This Study)
CTAB_F3	GGACAGTGCGGCGAGTG	Sequencing of A10F2 (This Study)
CTAB_R3	GTCGAACCATAAATTGAAC	Sequencing of A10F2 (This Study)
CTAB_F4	CAATTCCCATCACGGAGTTC	Sequencing of A10F2 (This Study)
CTAB_R4	CCTCTCTATTGGCATCTAAG	Sequencing of A10F2 (This Study)
CTAB_F5	CCTTCAGTACTTCTTTGTC	Sequencing of A10F2 (This Study)
CTAB_R5	GATGGTAGAACCTGACTTAG	Sequencing of A10F2 (This Study)
CTAB_F6	GCTTTGCCACCTCAACAC	Sequencing of A10F2 (This Study)
CTAB_R6	CCACTGCATAAACAGACC	Sequencing of A10F2 (This Study)
CTAB_F7	CAACCACTTAAAGATAAGC	Sequencing of A10F2 (This Study)
CTAB_R7	CATTGGCATATCTAAAGC	Sequencing of A10F2 (This Study)
CTAB_R8	CCTGAGCCAACAGTATTAG	Sequencing of A10F2 (This Study)
SeqW	GGTGGCTGGAGTTAGACATC	Sequencing out from ‘Entranceposon’ (Green <i>et al.</i> , 2012)
SeqE	CGACACACTCCAATCTTTCC	Sequencing out from ‘Entranceposon’ (Green <i>et al.</i> , 2012)
Isomerase_F	CGCGCGAAGCTTTTATGCAGTGGGGTTTGTT	Amplification of the Isomerase gene introducing a <i>Hind</i> III restriction site – This Study
Isomerase_R	CCCGCCGAATTCCCCTTCAGGAATAGATTCT	Amplification of the Isomerase gene introducing a <i>Eco</i> RI restriction site – This Study
<i>galE</i> _F	CGCGCGAAGCTTGCTCGTGCAAAGGATACACA	Amplification of <i>galE</i> introducing a <i>Hind</i> III restriction site – This Study
<i>galE</i> _R	GCGGCGGAATTC CA AAATCAAACCGATTCATGC	Amplification of <i>galE</i> introducing a <i>Eco</i> RI restriction site – This Study

7.3.3 Sequence Analysis of A10(F2)

The full A10(F2) sequence obtained was assembled and analysed using the *in silico* tools described in chapter 2.

7.3.4 Susceptibility of A10(F2) to CTAB

The susceptibility of *E. coli* EPI300 T1^R::pCC1BAC and A10(F2) was determined using the broth microdilution method described in 2.5.1. The concentration range tested for CTAB was 2 – 128 µg/ml. The agar dilution method was also employed to determine the susceptibility of *E. coli* EPI300 T1^R::pCC1BAC and A10(F2) to CTAB. 16 hour cultures of *E. coli* EPI300 T1^R::pCC1BAC and A10(F2) were adjusted to an OD₆₀₀ of 0.1. 10 µl of these suspensions were spotted on to the surface of LB agar containing CTAB (4 – 512 µg/ml) and plates were incubated for 16 hours at 37 °C.

7.3.5 Random Transposon Mutagenesis

To identify the gene(s) involved in CTAB resistance in A10(F2) using random transposon mutagenesis, the Template Generation System II Kit (Thermo Scientific; Paisley, UK) was used. This kit uses an ‘Entranceposon’ carrying a kanamycin resistance marker that is randomly inserted into the DNA sequence of interest. The sequence of the ‘Entranceposon’

is shown in Figure 7.4. 40 ng of A10(F2) pCC1BAC preparation was incubated with 1 µl of ‘Entranceposon’, 4 µl of MuA transposase buffer and 1 µl of MuA transposase (added last) in a final volume of 20 µl for 1 hour at 30 °C. The reaction was then heat-inactivated at 75 °C for 10 min before being diluted 1 in 10 using molecular grade water. The diluted reaction was electroporated into *E. coli* EPI300 T1^R, as described in 2.3.6, and after recovery the cells were incubated for 16 hours at 37 °C on LB agar (chloramphenicol 12.5 µg/ml, kanamycin 20 µg/ml; 100 µl cells per plate).

Figure 7.4

```
TGAAGCGGCGCACGAAAAACGCGAAAGCGTTTCACGATAAATGCGAAAACGATGTCTAACTCCAGCCACC GTTTAAACG
GATCCTAGTAAGCCACGTTGTGTCTCAAATCTCTGATGTTACATTGCACAAGATAAAAATATATCATCATGAACAATAAA
ACTGTCTGCTTACATAAACAGTAATAACAAGGGGTGTTATGAGCCATATTCAACGGGAAACGTCTTGCTCGAGGCCGCGAT
TAAATCCAACATGGATGCTGATTTATATGGGTATAAATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAATCTAT
CGATTGTATGGGAAGCCCGATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGATGTTACAGATGA
GATGGTCAGACTAAACTGGCTGACGGAATTTATGCCTCTCCGACCATCAAGCATTTTATCCGTA CTCTGATGATGCATG
GTTACTACCACTGCGATCCCCGGGAAAAACAGCATTCCAGGTATTAGAAGAATATCCTGATTCAGGTGAAAAATATTGTTGA
TGCGCTGGCAGTGTCTGCGCCGGTTGCATTCGATTCCTGTTTGAATTGTCCTTTTAAACAGCGATCGCGTATTTTCGTCTC
GCTCAGGCGCAATCACGAATGAATAACGGTTTGGTTGATGCGAGTGATTTTATGACGAGCGTAATGGCTGGCCTGTTGA
ACAAGTCTGGAAAGAAATGCATAAGCTTTTGCATTCTACCGGATTCAGTCGTCACCTCATGGTGATTTTCTCACTTGATAAC
CTTATTTTTGACGAGGGGAAATTAATAGGTTGATTGATGTTGGACGAGTCGGAATCGCAGACCGATACCAGGATCTTGC
CATCCTATGGAAGTGCCTCGGTGAGTTTTCTCCTTCATTACAGAAAACGGCTTTTTCAAAAATATGGTATTGATAATCCTGAT
ATGAATAAATTGCAGTTTCATTTGATGCTCGATGAGTTTTTCTAATCAGAATTGGTTAATTGGTTGTAACACTGGCAGAGC
ATTACGCTGACTTGACGGGACGGCGGCTTTGTTGAATAAATCGAACTTTATTCGGTTCGAAAAGGATCCGCGGCCGC CGAC
ACACTCCAATCTTTCCGTTTTTCGCATTTATCGTGAAACGCTTTTCGCGTTTTTCGTGCGCCGCTTCA
```

Figure 7.4 Entranceposon Sequence. The sequence of the ‘Entranceposon’ used in the creation of the A10(F2) transposon mutagenesis library is depicted here. The SeqW and SeqE primer binding sites used to sequence out from the transposon are highlighted in yellow and green respectively.

7.3.5.1 Screening of Random Transposon Mutagenesis Library

The clones that resulted from the 'Entranceposon' mutagenesis were picked onto LB agar (chloramphenicol 12.5 µg/ml, kanamycin 20 µg/ml) in a 96-grid system marked plate and to the corresponding wells of a 96-well plate that contained LB (chloramphenicol 12.5 µg/ml, kanamycin 20 µg/ml, CTAB 3.7 µg/ml). The plates were then incubated for 16 hours at 37 °C. Clones that grew on the agar plates but not in the 96-wells were selected. Plasmids were extracted from these clones of interest and the location of the 'Entranceposon' within the plasmid was determined by sequencing using the SeqW and SeqE primers, Table 7.1.

7.3.6 Subcloning of Epimerase and Isomerase Genes

To amplify *galE* from A10(F2), *galE*_F and *galE*_R primers were used, the Isomerase gene was amplified using the Isomerase_F and Isomerase_R primers. Both *galE* and the Isomerase gene were amplified using Isomerase_F and *galE*_R primers. These primers also introduced *Hind*III and *Eco*RI sites that flanked the promoter regions and translation stop sites of the genes of interest. The primers used in subcloning are listed in Table 7.1. The amplicons and pCC1BAC were double-digested by *Hind*III and *Eco*RI as described in chapter 2.3.2. After digestion, the free ends of pCC1BAC were dephosphorylated by CIAP as described in 2.3.3. The digestion reactions were purified using the QIAquick PCR Purification Kit. Following purification, the digested amplicons were ligated into pCC1BAC using T4 DNA ligase (NEB). Ligation reactions were desalted before electroporating into *E. coli* EPI300 T1^R.

After electroporation, cells were recovered in SOC for 1 hour at 37 °C before plating (100 µl) on LB agar supplemented with chloramphenicol (12.5 µg/ml) with or without CTAB (32 µg/ml). The constructs and strains created are detailed in Table 7.2.

Table 7.2 Strains and Constructs.

	Name	Reference	Source
Constructs	pCC1BAC::A10(F2)	pCC1BAC containing 19.1 Kb metagenomic DNA insert	This Study
	pCC1BAC:: <i>galE</i>	pCC1BAC containing 1, 054 bp <i>galE</i> amplicon	This Study
	pCC1BAC::Iso	pCC1BAC containing 1, 609 bp Isomerase amplicon	This Study
	pCC1BAC:: <i>galE</i> -Iso	pCC1BAC containing 2, 592 bp <i>galE</i> and Isomerase amplicon	This Study
Strains	A10(F2)	<i>E. coli</i> EPI300 T1 ^R ::[pCC1BAC::A10(F2)]	This Study
	<i>E. coli</i> ::pCC1 <i>galE</i>	<i>E. coli</i> EPI300 T1 ^R ::[pCC1BAC:: <i>galE</i>]	This Study
	<i>E. coli</i> ::pCC1iso	<i>E. coli</i> EPI300 T1 ^R ::[pCC1BAC::Iso]	This Study
	<i>E. coli</i> ::pCC1 <i>gal</i> -iso	<i>E. coli</i> EPI300 T1 ^R ::[pCC1:: <i>galE</i> -Iso]	This Study

7.3.7 LPS Extraction

E. coli::pCC1BAC, A10(F2) and *E. coli*::pCC1*galE* were grown for 16 hours in 10 ml LB containing chloramphenicol (12.5 µg/ml) and CTAB (3.7 µg/ml) when required. These cultures were centrifuged at 10,000g for five min and the resulting cell pellets washed twice with PBS (0.15M) by centrifuging as above. The pellets were resuspended in 4 ml of PBS and sonicated on ice for 10 min. Proteinase K (100 µg/ml) was then added to the sample incubated for 1 h at 65°C. The sample was then treated with DNase (20 µg/ml) and RNase

(40 µg/ml) with 20 % MgSO₄ (1 µl/ml) and chloroform (4 µl/ml) overnight at 37°C. The following day an equal volume of 90% phenol at 65°C was added to each sample and they were vigorously agitated at 65°C for 15 min before cooling on ice and centrifuging at 10,000 g for 10 min. Supernatants were transferred to 15 ml tubes and 300 µl of molecular grade H₂O was added to each to extract the LPS from the phenol phase. Sodium acetate at a final concentration of 0.5M and 10 volumes of 95% EtOH were added to the extracts and they were kept at -20°C overnight to allow LPS to precipitate. The samples were then centrifuged at 2000 g at 4°C for 10 min. The resulting pellets were dissolved in 1 ml molecular grade H₂O and dialysed against molecular grade H₂O using Pur-A-Lyzer™ 800 µl cassettes with 1kDa cut-offs (Sigma) to remove residual phenol.

7.3.8 Mass Spectrometry Analysis of Extracted LPS

The LPS extractions were analysed by LC-MS conducted by the UCL School of Pharmacy. The fragments identified by the mass spectrometry analyses were displayed as mass to charge ratios (m/z).

7.4 Results

7.4.1 Analysis of the A10(F2) Sequence

The A10(F2) insert was found to be approximately 19.1 Kb, Figure 7.5 (a).

Figure 7.5

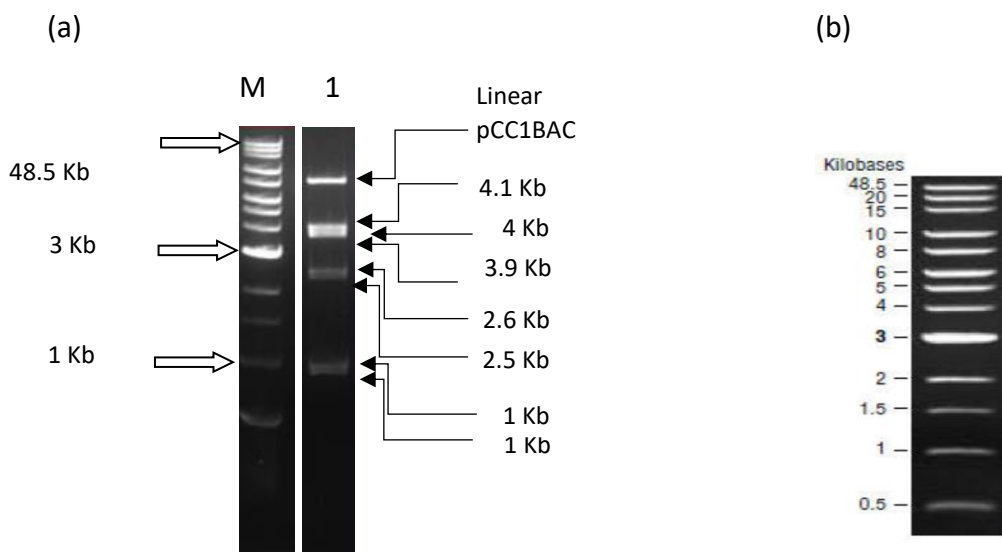


Figure 7.5 Agarose Gel Displaying *HindIII* Digested A10(F2). The lane marked M is the NEB 1Kb extended ladder, the lane Marked 1 contains the *HindIII* digested inserts. (a) Digestion of the A10(F2) insert with *HindIII* results in 7 fragments when viewed on a 0.9 % agarose gel stained with ethidium bromide. (b) An image depicting the NEB 1Kb extended ladder with size labels.

A total of 10,456 Kb of the insert was sequenced, 5,917 bp from the pCC1R primer binding side of the insert and 4,539 bp from the pCC1F primer binding side; the sequencing primers are listed in Table 7.1. The forward and reverse sequences obtained for A10(F2) are included in Appendix XII. BLASTN analysis of the 5,917 bp sequence revealed it to have similarity to

Veillonella parvum DSM 2008 (accession: CP001920.1, 88 % cover, 85 % ID). BLASTX revealed that this region of the insert contained 7 putative ORFs, encoding two nucleoside phosphate epimerases, a glucose-6-phosphate isomerase, two RNaseH domain containing proteins, a YbaK family deacylase protein and a protein of unknown function, Figure 7.6. The remaining 4,539 Kb had nucleotide sequence similarity to *P. melaninogenica* (accession: CP002122.1, 47 % cover, 81 % ID) according to BLASTN and BLASTX analysis indicated that it contained 4 ORFs encoding two putative ABC half transporters, a permease and an EngA type GTPase, Figure 7.6.

Figure 7.6

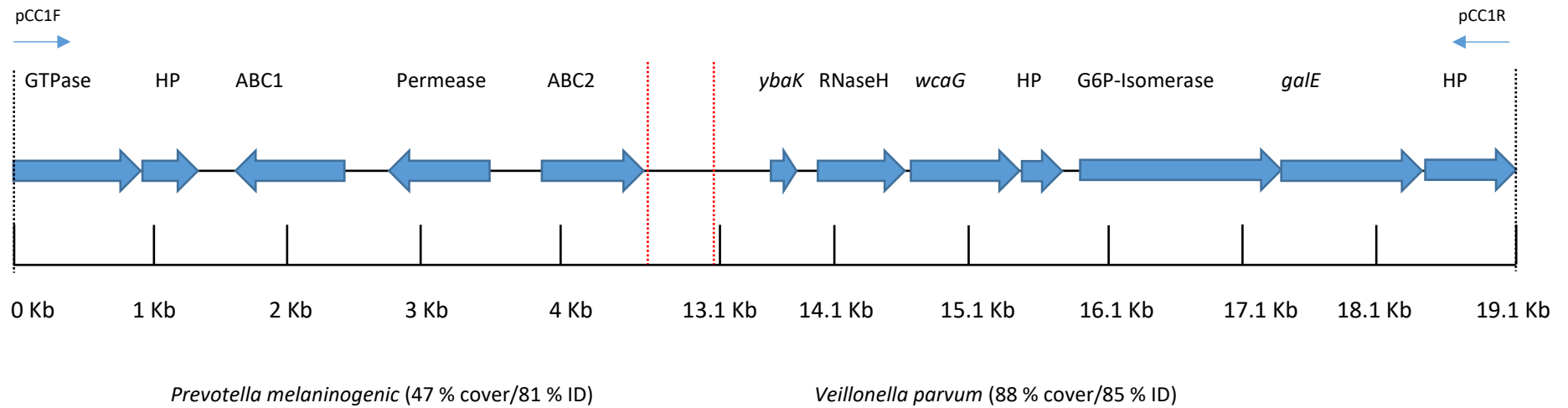


Figure 7.6 Schematic of the A10(F2) Insert. Diagram depicting the ORFs present in the sequenced regions of pCC1BAC::A10(F2). The 4,539 bp sequence adjacent to the pCC1F primer binding region is indicated on the left and the 5,917 bp region adjacent to the pCC1R primer binding site is indicated on the right. The region bordered by the vertical red dashed lines indicates the regions of the insert that has not been sequenced. The black vertical dashed lines indicate that regions of these genes are not present in the clone.

7.4.2 A10(F2) CTAB Susceptibility

The susceptibility of *E. coli* EPI300 T1^R::pCC1BAC to CTAB was 4 µg/ml using the microdilution method and 16 µg/ml using the agar dilution method. Using the microdilution method, the susceptibility of A10(F2) to CTAB was 8 µg/ml (2-fold increase) and 64 µg/ml (4-fold increase) using the agar dilution method.

7.4.3 Random Transposon Mutagenesis Results

In order to determine what genes were responsible for the observed reduced susceptibility to CTAB, random transposon mutagenesis was employed. Screening of the random transposon library revealed ten mutants that could repeatedly be shown to have lost their reduced susceptibility to CTAB phenotype. Sequencing of these ten mutants showed that the 'Entranceposon' had inserted into different locations in the A10(F2) insert, Figure 7.7. In one of the mutants, the transposon inserted into the glucose-6-phosphate (G-6-P) isomerase gene 324 bp from the stop codon of the gene. In the remaining 9 mutants, the transposon had inserted into different regions of *galE*; the relative positions of these insertions are shown in Figure 7.7.

Figure 7.7

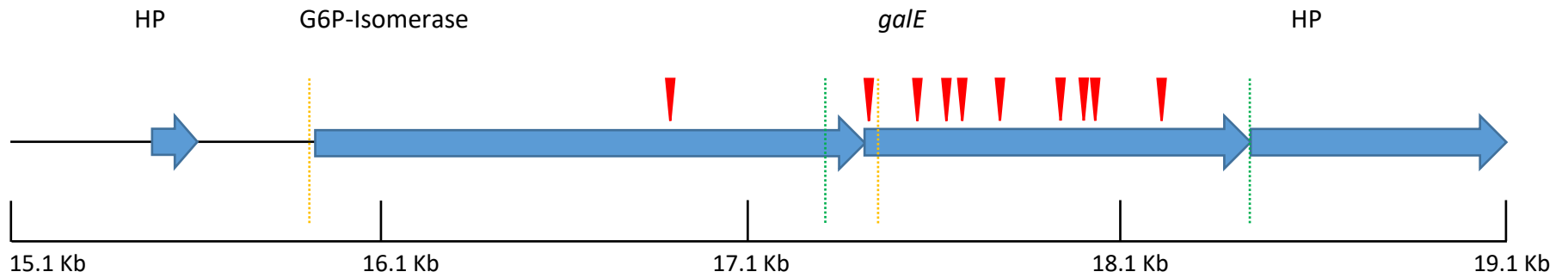


Figure 7.7 Schematic of A10(F2) 15.1 – 19.1 Kb. The red triangles indicate the insertion locations of the 'Entranceposons' that resulted in a loss of CTAB resistance in A10(F2). The vertical dashed orange lines indicate the subcloned region that contained the isomerase gene. The vertical dashed green lines indicate the subcloned region that contains *galE*. The subcloned region that contained both the isomerase and *galE* are represented by the vertical dashed orange and green lines that are furthest apart.

7.4.4 Subcloning of galE and Isomerase genes

To further confirm that *galE* expression was required for the observed reduction in CTAB susceptibility in *E. coli* EPI300 T1^R amplicons containing *galE*, the Isomerase gene and both *galE* and the Isomerase(*gal-iso*) were cloned into *E. coli* EPI300 T1^R using pCC1BAC, Figure 7.7 and Figure 7.8. *E. coli*::pCC1*galE* and *E. coli*::pCC1*gal-iso* were able to grown on LB agar containing CTAB (32 µg/ml). *E. coli*::pCC1iso was unable to grow on LB agar containing CTAB (32 µg/ml), indicating that *galE* was required for reduced susceptibility to CTAB in A10(F2).

Figure 7.8

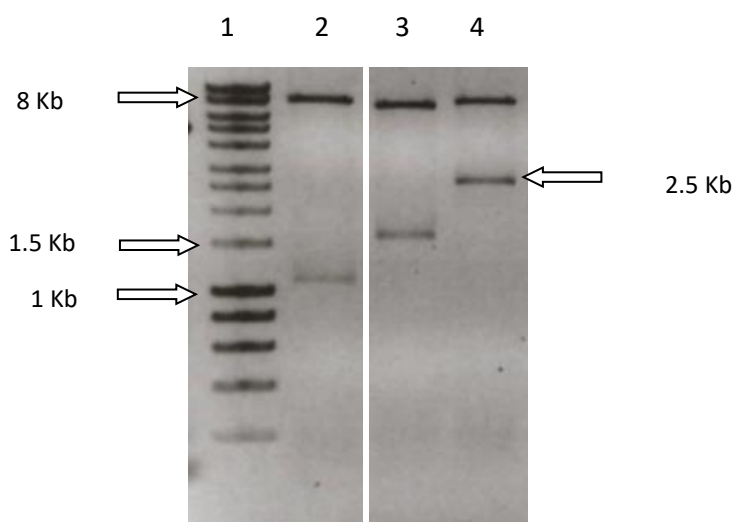


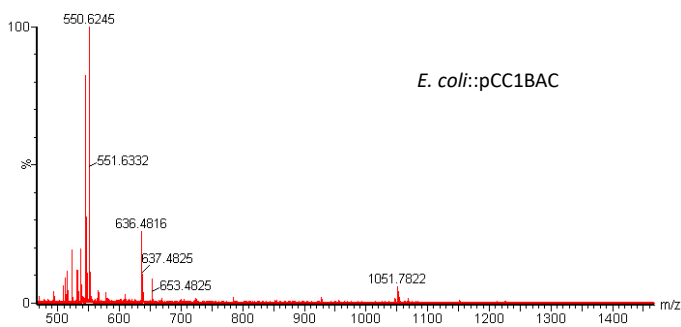
Figure 7.8 Agarose gel of A10(F2) Subclones. Agarose gel image showing the inserts of the *E. coli*::pCC1*galE*, *E. coli*::pCC1iso and *E. coli*::pCC1*gal-iso* after *Hind*III and *Eco*RI double digestion. Lane 1 contains HyperLadder I. Lane 2 shows the 1,054 bp inserts of the *E. coli*::pCC1*galE* subclone. Lane 3 shows the 1,609 bp insert of the *E. coli*::pCC1iso subclone. Lane 4 shows the 2,592 bp insert of *E. coli*::pCC1*gal-iso*. The 8 Kb fragment in lanes 2, 3 and 4 is the pCC1BAC backbone. The white line indicates the removal of lanes not included in this figure.

7.4.5 Mass Spectrometry Results

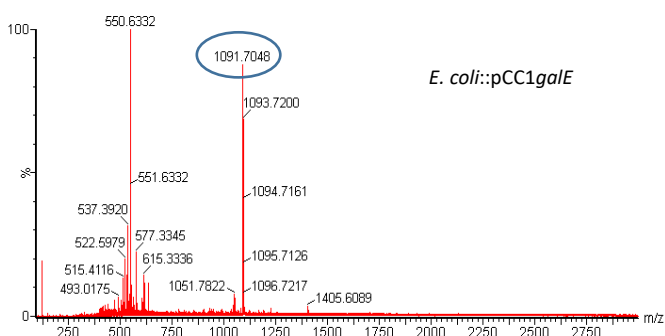
To gain an insight into the mechanism of reduced CTAB-susceptibility in A10(F2), LPS was extracted from *E. coli*::pCC1BAC, A10(F2) and *E. coli*::pCC1galE. All extractions contained fragments with mass to charge ratios between 490 and 650 m/z and a single peak at 1051 m/z, Figure 7.9(a-c). In the A10(F2) and *E. coli*::pCC1galE LPS extractions a peak at 1091 m/z was also present. Two fragments at 781 and 927 m/z were present in A10(F2) but not in the other two fractions. A peak at 1405 m/z was present in the *E. coli*::pCC1galE LPS extractions but not in the other two. These differences in mass-to-charge spectra between *E. coli*::pCC1BAC and both A10(F2) and *E. coli*::pCC1galE suggest that the latter two produce LPS with a different composition compared with *E. coli*::pCC1BAC.

Figure 7.9

(a)



(b)



(c)

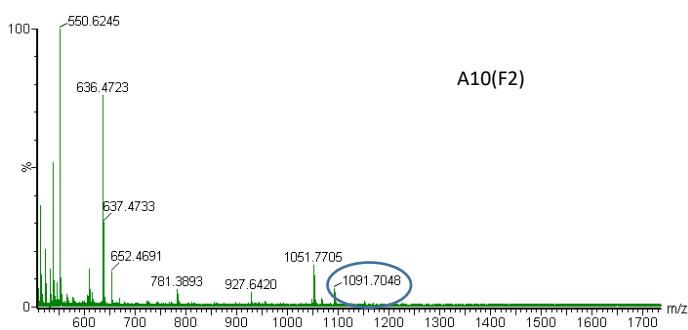


Figure 7.9 Mass Spectrometry of LPS. Graphs depicting the mass to charge ratios of the fragments in the LPS extractions of (a) *E. coli::pCC1BAC*, (b) *E. coli::pCC1galE* and (c) A10(F2). The fragment with a mass to charge ratio of 1091 m/z present in *E. coli::pCC1galE* and A10(F2) but not in *E. coli::pCC1BAC* are highlighted by a blue circle.

7.5 Discussion

A single clone with reduced susceptibility, A10(F2), was identified from the screening of a subset of the human saliva metagenomic library (1, 248 clones). To the best of our knowledge this is the first reporting of a CTAB-resistant clone identified from a functional metagenomic screen. The clone was likely a concatemer as sequencing revealed the insert to contain DNA from two different phyla, Firmicutes and Bacteroidetes. One side of the insert had similarity to *Veillonella parvum* (88 % cover, 85 % ID) and the other similarity to *P. melaninogenica* (47 % cover, 81 % ID) with a *Hind*III site marking the border. The insert contained putative ABC transporter genes, on the *P. histicola* side, as well as genes that have been shown to be involved in LPS synthesis, on the *V. parvum* side. Mechanisms of CTAB resistance being mediated by efflux and LPS expression have been described before (Ishikawa *et al.*, 2002; Ciric *et al.*, 2012).

Using the microdilution method, a 2-fold reduction in CTAB susceptibility was observed in A10(F2) compared with *E. coli* EPI300 T1^R and a 4-fold reduction was observed when the agar dilution method was used. These levels of susceptibility for *E. coli* EPI300 T1^R are in agreement with other studies (Ishikawa *et al.*, 2002; Zhang *et al.*, 2016). The greater reduction in susceptibility to CTAB observed for both *E. coli* EPI300 T1^R and A10(F2) when grown on agar compared with when they were grown in liquid culture (4-fold and 8-fold increase respectively) may be a result of the biofilm nature of their growth in colonies. Cells growing in biofilms are inherently more resistant to antimicrobials than are planktonically growing cells. That a greater reduction in susceptibility was observed for A10(F2) than for *E. coli* EPI300 T1^R may be a result of the presence of *galE* that leads to the production of an LPS that results in greater biofilm formation and thus reduced susceptibility to CTAB (Chalabaev *et al.*, 2014; Nakao *et al.*, 2012).

Transposon mutagenesis revealed a loss of CTAB resistance occurred when the 'entranceposon' inserted into either the G-6-P isomerase gene (A10(F2)-F8) or the *galE* (A10(F2)-G3) gene. As subsequent subcloning work revealed only that only *galE* was required for the CTAB resistance phenotype it is likely that insertion of the 'entranceposon' in the isomerase gene disrupted transcription of *galE* that is downstream of the G-6-P isomerase. Thus, although 4 transporter proteins were encoded on the side of the insert with highest nucleotide similarity to *P. melaninogenic* (47 % cover/81 % ID), none of these were required for CTAB resistance.

Although this, to the best of our knowledge, is the first time that a CTAB resistant clone has been isolated from a functional metagenomic library, previous functional metagenomic assays have identified *galE* as a resistance gene. Mori *et al.* isolated a menadione-resistant clone from a wastewater fosmid library expressing a *galE* gene that they hypothesised reduced the permeability of the OM (Mori *et al.*, 2008). In two separate functional metagenomic surveys of the human gut, *galE* expression was shown to be involved in salt tolerance and benzalkonium chloride resistance (Bülow, 2015; Culligan *et al.*, 2012).

The *galE* gene in A10(F2) was flanked by two genes encoding hypothetical proteins and a glucose-6-phosphate isomerase and *wcaG* (GDP-L-fucose synthase) on a fragment of DNA with highest similarity to *Veillonella parvum* (88 % cover/85 % ID). The latter gene has been shown to be involved in capsule synthesis in *E. coli*, *Klebsiella pneumoniae* and *Campylobacter jejuni*; however, it doesn't play such a role in *Veillonella* spp. as they don't produce a capsule (Mashima and Nakazawa, 2015; McCallum *et al.*, 2011; Shu *et al.*, 2009). As *Veillonella* spp. are also asaccharolytic these sugar-metabolising ORFs are unlikely to be involved in ATP generation and are more likely to provide building blocks for the synthesis of LPS and EPS polymers (Vesth *et al.*, 2013). However, as with all functional metagenomic surveys, taxonomic assignment is difficult and thus discussions on functions of genes in their native host is limited.

Although GalE in its native host may not function in reducing the cell's susceptibility to CTAB, in the context of the *E. coli* EPI300 T1^R host it does. Metabolic enzymes have been implicated in antimicrobial resistance before. FabI is an enoyl-acyl carrier protein reductase involved in fatty acid synthesis and is the target of the antimicrobial agent triclosan. In a study conducted by Heath *et al.* (2000), *B. subtilis* FabI was demonstrated to have a lower affinity for triclosan compared with *E. coli* FabI. They further showed that expression of *B. subtilis* *fabI* resulted in a 2-fold greater reduction in triclosan susceptibility compared with *E. coli* *fabI* expression in a *fabI* mutant *E. coli* strain (Heath *et al.*, 2000). Following my departure from the Eastman Dental Institute, clones from the human saliva metagenomic library with reduced susceptibility to triclosan were identified, all of which encoded *fabI* on their insert. This further suggests the importance of the heterologous expression of metabolic enzymes in conferring resistance, particularly given the recent identification of *fabI* on a mobile genetic element (Ciusa *et al.*, 2012).

It is likely that the expression of the metagenomic *galE* enzyme results in increased levels of UDP-galactose that can be incorporated into the LPS of A10(F2). LC-MS analysis of the LPS extractions from *E. coli*::pCC1BAC, A10(F2) and *E. coli*::pCC1*galE* revealed commonalities and differences between their mass-to-charge spectra. A10(F2) and *E. coli*::pCC1*galE* extractions each contained a peak at 1091 m/z not present in *E. coli*::pCC1BAC indicating the *galE* expression altered the host cell's LPS. The *E. coli*::pCC1*galE* spectrum also contained a fragment at 1405 m/z not found in the other extractions while A10(F2) showed to peaks at 781 m/z and 927 m/z that were not observed in the other spectra. The differences between the *E. coli*::pCC1*galE* and A10(F2) spectra may be a result of different LPS structures as A10(F2) carries the full 19.1 Kb insert that may encode genes for other enzymes capable of modifying the *E. coli* LPS without altering its CTAB susceptibility phenotype. Alternatively, these two peaks in A10(F2) may represent degraded fragments of a larger fragment indicated by the 1405 m/z peak in the *E. coli*::pCC1*galE* spectrum.

As we have shown that only *galE* of A10(F2) is required for the reduced CTAB susceptibility of A10(F2), the activity of endogenous glycosyltransferases to construct the modified LPS from the increased UDP-galactose pool must be required. For instance, WaaT, WaaB, WaaX, WaaW and WaaL are galactosyltransferases that are involved in the addition of galactose residues to the outer core polysaccharide of the 5 polysaccharide groups of *E. coli* (Amor *et al.*, 2000; Leipold *et al.*, 2007; Qian *et al.*, 2014). Further work to discern if such transferases play a role in CTAB resistance in A10(F2) is required. It may also be worthwhile determining if growing *E. coli* EPI300 T1^R on increasing galactose concentrations can reduce its susceptibility to CTAB.

The modified LPS structure of A10(F2) that results from the additional source of UDP-galactose may have a different charge than the *E. coli* EPI300 T1^R host resulting in a reduced affinity of CTAB for LPS. Additional galactose residues in the A10(F2) LPS may also produce structures that occlude the binding of CTAB. Interestingly, the A10(F2) clone was not identified when the library was screened for resistance to CPC, another QAC (quaternary ammonium compound), indicating that reduction in negative charge as well as structural alterations in the LPS may be responsible for the reduced CTAB susceptibility. Further work should be conducted to assess the susceptibility of other antimicrobials that target negative charges on the cell surface including colistin and cationic antimicrobial peptides such as defensins and histatins (Gupta *et al.*, 2009; Khurshid *et al.*, 2015; Peschel *et al.*, 1999).

Cytochrome C colorimetric titration assays could shed light on whether there is a cell surface charge difference between *E. coli*::pCC1AC and *E. coli*::pCC1*galE*. Cytochrome C is a positively charged protein that is able to interact with the negatively charged cell surface. When in an aqueous solution, it produces a red colour that becomes less intense when the cytochrome C comes out of solution and interacts with the cell surface. Thus, if *E. coli*::pCC1*galE* has a less negatively charged surface due to the production of an altered LPS more cytochrome C should remain in solution following incubation with these strains compared with *E. coli*::pCC1AC. Following my departure from the Eastman Dental

Institute this experiment was conducted by Mr. Supatthep Tansirichaiya. Figure 7.10 shows that following incubation with cytochrome C, more of the protein bound to the surface of *E. coli*::pCC1AC than *E. coli*::pCC1galE indicating that the latter strain had a less negatively charged cell surface.

Figure 7.10

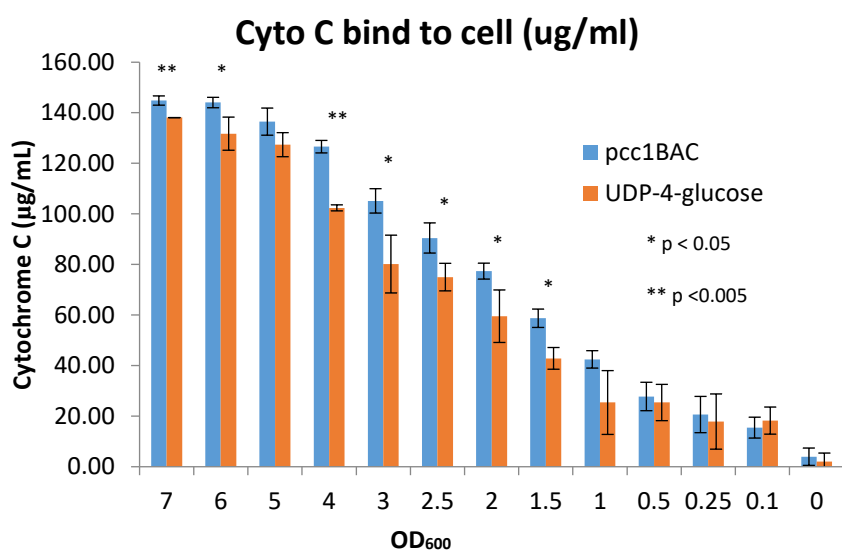


Figure 7.10 Cytochrome C Assay. The above graph depicts the results of a series of a cytochrome C surface charge assays indicating how much cytochrome C ($\mu\text{g/ml}$) is bound to the surface of *E. coli*::pCC1BAC (blue) and *E. coli*::pCC1galE (OD_{600} 0.1 – 7.0). At every OD_{600} tested *E. coli*::pCC1BAC was able to bind more cytochrome C than *E. coli*::pCC1galE indicating that *E. coli*::pCC1galE has a less negatively charged cell surface than *E. coli*::pCC1BAC. This diagram has been reproduced with permission from Mr. Supatthep Tansirichaiya.

To conclude, a *galE* gene cloned in A10(F2) was found to be solely required for the observed CTAB resistance phenotype likely through the production of increased levels of UDP-galactose that could be incorporated in the *E. coli* EPI300 T1^R LPS altering its cell surface charge. Further work is required to determine how relevant this gene is to CTAB resistance in its host cell and in the oral cavity.

Chapter 8

Final Discussion

The creation and screening of functional metagenomic libraries has the potential to identify novel genes from the yet-to-be cultured microorganisms of an environment. In this study two functional metagenomic libraries were created from human saliva and calf faeces, and subsequently screened for antimicrobial resistance and antimicrobial production. Both of these environments are known to harbour antimicrobial resistance genes and maintain dense populations of bacteria that interact with each other via the production of various molecules including antimicrobials such as bacteriocins. By creating and screening metagenomic libraries using bacterial DNA extracted from these environments, novel antimicrobial resistance and bacteriocin production genes may be identified.

The calf faecal metagenomic library was found to contain few clones harbouring inserts. Three clones from 10 contained inserts even though all 10 clones were white. For this reason, no further clones were analysed to determine if they contained inserts. Additionally, 16S analysis of the calf faecal metagenomic DNA extraction revealed it to contain mainly Proteobacteria (98.7 % of OTUs), with *E. coli* dominating (75.8 % OTUs). Although the library was not representative of the calf faecal microbiome and the majority of the clones contained no DNA, 2,840 clones were screened for ampicillin and tetracycline resistance. No clones of interest were identified.

The human saliva metagenomic library that was created consisted of 27,000 clones representing 97,074 Kb of bacterial DNA. The protocol used in this study to extract metagenomic DNA from human saliva resulted in a metagenomic DNA preparation consisting mainly of *Prevotella* spp., *Streptococcus* spp. and *Veillonella* spp. (according to Illumina 16S sequencing) which is in agreement with previous

phylotyping studies of human saliva (Dassi *et al.*, 2014; Segata *et al.*, 2012). Additionally, minimal loss of diversity was observed between the metagenomic extraction and the functional metagenomic library as both had similar profiles at the phylum and genus levels, indicating that our functional metagenomic library was representative of our metagenomic extraction.

Screening of the human saliva metagenomic library resulted in the identification of two tetracycline resistant clones. One of these clones, PS9, encoded two ABC half-transporter genes, *tetAB(60)* which had >90 % nucleotide identity to putative ABC transporter genes from *Streptococcus* spp. and *Granulicatella* spp. We demonstrated that both genes were minimally sufficient for the observed tetracycline resistance in this clone. Further to this, deletion of the Walker A motif-encoding region of either *tetA(60)* or *tetB(60)* resulted in a loss of tetracycline resistance, suggesting that *tetAB(60)* encoded a functional heterodimeric ABC transporter. Subsequent analysis showed that *tetAB(60)* specifically conferred resistance to tetracycline and tigecycline, and not to minocycline or the other classes of antibiotics tested. Tetracycline-specific ABC transporters have been identified from human saliva previously, although none confer such a high level of resistance to tigecycline (Warburton *et al.*, 2013). Growth curve assays showed that a fitness cost was associated with the expression of *tetAB(60)* in *E. coli* EPI300 T1^R. The results of subsequent ATP assays and the observation that one of the Walker A motif mutants [*E. coli*::pHSG396*tetA(60)*Δ*tetB(60)*] did not have a fitness cost indicated that the fitness cost was a result of the function of the ABC transporter rather than maintenance or transcription of the *tetAB(60)* genes. The results of the characterisation of this novel tetracycline and tigecycline ABC transporter have been recently published, Appendix XIII.

Interestingly, the tetracycline resistance phenotype was only observed when *tetAB(60)* were expressed from either pCC1BAC or pHSG396 in *E. coli* EPI300 T1^R. Tetracycline resistance was not observed when *tetAB(60)* were cloned using pGEM[®]-T Easy in *E. coli* EPI300 T1^R, or when *E. coli* Alpha-Select Silver was the host. Tetracycline resistance was also not observed in *S. mutans* UA159

when *tetAB(60)* were cloned using pVA838. These results highlight how important the choice of both host and vector is when creating a functional metagenomic library, as *tetAB(60)* would not have been identified if, for example, pHSG396 and *E. coli* Alpha-Select Silver were used to create the library.

The observed fitness cost associated with *tetAB(60)* in *E. coli* and the lack of their expression in the other hosts used in this study may suggest that their dissemination to human pathogens may be limited. However, the acquisition of compensatory mutations or genes that alleviate this fitness cost may aid their dissemination to human pathogens. Further work that is beyond the scope of this characterisation study is required to determine whether this is the case.

The second tetracycline-resistant clone, TT31, contained a 14 Kb insert. The left 7,226 bp of TT31 had 97 % nucleotide identity to the *tet(M)*, regulation and recombination modules of Tn916 and the right 7,000 bp had 99 % identity to *G. haemolysans*. As no *HindIII* site was found to divide the insert into these Tn916-like and *G. haemolysans* regions the insert was not a concatemer. Tn916-like elements have been isolated from the oral cavity in numerous studies (Ciric *et al.*, 2012; Brenciani *et al.* 2014). Although a previous study conducted by Cerdá Zolezzi *et al.* (2004) identified *int* and *tet(M)* in nasopharynx *Gemella* spp., to the best of our knowledge no Tn916-like elements from *Gemella* spp. have been fully characterised (Cerdá Zolezzi *et al.*, 2004).

The Tn916 region of the insert encoded *tet(M)* and *tet(L)* and two half ABC transporter genes were present on the *G. haemolysans* side of the insert. However, as the predicted start codon containing 62 bp of *tet(M)* were not cloned in TT31, it is likely that *tet(L)* and/or the ABC transporter(s) encoded by *G. haemolysans* were responsible for this clone's tetracycline resistant phenotype. The Tn916-like region of TT31 also had 99 % nucleotide identity to Tn6079, which encodes *tet(L)*, and to a region of the *L. johnsonii* strain BS15 that encodes a putative Tn916-like element. Compared with the Tn916-like region of TT31, the *L. johnsonii* Tn916-like element contains a 4,999 bp insert that contains pre/mob

and rep genes that have >99 % nucleotide identity to those encoded by Tn6079. This indicated to us that the Tn916-like element identified in TT31 may be an ancestral Tn916-like element and that *L. johnsonii* may be an 'evolutionary intermediate' between the TT31 Tn916-like element Tn916 and Tn6079.

Additionally, a truncated *orf9* gene, *orf9t*, was encoded by the Tn916-like region of the TT31. The first 270 bp of *orf9t* had 83.6 % nucleotide identity to the full length *orf9* found in Tn916. Interestingly, the 4,999 bp insert in *L. johnsonii* was in the *orf9t* gene, and the insert was found to contain the 3'-end of the full length *orf9* in frame with the 5'-end of *orf9t*; such that *L. johnsonii* encoded the full length *orf9*. The 3'-end sequence of *orf9t* is still present. This suggested that TT31 Tn916-like element was an ancestor of Tn916 and that *L. johnsonii* encoded an evolutionary intermediate between the two.

Further work is required to discern whether the Tn916-like region of TT31 is a part of a larger Tn916-like element as no conjugation module was cloned in TT31. This is important to justify our hypothesis detailed in chapter 6 as without it, doubt could be cast on whether the TT31 Tn916-like element exists.

Screening of a subset of the human saliva library (1,248 clones) also led to the identification of a clone, A10(F2), with reduced susceptibility to CTAB. The clone contained a 19.1 Kb insert of which 10,456 Kb was sequenced. From the pCC1R primer side of the pCC1BAC cloning site, 5,917 Kb of the insert had 88 % nucleotide identity to *V. parvum* DSM 2008 and from the pCC1F primer side the 4,529 Kb sequence had 99 % nucleotide identity to *P. histicola* F0411. Random transposon mutagenesis and subcloning revealed that a *galE* gene (encoding a UDP-glucose 4-epimerase) on the *V. parvum* side of the A10(F2) insert was responsible for the reduced CTAB susceptibility. As CTAB binds to the *E. coli* cell via its negatively charged LPS we hypothesised that the heterologous GalE produces additional galactose in the *E. coli* host which is then used to produce a modified and less negatively charged LPS.

Mass spectrometry of the LPS produced by *galE* expressing clones indicated that it had a different composition to the *E. coli* host harbouring only the empty vector. Additionally, following my departure from the Eastman Dental Institute, cytochrome C assays were conducted and the clones expressing *galE* were found to have more positively charged cell surfaces compared with *E. coli* harbouring only the empty vector. Whether *galE* contributes to reduced susceptibility in its native host is unclear. The results of this work have also been published recently, Appendix XIII.

The genes identified as conferring tetracycline resistance [*tetAB(60)*] and reduced CTAB susceptibility (*galE*) in this study were characterised as such in a heterologous host (*E. coli*). It is, however, possible that TetAB(60) does not function to export tetracyclines in its native host and that GalE does not alter susceptibility to CTAB except when expressed in our *E. coli* EPI300 T1^R host. Similarly, the MFS [*tet(L)*] and ABC transporters that likely play a role in tetracycline resistance in TT31 may have alternative substrates in their original host. This raises an important question; if a gene plays no role in antimicrobial resistance in its native host should it be called a resistance gene if it confers resistance when heterologously expressed in a different host?

As discussed previously, an opinion piece by Martínez *et al.* (2015), suggested that housekeeping genes identified in metagenomic studies should not be described as resistance genes unless they are demonstrated to be transferable (Martínez *et al.*, 2015). Although this seems a logical statement to make, it may also be an excessive one. As any gene has the potential to be incorporated into a MGE, any gene identified as conferring resistance in a functional screen should be classified as a resistance gene. Indeed, in a correspondence to this article, Bengtsson-Palme and Larson (2015) comment, "In principle, resistance need only emerge once in a single cell in order for it to have potential to rise in frequency and be transferred" (Bengtsson-Palme and Larsson, 2015). In this respect, functional metagenomics allows us to conduct prospective studies on what genes (housekeeping or otherwise) in any environment could pose a threat to the success of antimicrobial use.

Looking at the recent identification of the plasmid-borne colistin-resistance gene, *mcr-1*, in *E. coli*, it is possible that a functional metagenomic survey using a large insert vector such as pCC1BAC may have identified this gene in its plasmid context prior to identification in *E. coli* (Liu *et al.*, 2015). Indeed, following the identification of this plasmid-borne *mcr-1*, subsequent studies have identified the gene in diverse phylotypes of food-producing animal *E. coli* isolates dating back to 2004. This indicates that the gene was spreading via HGT long before it was first described (El Garch *et al.*, 2017; Skov and Monnet, 2016). Similarly, serine β -lactamases have been identified in *Kluyvera* spp. that typically reside in soil. In fact, it is likely that human pathogens have acquired β -lactamases genes from *Kluyvera* spp. This was not hypothesised until after these resistance genes had already disseminated to pathogens (Lartigue *et al.*, 2006). It may be that the identification of *tetAB(60)* in our human saliva metagenomic library will be followed by its identification in a human pathogen, if there is a great enough selection for its maintenance.

Conforming to Martínez's definition, *fabI* which encodes a reductase involved in fatty acid synthesis has also been described as conferring reduced susceptibility to triclosan when heterologously expressed in certain hosts (Heath *et al.*, 2000). Recently, *fabI* has been identified on a MGE in an *S. aureus* isolate that had reduced susceptibility to triclosan, indicating that there was a selective advantage for this isolate to maintain the acquired *fabI*. The authors concluded that as the *S. aureus* isolate had no mutations in its native *fabI*, the observed reduced susceptibility was a result of increased FabI production from the two genes. A similar scenario can be envisaged for *galE*, whereby a cell acquires *galE* via HGT allowing it to produce surplus UDP-galactose that can be incorporated into an LPS structure that has a reduced affinity for CTAB. Following my departure from the Eastman Dental Institute, clones with reduced triclosan susceptibility were also identified from the human saliva metagenomic library. As CTAB and triclosan are used in dental hygiene products and as oral

treatments there would be a selection pressure for cells heterologously expressing these genes to maintain them, if they had these resistant or reduced susceptibility phenotypes.

The acquisition of heterologous housekeeping genes may contribute to the stepwise evolution of antimicrobial resistance in some strains as they may confer small, incremental changes in susceptibility. It is possible that the expression of *galE* in a heterologous host may allow it to persist in low CTAB concentrations making it more likely to acquire additional genes or mutations that would result in a further decreased susceptibility which would not be seen without the previously acquired housekeeping gene. These acquired genes or mutations may have an epistatic relationship with *galE* that allows the cell to survive in higher CTAB concentrations. The stepwise evolution of resistance to some antibiotics has been demonstrated. For example, Toprak *et al.* demonstrated, *in vitro*, that resistance to trimethoprim developed in a stepwise manner in *E. coli* due to the slow acquisition of mutations in the gene encoding the antibiotics target, dihydrofolate reductase. The successive acquisition of mutations in this gene allowed the cell to survive in higher concentrations of trimethoprim, thus making it more likely to obtain additional mutations that would further increase its resistance (Toprak, 2011).

To conclude, classifying a gene as a resistance gene should depend on the context of its expression. If expression of such a gene confers resistance or reduced susceptibility in a heterologous host but not in its native host it should be referred to as a resistance gene in the former but not the latter. This distinction is particularly important considering that, theoretically, any gene has the potential to be mobilised, transferred and expressed in multiple hosts.

Chapter 9

References

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Appendix I – XIII

All Appendices are included with the compact disk included with this thesis.

Appendix I Ethics Form for Human Saliva Collection

Appendix II QIIME Script

Appendix III Nucleotide Sequence of PS9 Insert (CD)

Appendix IV PS9 Alignment with *Granulicatella adiacens* ATCC 49175 (CD)

Appendix V PS9 Alignment with *Streptococcus* sp. 263_SSPC (CD)

Appendix VI *tetAB*(60) sequence (CD)

Appendix VII Nucleotide Sequence of TT31 Insert (CD)

Appendix VIII Alignment of TT31 with Tn916 (CD)

Appendix IX Alignment of TT31 with Tn6079 (CD)

Appendix X Alignment of TT31 with *L.johnsonii* strain BS15 genome (CD)

Appendix XI Alignment of Tn916 with *L. johnsonii* strain BS15 genome (CD)

Appendix XII A10(F2) Forward and Reverse Sequences (CD)

Appendix XIII Publication that resulted from this PhD

Appendix I Ethics Form for Human Saliva Collection

UCL RESEARCH ETHICS COMMITTEE
GRADUATE SCHOOL OFFICE



Professor Dave Spratt
Eastman Dental Institute
256 Gray's Inn Road
UCL

9 September 2013

Dear Professor Spratt

Notification of Ethical Approval

Project ID: 5017/001: Understanding oral microbial ecology

I am pleased to confirm that in my capacity as Chair of the UCL Research Ethics Committee I have approved your study for the duration of the project i.e. until September 2018.

Approval is subject to the following conditions:

1. You must seek Chair's approval for proposed amendments to the research for which this approval has been given. Ethical approval is specific to this project and must not be treated as applicable to research of a similar nature. Each research project is reviewed separately and if there are significant changes to the research protocol you should seek confirmation of continued ethical approval by completing the 'Amendment Approval Request Form'.

The form identified above can be accessed by logging on to the ethics website homepage: <http://www.grad.ucl.ac.uk/ethics/> and clicking on the button marked 'Key Responsibilities of the Researcher Following Approval'.

2. It is your responsibility to report to the Committee any unanticipated problems or adverse events involving risks to participants or others. Both non-serious and serious adverse events must be reported.

Reporting Non-Serious Adverse Events

For non-serious adverse events you will need to inform Helen Dougal, Ethics Committee Administrator (ethics@ucl.ac.uk), within ten days of an adverse incident occurring and provide a full written report that should include any amendments to the participant information sheet and study protocol. The Chair or Vice-Chair of the Ethics Committee will confirm that the incident is non-serious and report to the Committee at the next meeting. The final view of the Committee will be communicated to you.

Reporting Serious Adverse Events

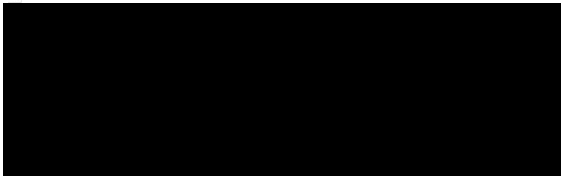
The Ethics Committee should be notified of all serious adverse events via the Ethics Committee Administrator immediately the incident occurs. Where the adverse incident is unexpected and serious, the Chair or Vice-Chair will decide whether the study should be terminated pending the opinion of an independent expert. The

adverse event will be considered at the next Committee meeting and a decision will be made on the need to change the information leaflet and/or study protocol.

On completion of the research you must submit a brief report (a maximum of two sides of A4) of your findings/concluding comments to the Committee, which includes in particular issues relating to the ethical implications of the research.

With best wishes for the research.

Yours sincerely



Professor John Foreman
Chair of the UCL Research Ethics Committee

Cc:
Adam Roberts, Applicant
Professor Stephen Porter, Director, Eastman Dental Institute

Appendix II QIIME Script

To pair-end forward and reverse reads in the FASTQ files.

```
join_paired_ends.py-f LIAM1_S78_L001_R1_001.fastq.gz -r LIAM1_S78_L001_R2_001.fastq.gz -o LIAM1
```

```
join_paired_ends.py-f LIAM2_S79_L001_R1_001.fastq.gz -r LIAM2_S79_L001_R2_001.fastq.gz -o LIAM2
```

To rename the files following pairing to sample_name_paired.fastq and copy them to the working directory (Desktop/QIIME/Run1)

```
cp ~/Desktop/QIIME/Liam/LIAM1/fastqjoin.join.fastq LIAM1_paired.fastq
```

```
cp ~/Desktop/QIIME/Liam/LIAM2/fastqjoin.join.fastq LIAM2_paired.fastq
```

To quality trim sequences with low base confidence scores (Phred count <20)

```
split_libraries_fastq.py -i LIAM1_paired.fastq --sample_ids LIAM1 -o quality_filtered_q20_LIAM1/ -q 19 --barcode_type 'not-barcoded'
```

```
split_libraries_fastq.py -i LIAM2_paired.fastq --sample_ids LIAM2 -o quality_filtered_q20_LIAM2/ -q 19 --barcode_type 'not-barcoded'
```

#To copy the resultant files into the working directory and rename them as seqs_quality_filtered.fna

```
cp ~/Desktop/QIIME/Liam/quality_filtered_q20_LIAM1/seqs.fna seqs_quality_filtered_1.fna
```

```
cp ~/Desktop/QIIME/Liam/quality_filtered_q20_LIAM2/seqs.fna seqs_quality_filtered_2.fna
```

#To pick OTUs using the open reference method and save the resulting files in Desktop/QIIME/Run2/otu_picking_stage. Read sequences are compared with the green genes coresets (closed reference method). Unidentified reads are compared with each other and those that match are deemed an OTU. Only OTUs identified by the closed reference methods are aligned by PyNAST.

```
pick_open_reference_otus.py -i seqs_quality_filtered_1.fna -o otu_picking_stage_1/
```

```
pick_open_reference_otus.py -i seqs_quality_filtered_2.fna -o otu_picking_stage_2/
```

To summarise the taxa present in the samples and store results in a new table

```
summarize_taxa.py-i
```

```
otu_picking_stage_1/otu_table_mc2_w_tax_no_pynast_failures.biom -o
```

```
summarised_taxa1
```

```
summarize_taxa.py-i
```

```
otu_picking_stage_2/otu_table_mc2_w_tax_no_pynast_failures.biom -o
```

```
summarised_taxa2
```

```
# Move the appropriate file across from the folder into the working directory and group the aligned reads based on taxa from phylum to genus
```

```
Cp
~/Desktop/QIIME/Liam/summarised_taxa1/otu_table_mc2_w_tax_no_pynast_failures_L2.txt
phylum1.txt
cp
~/Desktop/QIIME/Liam/summarised_taxa2/otu_table_mc2_w_tax_no_pynast_failures_L2.txt
phylum2.txt
cp
~/Desktop/QIIME/Liam/summarised_taxa1/otu_table_mc2_w_tax_no_pynast_failures_L3.txt
class1.txt
cp
~/Desktop/QIIME/Liam/summarised_taxa2/otu_table_mc2_w_tax_no_pynast_failures_L3.txt
class2.txt
Cp
~/Desktop/QIIME/Liam/summarised_taxa1/otu_table_mc2_w_tax_no_pynast_failures_L4.txt
order1.txt
cp
~/Desktop/QIIME/Liam/summarised_taxa2/otu_table_mc2_w_tax_no_pynast_failures_L4.txt
order2.txt
Cp
~/Desktop/QIIME/Liam/summarised_taxa1/otu_table_mc2_w_tax_no_pynast_failures_L5.txt
family1.txt
Cp
~/Desktop/QIIME/Liam/summarised_taxa2/otu_table_mc2_w_tax_no_pynast_failures_L5.txt
family2.txt
Cp
~/Desktop/QIIME/Liam/summarised_taxa1/otu_table_mc2_w_tax_no_pynast_failures_L6.txt
genus1.txt
Cp
~/Desktop/QIIME/Liam/summarised_taxa2/otu_table_mc2_w_tax_no_pynast_failures_L6.txt
genus2.txt
```

```
## To create pie charts showing the taxonomic break down of the samples at various levels
```

```
plot_taxa_summary.py-i
phylum1.txt,phylum2.txt,class1.txt,class2.txt,order1.txt,order2.txt,family1.txt,family2.txt,genus
1.txt,genus2.txt -l Phylum,Phylum,Class,Class,Order,Order,Family,Family,Genus,Genus -c pie -o
charts/
```

Appendix II. This script details how the demultiplexed FASTQ data obtained from Illumina Miseq sequencing of the V5-V7 region of the 16S gene was processed using QIIME. Lines preceded by a # are descriptions of the script that follows.

Sample name LIAM1 refers to the calf faecal metagenomic preparation and LIAM2 refers to the human saliva metagenomic preparation.

Appendix XIII Publications Resulting from this Study

Reynolds LJ, Roberts, AP, Anjum MF (2016) Efflux in the Oral Metagenome: The Discovery of a Novel Tetracycline and Tigecycline ABC Transporter. *Front Microbiol.* 2016; 7: 1923.

Supathet T, Reynolds LJ, Cristarella G, Wong LC, Rosendahl K, Roberts, AP (2017) Reduced Susceptibility to Antiseptics is Conferred by Heterologous Housekeeping Genes. *Microb Drug Resist.* 2017.