ANGLIA RUSKIN UNIVERSITY

THE MOLECULAR EPIDEMIOLOGY OF ANTIMICROBIAL RESISTANCE GENES AND THE CHARACTERISATION OF THEIR GENETIC SUPPORT

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A thesis in partial fulfilment of the
requirements of Anglia Ruskin University
for the degree of Master of Philosophy

Submitted: 14th JANUARY 2017

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ABSTRACT

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One of the growing public health concerns in the 21st century is the increasing prevalence of bacteria that are resistant to antibiotics. This has been due to the overuse and misuse of antibiotics in healthcare and agriculture. Increased levels of antibiotic resistant bacteria have been observed and new antibiotic-resistant strains of bacteria will continue to arise.

Bacterial resistance to antimicrobials is typically gained through the acquisition of genes that confer antibiotic resistance. Such genes are often associated with mobile genetic elements (MGEs) such as plasmids and transposons and are capable of moving between different bacteria both within a species and in between different species.

In order to attempt to curb the spread of antimicrobial resistance a greater understanding of the effect of antibiotics on both commensal and pathogenic strains of bacteria is required, including the investigation into the genetic basis of antibiotic resistance.

The presence of mobile genetic elements was investigated in bacteria that were obtained from swabs from infected wounds and saliva from healthy volunteers. These isolates were predominantly members of the *Streptococcus* and *Staphylococcus* genus. Two isolates from wound sites confirmed to be *Corynebacterium striatum* demonstrated resistance to tetracycline (MIC >512ug/ml). Further investigation has revealed that the resistance gene present is *tetW*. A ribosomal protection protein that has only been observed once before in this species of bacteria in China. To date there have been no such observations in the EU or US. This suggests that the *Corynebacterium striatum* isolates acquired the *tetW* gene via a plasmid obtained by horizontal gene transfer. Sequence analysis of the DNA regions flanking this gene indicate that it may be located upon a transposon that belongs to the Tn*5253* family.

The presence of novel plasmids in metagenomic DNA from saliva was investigated using transposon aided capture (TRACA), the plasmids that were discovered mostly belonged to the pTRACA42 family, plasmids were found that carried antibiotic resistance genes that confer resistance to chloramphenicol, ampicillin and kanamycin, clonal resistance was confirmed via minimum inhibitory concentration (MIC) testing. In order to detect the transposon Tn*916*, a screening method based upon the characterisation of the digestion profile of two polymerase chain reaction (PCR) amplicons with the restriction enzyme *Hinc*II. This method was used to successfully detect Tn*916* in the control strain *Bacillus subtilus* 34A.

Overall, the data generated throughout this study adds to the body of evidence supporting that there is a large reservoir of antibiotic resistance genes that are located upon mobile elements, in particular those that belong to the Tn*916* family play a significant role in the maintenance of tetracycline and erythromycin resistance genes.

Key words: Antimicrobial resistance, mobile genetic elements, tetracycline, erythromycin, horizontal gene transfer, molecular microbiology, transposon, plasmid.

ACKNOWLEDGEMENTS

The author would like to thank his supervisory team Ben Evans and Peter Coussons, for their support and motivation.

I would also like to thank my fellow postgraduate students, Alex Dietrich, Mark D'Arcy, Dipak Meshram, Himel Chowdhury, Aparna Shil and Silvia Caprari for their support as well as the technical staff at Anglia Ruskin University, in particular Imi Duncan, Natalie Lambert and Carolyn Bugg.

Lastly, thanks to all my friends and family particularly Kim, Peter and Liam Whelan, Shirin Naserikarimvand, Tom Brown, Joshua de Boer, Frederik Feldmann and Ewa Sierawska.

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Chapter 1

Introduction

1.1 Antibiotic Resistance

Antibiotics refer to substances that either kill (bactericidal) or inhibit the growth of bacteria (bacteriostatic) (Memeth *et al.*, 2014). The discovery and subsequent clinical application of antibiotics has resulted in a major improvement in infectious disease treatment. However, the continuous exposure to antibiotics has provided a selection pressure that has facilitated an acceleration in the emergence of antibiotic resistance, particularly in the case of pathogens that cause nosocomial infections (Hsueh *et al.*, 2002).

The continued emergence of strains of pathogenic bacteria that are resistant to antimicrobial therapies is a significant problem in public healthcare. Historically bacterial infections, with pathogens such as *Staphylococcus aureus*, were difficult to treat and were potentially fatal prior to the discovery of penicillin (Flemming, 1929). The introduction of penicillin and subsequent antibiotics revolutionised the treatment of bacterial infections. Since the emergence of antimicrobial resistance the effect of antibiotics as clinical

treatment has been reduced. Examples illustrating this include the case of a 33 year old woman who was dying as a result of Streptococcal sepsis in 1942, at the beginning of the antibiotic era whereby treatment resulted in a full recovery and the patient surviving until age 90. In the space of 66 years and the rise of antimicrobial resistance saw a different outcome for man who was afflicted with endocarditis as a result of infection by *Enterococcus faecium*, antimicrobial treatment failed due to resistance that the infecting strain had acquired and as such the clinicians were unable to treat his infection using antibiotics and he subsequently died as a result of the infection (Arias and Murray, 2009). Such cases demonstrate how, for patients who are infected with multidrug resistant bacteria, treatment is similar to that in the pre-antibiotic era.

The evolution of methicillin resistant *Staphylococcus aureus*(MRSA) shows the adaption of an organism into a multi-drug resistant pathogen. Shortly after the introduction of penicillin and other β-lactam antibiotics, *S. aureus* developed resistance to β-lactams, by the year 2003 half of the *S. aureus* isolates recovered from U.S. hospitals were resistant to methicillin (Arias and Murray, 2009).

The problems that are presented by antibiotic resistance are wider reaching than just the curing of bacterial diseases, antibiotics are crucial in enabling the application of medical and surgical techniques to patients who are weak, elderly or immunocompromised, therefore the increase in antimicrobial resistance in pathogenic bacteria also impacts medical surgery, anticancer chemotherapy and patient immunosuppression following transplant (Levy, 1998; Smith *et al.*, 2004). A statement from the World Health Organisation highlights the severity of antimicrobial resistance: medical procedures once taken for granted could be conceivably consigned to medical limbo. The

repercussions of which are almost unimaginable (World, 2000). Therefore it is necessary to conduct research that addresses means that may aid in the facilitation of predicting and possibly limiting antibiotic resistance (Martinez *et al.*, 2007). In order to achieve this, two distinct problems need to be addressed, these are the acquisition of antibiotic resistance genes by previously susceptible bacteria (Davies, 1997; Martinez and Baquero, 2000) and intrinsic resistance (Fajardo *et al.*, 2008).

1.1.1 The Resistome

The concept of the resistome refers to an aggregate of all the antibiotic resistance mechanisms (Wright, 2007; D'Costa *et al.*, 2007). These mechanisms are not confined to the pathogenic bacteria rather they also include the microorganisms that are not typically associated with disease, an example being bacteria that produce antibiotics where the co-evolution of resistance is required in order to prevent auto-toxicity. Previous research has shown that in multiple occasions the antimicrobial resistance mechanisms that emerge clinically have originated from non-pathogenic bacteria (Thaker and Spanogiannopoulos, 2010). Examples of this include glycopeptide resistance due to altered cell wall biosynthesis (Marshall *et al.*, 1998), inactivating enzymes causing aminoglycoside resistance (Davis and Wright, 1997) and the CTX class of β-lactamases that confer β-lactam resistance (Canton and Coque, 2006) as well as ribosomal methylation (Liou *et al.*, 2006). The understanding of antimicrobial resistance in its totality rather than the focusing on mechanisms only from pathogenic bacteria that have emerged as clinical problems it is possible to better understand the evolution of resistance allowing for the anticipation of new resistance mechanisms coming from the environment into the clinic and use this information to guide drug

discovery research (Thaker and Spanogiannopoulos, 2010).

1.1.1.1 The Tetracycline Resistome

The tetracycline resistome is large and diverse, it includes over 1000, reported resistance genes that span over 80 genera and 350 species, this resistome may be the largest against a single class of antibiotics (Thaker and Spanogiannopoulos, 2010). The reason that there may be such a large distribution of tetracycline resistance genes may be due to there being a large amount of naturally occurring tetracycline like molecules in the environment. It has been estimated that 1 out of 1000 actinomycetes produces tetracycline (Baltz, 2007). An average soil sample contains approximately 10^6 - 10^8 bacteria. Tetracycline resistance has been demonstrated in a diverse range of soil sample from across the globe (D'costa *et al.*, 2006). One theory (Baltz, 2007) explaining the prevalence of tetracycline resistance is that the 1:1000 actinomycetes produces tetracycline and approximately 5%-6% of the bacteria that reside within the soil are actinobacteria thus there is a large amount of naturally occurring tetracycline molecules creating an evolutionary selective pressure favouring the development and acquisition of tetracycline resistance (Thaker and Spanogiannopoulos, 2010).

1.2 Nosocomial Infections

Nosocomial infections refer to infections that have been acquired either in a hospital setting or as a result of healthcare related interventions. For an infection to be classed as nosocomial, they must arise between 48 and 72 hours following hospital admission or within thirty days following discharge (Breathnach, 2009). The highest frequency of nosocomial infections in British

hospitals occurs within intensive care units and surgical wards (Majumdar and Padiglione, 2012). Antibiotic resistant nosocomial infections are associated with an increase in the morbidity and mortality of patients (Woodford and Livermore, 2009).

1.3 Mechanisms of Antibiotic Resistance

1.3.1 The Ancient Resistome

The development of resistance to antibiotics is the product of evolution over billions of years (Blair *et al.*, 2014). Evidence of this stems from observing bacteria that reside at pristine sites where there has been no human activity, yet antibiotic resistance persists (Bhullar *et al.*, 2012). The majority of antibiotics that are employed for use as medication are derived from antimicrobial compounds that are produced naturally by microorganisms. As these microorganisms produce antimicrobial compounds, other microbial species that reside in close proximity are exposed to these compounds, therefore creating a selection pressure for the development and maintenance of antimicrobial resistance in a setting unrelated to human activity (Blair *et al.*, 2014). The result of this, is a situation where a natural reservoir of antimicrobial resistance genes that have the potential to transfer into human pathogens and thereby reduce the effect of treatment (Wellington *et al.*, 2013). Since their discovery, the excessive usage of antibiotics by humans in the fields of medicine and agriculture has provided a selective pressure on genes that confer resistance to transfer from these ancient resistomes such as the "soil resistome" into pathogenic bacteria, this is demonstrated by the fact that the same resistance genes are present in both soil microflora and human pathogens (Blair *et al.*, 2014). Further evidence supporting this is demon-

strated by genes that confer resistance to antimicrobials prior to their first clinical use, for example the recently licensed daptomycin (D'Costa *et al.*, 2012; Perry and Wright, 2013).

1.3.2 Obstruction of Target

1.3.2.1 Decreased Permeability

An obvious strategy in the development of antimicrobial resistance is to prevent antimicrobial agents from reaching their target. There are a variety of ways that this can be achieved, one such way is reduced permeability. This is evidenced when comparing Gram positive and Gram negative species. The reduced permeability of Gram negative bacteria, as a result of the outer membrane grants these species intrinsic resistance to some antibiotics, with a notable example being penicillin (Kojima and Nikaido, 2013; Vargiu and Nikaido, 2012). Some species of bacteria, such as *Enterobacteriaceae*,*Pseudomonas* spp. and *Acinetobacter* spp. are able to reduce their permeability via the down regulation or replacement of porins. It has been noted in recent studies that porin expression reductions have allowed these species to demonstrate resistance to later generation antibiotics such as cephalosporins and carbepenems, resistance to these antibiotics typically occurs via enzymatic degradation (Lavigne *et al.*, 2013). An example of this has been observed in *Enterobacteriaceae* where in the absence of carbapenem, mutations reducing porin production have resulted in the development of clinically relevant levels of resistance to carbapenem (Poulou *et al.*, 2013). Exposure to carbepenems creates a selection pressure that favours such mutations in genes that encode porins or are involved with porin expression, this has been demonstrated by an accumulation of mutations in such genes

following carbepenem exposure in *E. coli* and *Enterobacter* spp. (Tangden *et al.*, 2013).

1.3.2.2 Efflux Pumps

Efflux pumps are responsible for the active transportation of antibiotics out of the cell. Some types of efflux pumps are specific and are only able to remove a certain type of antibiotic, whereas other efflux plumps are able to remove a range of structurally dissimilar antibiotics from the cell, such pumps are referred to as multidrug resistance (MDR) efflux pumps (Blair *et al.*, 2014).

Efflux pumps that transport multiple substrates have not evolved in response to selection pressure bought about by the antibiotic era (Webber and Piddock, 2001). All bacterial chromosomes contain multiple genes that encode multi-substrate efflux pumps which highlights their ancient origin (Webber and Piddock, 2001).

1.3.3 Modification of Antimicrobial Targets

1.3.3.1 Structural Changes Based upon Mutation

Antibiotics typically have a high affinity for their target, therefore preventing the normal activity of the target once bound. Because of this, mutations that alter the structure of the target may still allow the target to function correctly yet prevent binding by the antibiotics. Strains that are carrying this mutation are then able to proliferate, the presence of the antibiotic causes a selection pressure that encourages the proliferation of strains containing the mutation. An example of this occurring has been observed from clinical usage of the antibiotic linezolid, that targets the 23S rRNA of Gram positive bacteria,

has created a selection pressure favouring a mutant allele in *S. pneumoniae* and *S. aureus* as possession of this allele confers resistance (Gao *et al.*, 2010; Leclercq, 2002).

1.3.3.2 Direct Modification of Targets

Another mechanism of antibiotic resistance involves the modification of the target without relying on mutation (Blair *et al.*, 2014). This mechanism is relevant in the case of several antibiotics, with two examples being tetracycline and erythromycin. Prominent examples include, in the case of erythromycin, the *erm* family of genes which confer resistance by methylating the 16S rRNA which prevents erythromycin from binding to its target site (Blair *et al.*, 2014). In the case of tetracycline, the gene *tetM* provides protection to the ribosome by encoding a soluble protein that prevents tetracycline from binding to the ribosome, as such TetM belongs to a family of proteins called ribosomal protection proteins (RPPs) (Donhofer *et al.*, 2012). Original models describing the mechanism by which TetM prevents tetracycline from binding to the ribosome described an indirect situation where the TetM protein prevents tetracycline binding by causing conformational changes to the structure of the ribosome. Since then, an alternative model has been presented where TetM directly dislodges tetracycline from its binding site on the ribosome to confer resistance (Donhofer *et al.*, 2012).

1.3.4 Modification of Antimicrobial Agents

Some mechanisms of antibiotic resistance rely upon the direct modification of antimicrobial agents to render them ineffective. This was the first mechanism of antimicrobial resistance discovered in 1940 with the discovery of β-lactamase (Blair *et al.*, 2014). There have been thousands of enzymes

identified that confer resistance to antimicrobial agents of unrelated classes. In addition to this there are also subclasses of enzymes that are able to inactivate antibiotics within the same class. An example can be seen in the β-lactam antibiotics where different types of β-lactams such as penicillins, carbepenems, cephalosporins, monobactams and clavams are rendered ineffective due to hydrolysis by different β-lactamase enzymes (Voulgari *et al.*, 2013).

1.3.5 Resistance to Tetracycline Antibiotics

Genes that confer resistance to tetracycline are named as *tet* genes referring to tetracycline resistance or *otr* genes referring to oxytetracycline resistance, the difference in nomenclature is due to the oxytetracycline resistance genes being discovered in bacteria that produce oxytetracycline, there is no inherent difference between *tet* and *otr* genes (Chopra and Roberts, 2001).

1.3.5.1 Tetracycline Efflux

To date there have been 26 different classes of tetracycline efflux pumps that have been discovered (Thaker and Spanogiannopoulos, 2010), these have been classified into 11 groups (Chopra and Roberts, 2001; Roberts, 2005). Efflux proteins are found across several microbial genera, and are membrane proteins that span across the lipid bilayer of the inner cell membrane 12 to 14 times (Thaker and Spanogiannopoulos, 2010).

1.3.5.2 Ribosomal Protection

The RPP represent a class of tetracycline resistance determinants that is widely distributed spanning both Gram positive and Gram negative species. There are 11 different types of RPPs with Tet(M) and Tet(O) being the most

prevalent and heavily studied classes. Tet(M) has been detected within 24 genera and Tet(O) has been detected in 8 genera. There are three groups of RPPs that have been classified based upon their amino acid sequences, these groups are shown in Table 1.1. RPPs confer resistance to tetracyclines by weakening the interaction between tetracycline and the ribosome resulting in the subsequent antibiotic release, freeing the ribosome from the inhibitory effects caused by tetracycline (Connell *et al.*, 2003). This mechanism confers resistance to first and second generation tetracyclines but not the third generation. For example the third generation tigecycline has been shown to exhibit antibacterial activity against Tet(M)-protected ribosomes possibly because the drug has a stronger binding to its target (Rasmussen *et al.*, 1994).

Table 1.1: RPP class of tetracycline resistance determinants further categorised into three groups based upon amino acid sequence (Thaker and Spanogiannopoulos, 2010)

Group 1	Group 2	Group 3
Tet(M)	TetB(P)	Tet(Q)
Tet(O)	Otr(A)	Tet(T)
Tet(S)	Tet	
Tet(W)		
Tet(32)		
Tet(36)		

1.3.5.3 Enzymatic Inactivation

There have been relatively few tetracycline inactivators that have been reported, this is in contrast to other classes of antibiotics such as aminoglycosides and β-lactams where modification and inactivation are the predominant mechanisms of resistance, as of 2010 only three such genes had been identified within the tetracycline resistome (Thaker and Spanogiannopoulos, 2010) however 10 more such genes have been discovered since then. The first tetracycline resistance determinant that confers resistance to tetracycline via this mechanism was discovered in 1989 was Tet(X) from *Bacteroides fragilis* (Speer and Salyers, 1989).

1.3.5.4 Mosaic Elements

Mosaic derivatives of known tetracycline resistance genes have recently been discovered, in mosaic genes at least one section of the gene shows a homology that is above 80% to a known tetracycline resistance gene and the other section shows homology to either a different known tetracycline resistance gene or a new determinant (Thaker and Spanogiannopoulos, 2010). These mosaic elements are composed of determinants from the RPP group and have involved Tet(M), Tet(O), Tet(W) and Tet(32). Most of the bacteria that have been found carrying mosaic elements have been isolated from either farm animals or humans. All of the mosaic genes that have been identified to date have shown different patterns of mosaicism however the final size of the gene remains unaltered (Thaker and Spanogiannopoulos, 2010).

1.4 Horizontal Gene Transfer

Horizontal gene transfer (HGT) refers to the transfer of genetic material between bacteria without reproduction as in vertical gene transfer (Poole, 2009). HGT is an important mechanism in the dissemination of antibiotic resistance genes. HGT occurs via three different mechanisms. The three main mechanisms facilitating HGT are conjugation, transformation and transduction. Conjugation refers to the direct transfer between donor and recipient cells in a manner that is insensitive to DNase enzymes, this mechanism facilitates the transfer of conjugative plasmids and conjugative transposons (Roberts and Kreth, 2015). Transduction refers to the transfer of host genomic DNA by bacteriophages that package host DNA into the bacteriophage head structures (Roberts and Kreth, 2015). Whereas transformation refers to the uptake of exogenous extracellular DNA present in the environment, often this DNA has been released by dead bacteria (Roberts and Kreth, 2015).

1.5 Mobile Genetic Elements

Mobile genetic elements refer to sections of DNA that are able to move as a result of genes that code for proteins that facilitate the movement of the DNA both intra-cellularly (within the same cell, between different parts of the bacterial genome, and between plasmids and the chromosome), and extracellularly (in between different bacterial cells both belonging to the same species, and other species of bacteria) (Frost *et al.*, 2005). The transfer of antibiotic resistance genes in bacteria typically occurs as a result of mobile genetic elements such as transposons and plasmids. Such mobile elements have the capability of moving between different bacteria both within the same species and in between different species, these elements are responsible

for increased levels of antibiotic resistant bacteria in patients who are undergoing antimicrobial therapy (Warburton *et al.*, 2007). In addition to this, research has resulted in the identification of mobile elements that carry multiple antibiotic resistance genes that confer resistance to unrelated antibiotics (Henderson-Begg *et al.*, 2009; Warburton *et al.*, 2007).

1.5.1 Conjugation

Conjugation refers to cell to cell DNA transfer that may occur, sometimes through a pilus that connects the two cells and acts as a tunnel allowing genetic material to pass through. Conjugation is also possible via cell to cell contact without the presence of a pilus. Conjugation usually involves conjugative elements that are self transmissible such as plasmids and conjugative transposons (Frost *et al.*, 2005).

1.6 Plasmids

1.6.1 Overview

Plasmids are DNA molecules that are able to replicate and exist independently from the bacterial genome, some exist in a circularised form whereas others are linear. They are ubiquitous and exist naturally within prokaryotes, arcehea and eukaryotyes (Meinhardt *et al.*, 1990; Zillig *et al.*, 1996; Couturier *et al.*, 1988). They vary in size and may confer selective advantages to their host bacteria, for example genes that confer resistance to antibiotics or genes that enable the host bacteria to metabolise nutrients more effectively. When the host cell divides and the bacterial chromosome is replicated, any plasmids that are present within the bacteria are also replicated. These elements are

able to transfer between different bacterial cells via the process of conjugation (Arias and Murray, 2009).

1.6.2 Plasmid Types

1.6.2.1 Linear Plasmids

Linear plasmids have been discovered in both Gram positive and Gram negative bacterial species (Hinnebusch and K., 1993), they were first discovered in *Streptomyces* sp. in 1979 (Hayakawa *et al.*, 1979). Research has uncovered linear plasmids in the tick born spirochaete pathogen *Borrelia turicatae* (Kingry *et al.*, 2016) The linear plasmids of *Borrelia* have a covalently closed hairpin loop at each end, whereas the linear plasmids found in *Streptomyces* sp. have a covalently attached protein at each end (Hinnebusch and K., 1993).

1.6.2.2 Conjugative Plasmids

Conjugative plasmids are plasmids that are able to initiate and use bacterial conjugation in order to transfer between different cells (Norvick *et al.*, 1976). Bacterial conjugation refers to the process whereby genetic information is exchanged between a donor and recipient bacterial cell via cellular contact (Norvick *et al.*, 1976). Conjugative plasmids are large compared with non-conjugative plasmids, conjugative plasmids greater than 100kb exist. This larger size reflects the DNA that they carry that is required to encode the proteins that are involved with conjugation, this DNA can be around 20-30kb in size (Bennett, 2008). Different conjugative plasmids exhibit either a broad or narrow range in their host bacteria with both types being common, an example of a plasmid with a broad host range is RP1 which was first detected

in *Pseudomonas aeruiginosa*, this plasmid is able to transfer to most Gram negative species (Bennett, 2008).

1.6.2.3 Nonconjugative Plasmids

These plasmids lack the necessary genes to be able to initiate bacterial conjugation in order to facilitate transfer, however, some nonconjugative plasmids may mobilise via conjugative factors that co-exist within the host cell, these are known as mobilisable plasmids (Norvick *et al.*, 1976). Nonconjugative plasmids are typically smaller than conjugative plasmids, often smaller than 10kb, these plasmids lack the necessary genes that are required to initiate conjugation, however they do carry genes that facilitate the transfer of their own DNA as these plasmids are mobilisable when they are co-resident with a conjugative plasmid that is able to initiate conjugation (Bennett, 2008).

1.6.3 Plasmid Incompatibility

Plasmid incompatibility occurs when two plasmids that are present within the same bacterial host cell are not able to both be successfully inherited by the daughter cells resulting in the two plasmids being inherited by different daughter cells. Incompatibility arises as plasmids that have the same origin of replication systems, cannot co-exist within the same cell (Norvick *et al.*, 1976). Plasmids that are not compatible with one another have been placed into "incompatibility groups" as a means of plasmid classification, of which over 30 of such groups exist presently (Shintani *et al.*, 2015).

1.7 Maintenance and Stability Systems

While the advantages of HGT are clear, it is still not sufficient alone to understand how plasmids are able to persist over a long period of time in bacterial populations (Millan *et al.*, 2016). Classical models predict that HGT via conjugation is required in order to maintain the plasmid population due to the fitness cost that is associated with carrying plasmids (Vogwill and MacLean, 2014; Baltrus, 2013). More recent research show that there is a compensatory adoption that alleviates plasmid fitness cost which allows plasmids to persist within a population for hundreds of generations without the necessity of HGT (Harrison and Brockhurst, 2012; Peria-Miller *et al.*, 2015). It is also possible for beneficial genes that have originated on plasmids to transfer to the bacterial chromosome via transposons which allows the bacteria to keep the benefits that are associated with a particular gene whilst eliminating the fitness cost from the plasmid (Modi *et al.*, 1992). Although this is the case, it has been demonstrated that there are advantages to carry a gene on a plasmid rather than the bacterial chromosome, one advantage is that if the gene is being carried on a multi copy plasmid then higher gene expression will be produced (Latorre *et al.*, 2005), another possible advantage is the variation in plasmid copy number in the different cells of the population causing gene expression heterogeneity which may provide a beneficial phenotypic plasticity (Zhang *et al.*, 2009). Another advantage to carrying genes on plasmids that was recently demonstrated is that multicopy plasmids function as platforms for evolution via increasing the mutation rate because of the increased gene copy per cell (Millan *et al.*, 2016).

1.7.1 Toxin-Antitoxin Systems

Toxin-Antitoxin (TA) systems refer to a pair of complimentary gene codes, one of which codes for a toxin and the other codes for an antitoxin. The TA genes are located upon plasmids and collectively create an environment in which for the bacteria to survive it is essential for the plasmid to be inherited when the bacteria divide. This occurs because in the absence of the plasmid, the unstable antitoxin is no longer coded for and therefore is not replenished, thus the bacteria continues to live in the presence of the stable toxin eventually leading to a form of toxicity, referred to as post-segregational killing (PSK) (Hayes, 2003). The first TA system to be discovered was *ccdA/ccdB*, this module was shown to enhance F plasmid stability by the post segregational killing of plasmid free daughter cells (Unterholzner *et al.*, 2013). Another role of TA systems is in the exclusion of co-existent compatible plasmids (Cooper and Heinemann, 2000). During the process of conjugation, bacterial cells may arise that contain two plasmids that belong to the same compatibility group, when this occurs the two plasmids can not be stably maintained within the host. The TA module ensures that the plasmid that it is located upon is the one that is maintained within the bacterial population, as the loss of the plasmid that contains the TA module will result in the death of the daughter cell whereas if the cell loses the other plasmid it is unaffected, following several rounds of conjugation and exclusion, the plasmid that contains the TA module is able to out-compete the second plasmid from the population of host bacteria (Unterholzner *et al.*, 2013).

The toxins of all bacterial TA systems that have been characterised are proteins, whereas the antitoxins can be either proteins or small RNA molecules. Five categories of TA modules exist (Types I-V). These are classed based upon their regulation and genetic structure. Type I and type III modules

have antitoxins that are small noncoding RNA molecules. The type II, IV and V the antitoxins are modules (Unterholzner *et al.*, 2013).

1.7.1.1 Type I

Type I TA modules have been found on both plasmids and bacterial chromosomal DNA (Unterholzner *et al.*, 2013). In type 1 TA modules the antitoxins are unstable antisense RNA molecules, the expression of the toxin is generally downregulated by the base pairing of the antitoxin RNA with the stable toxin mRNA (Brantl, 2012). Downregulation occurs as this interaction prevents the binding of the mRNA to the ribosome therefore inhibiting the translation of the toxin mRNA (Fozo *et al.*, 2008). The *symR/symE* module from *E. coli* is an example of a type I TA system (Kawano *et al.*, 2007). In this example, additional regulation over the expression of the toxin *symE* exists in the form the SOS-response regulated transcriptional repressor LexA as well as the protease Lon which degrades SymE LexA is responsible for SymE synthesis at the level of transcription, SymR RNA represses SymR synthesis at the mRNA level and Lon represses SymR at the stable protein level (Fernández de Henestrosa *et al.*, 2000). Other examples of type I TA modules include *fstI/rnaII* on the plasmid pASD1 (Greenfield *et al.*, 2000) as well as *ibs/sib* and *tisB/istR-1* which are both found upon the *E. coli* chromosome (Fozo, 2012)). The type I antitoxin RNA molecules encoded by plasmids have an extensive complementarity with the toxin mRNA whereas in many cases the type I antitoxins that are chromosomally encoded act by base pairing with targets that have limited complementarity (Park *et al.*, 2013). It has been hypothesised that an extensive complementarity could limit the dissociation of RNAs although the level of complementarity that is required for regulation is not known (Fozo *et al.*, 2008).

1.7.1.2 Type II

In type II TA modules, both the toxin and the antitoxin molecules are proteins. The antitoxin neutralises the toxin via the formation of a protein-protein complex which is subsequently degraded by the protease Lon (Van Melderen *et al.*, 1996) or by proteases of the Clp family (Diago-Navarro *et al.*, 2013; Lehnherr and Yarmolinsky, 1995). In many cases type II TA modules are composed of an operon that contains two open reading frames, of which the gene upstream codes for the antitoxin protein (Unterholzner *et al.*, 2013). There are however exceptions to this, one example being the type II TA module *higB/higA* from *Vibrio cholerae*. In this module the toxin encoding gene *higB* is located upstream of the antitoxin encoding gene *higA* (Tian *et al.*, 2001). The translation of the type II TA operon is typically regulated automatically by the binding of either the antitoxin or the antitoxin-complex to the promoter (Bukowski *et al.*, 2011).

1.7.1.3 Type III

As is the case with type I TA systems, type III system use RNA molecules as the antitoxin however the mechanism of interaction is different to the RNA molecules in type I systems (Unterholzner *et al.*, 2013). An example of a type III TA module is *toxI/toxN* of plasmid pECA1039 isolated from the bacteria *Pectobacterium carovoum* (Blower *et al.*, 2012). This was originally described as a mechanism that protected bacteria against infection from bacteriophages (Fineran *et al.*, 2009). In this module the *toxN* gene is preceded by a tandem array of direct repeats and a short inverted repeat. The inverted repeat functions as a terminator in the regulation of the levels of antitoxin RNA and toxin mRNA (Unterholzner *et al.*, 2013). The toxin protein ToxN has RNase activity and cleaves the *toxI/toxN* transcript at the direct repeats

which releases the active 36 nucleotide antitoxin RNA (Blower *et al.*, 2012). This interaction between the protein and RNA leads to the formation of a heterohexameric triangular assembly of three ToxN proteins, these proteins are interspaced by three 36 nucleotide ToxI RNA pseudoknots (Unterholzner *et al.*, 2013; Blower *et al.*, 2011) and this binding interaction has a high molecular specificity (Short *et al.*, 2013).

1.7.1.4 Type IV

In the case of type IV TA systems the toxin and the antitoxin do not directly interact (Unterholzner *et al.*, 2013). An example of a type IV TA system is *yeeU/yeeV* of the bacteria *E. coli* (Masuda *et al.*, 2012; Unterholzner *et al.*, 2013). In this system, the toxin YeeV interacts with the proteins FtsZ and MerB by interfering with their polymerisation and by extension cytoskeleton assembly. The antitoxin YeeU counteracts the effects of YeeV via the stabilisation of FtsZ and MreB polymers, thus neutralising the effect of the toxin indirectly (Unterholzner *et al.*, 2013). The TA module *cptA/cptB* also from *E. coli* is reported to operate according to a similar mechanism (Masuda *et al.*, 2012).

1.7.1.5 Type V

The TA module *ghoS/ghoT* from *E. coli* has been designated as type V. In this module the GhoS antitoxin protein has endonuclease activity that is sequence specific for the cleavage of GhoT toxin mRNA which therefore results in the prevention of GhoT translation (Unterholzner *et al.*, 2013; Wang *et al.*, 2012).

1.8 Plasmid Replication

1.8.1 Plasmid Replication Mechanisms

Plasmid replication occurs in a manner that is autonomous and self controlled, however during replication plasmids make extensive use of the host machinery that is responsible for chromosomal DNA replication (Del Solar *et al.*, 1998). There are three primary mechanisms that are employed in the replication process of circular plasmids, these are rolling circle (RC) theta type replication and strand displacement (Del Solar *et al.*, 1998). Different mechanisms are involved in the replication of linear plasmids, which replicate by either using concatemeric intermediates or by using a protein priming mechanism (Salas, 1991). Plasmids contain an essential location that contains the genes that are necessary for replication to take place (Del Solar *et al.*, 1998). The first of these essential genes is termed *ori*, and encodes a protein that is involved in the initiation of replication (Del Solar *et al.*, 1998). The essential location also contains genes that are involved in the control of replication. This requirement for plasmid associated initiation genes is reflected in that there are DNA cognate sites present in the origin of replication which is where the interaction between DNA and protein takes place (Del Solar *et al.*, 1998).

1.8.2 Origins of Replication

The origins of replication contain sites that are necessary for interactions with proteins that are both plasmid encoded and host encoded. The plasmid origin of replication is the minimal *cis*-acting region that is able to support the autonomous replication of the plasmid, i.e. the region where DNA is melted initiating the process of replication at the location where DNA synthesis of

the leading strand begins (Del Solar *et al.*, 1998).

1.8.3 Mechanisms of Replication Initiation Regulation

Replication initiation frequency is regulated by negative feedback loop mechanisms (Nordstrom, 1994). Such mechanisms allow for rapid expansion when the plasmids colonise a new compatible cell, known as the establishment phase. Once the cellular plasmid population has been established, the frequency of plasmid replication decreases so that there is approximately one replicative event per copy number per cell cycle therefore minimising fluctuations in copy number (Nordstrom, 1994). Regulation of plasmid copy number relies upon mechanisms to monitor the copy number via a "sensor" as well as mechanisms that modulate the initiation of replication through an "effector" (Del Solar and Espinosa, 2000). The sensory mechanism is dependent upon molecules where concentration in the cytoplasm is proportional to the copy number. In the case of plasmids that replicate via theta replication, inhibition of replication occurs during the initiation step and is dependent upon three different mechanisms, these mechanisms are: (i)The binding of Rep proteins to iterons that are located in the Rep promoter; antisense RNA molecules that hybridise to a complementary section in an RNA that is essential for initiation; (iii) steric hindrance between plasmids via the interaction of Rep proteins that are bound to different plasmids. In each of the cases described above, the same molecule is responsible for both the sensor and effector functions (Lilly and Camps, 2015).

1.8.3.1 Inhibition of Countertranscribed RNA

Each of these feedback mechanisms contains the following elements: two promoters facing opposite directions, one promoter directs RNA synthesis,

the other directing the synthesis of an inhibitor countertranscribed RNA (ctRNA). This ctRNA is complementary to a region that is located close to the 5' end of the essential RNA. The ctRNA is usually strongly expressed yet has a short half-life whereas the RNA that it targets is expressed at lower levels but is constitutive (Hayes, 2003). Examples of this mechanism can be seen in ColE1 plasmids where ctRNA targets a primer that is required for replication initiation. Other examples of ctRNA targets include the inhibition of RepA translation in R1 plasmids, and the premature termination of an mRNA encoding a Rep protein in class D theta plasmids (Wagner *et al.*, 2002). Initial contact between the sense and antisense RNAs occurs via the pairing of complementary sequences at the loop portion of stem loops, this is a rate limiting step that is known as the "kissing complex formation" (Lilly and Camps, 2015). There are many ctRNAs that have a short half-life due to the presence of an RNase E cleavage site which contains a U rich sequence as well as a hairpin structure at the 3' end (Hayes, 2003). Cleavage by RNase E results in the production of monophosphorylated decay intermediates that lack short portions of the 5' end (Lilly and Camps, 2015).

1.8.3.2 Single Mechanisms Involving ctRNA

In the plasmid ColE1, the ctRNA (RNAI) is from a promoter (P1) that is located 108bp downstream from the sense promoter P2. Three stem loops are formed from both the primer and the preprimer, the loop is composed of 6 to 7 unpaired residues (Lilly and Camps, 2015). The pairing of these residues with their counterparts is responsible for the initiation of hybridisation. Following pairing, the 5' end of RNAI nucleates the hybridisation between the two RNAs resulting in the formation of a duplex (Lilly and Camps, 2015). Hybridisation occurs between the ctRNA and the preprimer which leads to

conformational changes in the preprimer, these changes are mediated by the interaction of the sequence domain β in the preprimer with the γ sequence that is located further downstream which results in the preprimer becoming incompetent for R-loop formation further downstream (Polisky, 1988). As well as being short lived, RNAI from ColE1 has a short window of action due to RNA II being transcribed 200 bp downstream of the DNA/RNA switch. The hybridisation of the β domain with the α domain results in the formation of a new loop making RNAII refractory to the inhibition of RNAI (Lilly and Camps, 2015). The competitive hybridisation between tRNA and RNAI/RNAII interferes with RNAI/II hyrbid formation (Lilly and Camps, 2015). In addition the uncharged tRNA is responsible for the cleavage of RNAI, as well as this the uncharged tRNAs contain a 3'-CAA terminus that is able to hybridise stably with RNAI (Wang *et al.*, 2002). The functional interaction between RNAI and tRNAs influences the deregulation of the copy number that is associated with the amino acid starvation of *rel*A strains that are used in recombinant gene expression (Lilly and Camps, 2015). In the case of ColE2 plasmids, the ctRNA is also known as RNAI and it has a secondary complex structure. The RNAI is complementary to the 5' end of rep mRNA which contains an untranslated sequence, inhibition in this case is likely caused by the structural disruption of secondary and tertiary structures that are required for translation as the 5' end of RNAI does not cover the initiation codon of Rep (Hiraga *et al.*, 1994).

1.8.3.3 Dual Mechanisms Involving ctRNA

These are mechanisms that regulate the plasmid copy number using two elements, these elements include a transcriptional repressor protein and a ctRNA (Del Solar and Espinosa, 2000). In dual systems, the expression of

Rep proteins is controlled by a strong, repressor regulated promoter causing a high rate of Rep transcription when the repressor does not operate (Lilly and Camps, 2015). Dual mechanism of regulation have been most extensively studied in the plasmids R1 and pIP501. In the plasmid R1, the ctRNA is CopA and the repressor is CopB (Cervantes-Rivera *et al.*, 2010). In the case of the plasmid pIP501 the ctRNA is RNAIII and the repressor is CopR. Such dual mechanisms may confer an advantage to the plasmids during the establishment phase, particularly in the case of mobilisable plasmids such as class D theta plasmids (Sharma *et al.*, 2004). In the case of the plasmid R1, the protein RepA may be transcribed either from an upstream promoter P1 or from an alternative promoter located downstream called P2. The expression of *repA* is coupled to a small leader peptide called tap (Del Solar and Espinosa, 2000). CopA inhibits the expression of RepA via the inhibition of the transcription of tap. CopB is a transcriptional regulator of P2, when CopB levels are high, tap and RepA are transcribed as polycystron cobB-tap-RepA RNA from P1, as P2 is silenced by CopB. When CopB levels are low, the P2 promoter becomes depressed and tap and RepA may also be expressed from this alternative promoter which results in the transient increase in the expression of tap and RepA (Malmgren *et al.*, 1996). In class D theta plasmids there is a cop-ctRNA-rep modular structure (Lilly and Camps, 2015).

1.8.3.4 Regulation via Rep Binding

In the case of some class A theta plasmids, a mechanism copy number regulation is dependent upon the inhibition of Rep transcription by Rep itself (Lilly and Camps, 2015). In such plasmids, iterons are located within the promoter of the Rep operon outside of *ori*. Rep binds to these cognate se-

quences resulting in the inhibition ore Rep expression therefore acting as an auto-regulatory mechanism. The binding of Rep to two alternative binding sites results in changes to the conformational structure of Rep. This mechanism has been studied in detail in the Rep A protein that is associated with the plasmid pPS10 (Giraldo *et al.*, 2003). This Rep protein is composed of two winged helix domains named WH1 and WH2. When the Rep A protein is in dimeric form it acts as a transcription repressor where the WH1 domain functions as a dimerisation interface (Lilly and Camps, 2015). Low concentrations of the protein Rep A are linked to the dissociation of Rep dimers into monomers, these are the only form that is active as an initiator. The monomerisation of Rep involves the conversion of the dimerisation domain to a second sequence binding to *ori* and the remodelling of the WH1 sequence causing it to bind to the opposite iteron end (Giraldo *et al.*, 2003). In some plasmids, the monomerisation of Rep is assisted by chaperone proteins or by the alliosteric effect of binding iterons at *ori* (Diaz-Lopez *et al.*, 2003).

1.8.3.5 Steric Hindrance

Steric hindrance is another feedback mechanism that is associated with the regulation of plasmid copy number, this mechanism was originally proposed for P1 and RK6 plasmids however they could operate in more iteron containing plasmids (Gasset-Rosa *et al.*, 2008). In this model, as the copy number of plasmids per cell increases the Rep proteins that are bound to the iterons of one *ori* begin to interact with similar complexes that have formed in other origins, such pairing is known as handcuffing (Lilly and Camps, 2015). This pairing produces Rep-Rep interactions which results in steric hindrance to both *ori*s therefore interfering with the melting of *ori* (Zzaman and Bastia, 2005). The Rep molecules are paired through the zipping up of DNA

bound Rep A monomers (Lilly and Camps, 2015). One difference between this model of copy number regulation and the auto-regulation model is that replication rate is dependant upon the concentration of iterons as opposed to the Rep expression level. Both of these mechanisms of regulation could be working together for initiators that are limiting (Das *et al.*, 2005).

1.8.3.6 Summary

Plasmids contribute to bacterial adaptation to environments that are constantly changing through their mobilisation and amplification of selected genes (Samalla and Sobercky, 2002). Different plasmids show differences in duplex melting as well as leading strand priming and the synthesis of the lagging strand. The maintenance of a stable plasmid copy number is critical for the host bacteria as the loss of the plasmid results in the loss of the adaptive functions that are carried upon the plasmid, and runaway plasmid replication is lethal for the host. As such, mechanisms that regulate plasmid replication represent potential targets for antimicrobial intervention (Lilly and Camps, 2015).

1.8.4 Rep Proteins

Rep proteins are responsible for the initiation of replication and are encoded by the plasmid, however in order to replicate some theta plasmids rely exclusively upon host initiation factors (Giraldo, 2004). The recognition sites of Rep proteins contain direct repeats known as iterons, of which the specific sequence and spacing are important for initiator recognition. Rep proteins are also rate limiting in terms of plasmid copy number, for example controlled expression of Rep proteins π of R6K and RepA of ColE2 can result in a variation in the plasmid copy number that ranges between 1 and 250 copies

(Kittleson *et al.*, 2011). The structure of plasmid replicons is typically modular, containing motifs that are recognised by plasmid encoded Rep proteins (Lilly and Camps, 2015).

1.8.5 Plasmids and Antibiotic Resistance

Plasmids mediate the HGT of genes between bacteria therefore having an important role in driving bacterial evolution (Millan *et al.*, 2016). Bacterial sexual activity was first observed in *E. coli* in 1946 (Lederberg and Tatum, 1946) over a decade before antibiotic resistance was first reported. In the 1960s, the transmission of antibiotic resistance genes on large R-plasmids was observed within *Enterobacteriaceae* via a contact based mechanism called conjugation. During the same period an increase in antimicrobial resistance amongst Gram positive bacteria was also being reported however any such R-plasmids were not detected until the discovery of a R-plasmid moving via conjugational transfer was identified in *Enterococcus faecalis*.

The transfer of plasmids is one mechanism by which bacterial genes are transferred horizontally. Plasmid mediated transfer is problematic in healthcare as plasmids are able to transfer between different species, allowing resistance genes a mechanism to cross species and genus barriers, it has been reported that the rate of plasmid transfer is increased in heterogeneous environments (Dionisio *et al.*, 2002). This leads to plasmids being able to spread resistance genes into niche areas that have had had limited exposure to antibiotics (Hughes and Datta, 1983). Many plasmids carrying antibiotic resistance genes are conjugative (Bennett, 2008). Plasmid mediated antibiotic resistance encompasses most of the antibiotic classes that are in use clinically (Bennett, 2008).

The first plasmids that were isolated prior to the discovery and widespread

use of antibiotic therapy were commonly found however during this era the presence of antibiotic resistance genes upon the plasmids was rare. In order to understand the significance of the resistome that existed in the pre-antibiotic era there have been retrospective studies analysing samples from around this time. These studies have demonstrated that antibiotic resistance genes were present in non-antibiotic producing bacterial strains prior to the widespread dissemination of antimicrobial agents (Allen *et al.*, 2010). Thirty strains of *E. coli* were lyophelized prior to 1950, these were tested for susceptibility against 8 antibiotics and 4 strains were reported to be resistant, all resistance elements found were able to conjugate into *E. coli* (Smith and Halls, 1967). Another retrospective study analysing 433 strains of *Enterobacteriae* that had been collected from a variety of locations around the world between 1917 and 1952 found that 24% of these strains could transfer plasmids and furthermore 11 strains were resistant to either tetracycline or ampicillin however in all cases the resistance was not associated with a conjugative element (Hughes and Datta, 1983), since then another study has investigated historical *Enterobacteriae* samples finding similar results (Hount and Ochman, 2000). These studies confirm that antibiotic resistance determinants existed naturally in the pre-antibiotic era and were likely mobile via horizontal gene transfer before the dramatic increase in selection pressure favouring the acquisition of antimicrobial resistance determinants was imposed following the introduction of antibiotic use in therapy (Allen *et al.*, 2010).

Plasmids have frequently evolved to carry antibiotic resistance genes as mobile genetic elements may evolve to provide benefits to the host organism, this is likely due to the fitness cost that harbouring mobile elements such as large plasmids has upon the host, plasmids that do not offer an environmental advantage may be ejected or silenced by degrading mechanisms such as

methylation or restriction (Rankin *et al.*, 2011).

1.9 Transposons

The definition of a transposon is of a genetic element that is able to change its position in a DNA segment by moving dependent on excision and integration reactions. When transposons were first identified, it appeared that they were simple elements, however further investigation has revealed the presence of more complicated transposons that are diverse in their structures and properties (Ochman *et al.*, 2000). In addition to this, the first transposons discovered were small, being less than 15kb and were only able to change their position within the same cell in contrast to the larger transposons that are able to travel between different cells via the process of conjugation (Ochman *et al.*, 2000). Transposon site selectivity varies between the different elements, for example the transposon Tn*5* and bacteriophage Mu are able to integrate into a wide range of different sites whereas other transposons are able to integrate into a more limited number of sites.

The function of transposons varies between different elements. Some transposons are segments of DNA of which their only function is the act of moving from one place in the cellular DNA to another whereas other transposons also carry additional genes that confer a fitness advantage for the bacterial host. The least complicated type of transposable elements are the insertion sequences (IS), these only carry genes that are necessary for transposition. Larger more complicated transposons also carry a variety of accessory genes that can have a range of different functions such as genes that confer resistance to antibiotics or heavy metals, nitrogen fixation genes and genes that are involved in the synthesis of vitamins (Ochman *et al.*, 2000). Transpos-

able elements are widely dispersed in nature (Stewart and Costerson, 2001), this distribution suggests they have contributed to the evolution and diversity of microbial genomes as they facilitate the acquisition of new genes and promote rearrangements in DNA that are central to the process of evolution (Hallet and Sherratt, 1997).

1.9.1 Common Themes in Transposition

There are some features that are common to many transposons. The first step of transposition typically involves the recognition and cleavage of the transposon ends by a transposase, causing the generation of an active 3'-OH group. Once the transposon ends have undergone cleavage the activated 3'-OH groups initiate a nucleophilic attack either on the opposite strand intramolecularly causing a hairpin structure to form, or intermolecularly on a target DNA molecule resulting in the generation of a cointegrate or deletions (Barabas *et al.*, 2008). This nucleophilic attack may also occur intramolecularly on the other end of the transposon resulting in the formation of a figure 8 structure (Ochman *et al.*, 2000).

1.9.2 Types of Transposition

Four mechanisms of transposition have been discovered, these are conservative transposition, replicative transposition, excisive transposition and retrotransposition (Ochman *et al.*, 2000).

1.9.2.1 Conservative Transposition

Conservative transposition is a cut and paste mechanism whereby the transposon is excised from the chromosome (or plasmid) and integrates into target

DNA. The transposon is lost from the original via this mechanism. The steps taken during conservative transposition are illustrated in Fig.1.1.

1.9.2.2 Replicative Transposition

Two models of replicative transposition exist, the first such model suggests that the transposon is replicated during the cellular DNA replication process when it transposes from a site that has already undergone replication to a site that is yet to undergo replication (Chen *et al.*, 1992). This model suggests that transposition is more likely to occur following DNA replication.
Another model for replicative transposition is based on the formation of a double stranded DNA break that occurs at the site of excision leading to the repair of the DNA based on a homologous template. This template can either be DNA from the original chromosome in which case the transposon is lost, however if the template is the sister chromatid containing the transposon, then the transposon will be restored (Skipper *et al.*, 2013).

1.9.2.3 Retrotransposons

Retrotransposons transpose either by the process of reterohoming or retero-transposition (Beauregard *et al.*, 2008). In the process of reterohoming the excised RNA reverse splices into one strand of the DNA target site, after which it is reverse transcribed producing the DNA copy (Beauregard *et al.*, 2008). During the RNA splicing, a ribonucleoprotein complex is formed that mediates the process. As well as being responsible for the reverse transcription of the bacterial interon, the interon encoded protein (IEP) also functions as a DNA endonuclease cleaving the target DNA site and creating a primer for the reverse transcription process, and also as an RNA maturase which stabilizes the catalytically active RNA for splicing (Beauregard *et al.*, 2008).

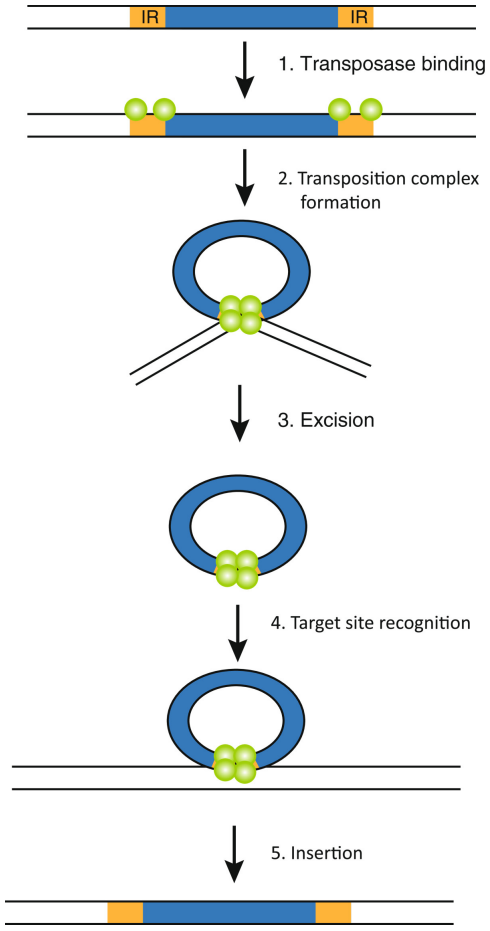


Figure 1.1: Orange = inverted terminal repeats, blue=central regions, green circles=transposase enzymes. Diagram illustrating the steps in conservative transposition. First, the transposase enzymes bind to the two inverted terminal repeats that flank the transposon forming the transposition complex, following this the DNA bends allowing the transposes to interact, this activates the complex causing the transposase enzymes to cut the DNA and the transposon is excised. The transposon remains circular until the transposase binds to the target DNA and cuts the target site allowing for the integration of the transposon(Skipper *et al.*, 2013).

Following reverse splicing into DNA, the second strand is cleaved downstream of the intron insertion site and full length cDNA synthesis can occur (Beauregard *et al.*, 2008). Some group II interons do not contain the endonuclease domain which leaves them unable to cleave the target DNA site, following the first steps, these group II interons recruit a variety of host functions in order to complete the process (Beauregard *et al.*, 2008).

The process of retrotransposition begins with the transcription of transposon DNA to RNA. Retrotransposition requires at least two enzymes, an integrase which cuts the DNA integration site forming a staggered break, and reverse transcriptase that reverse transcribes RNA transcript to DNA. Retrotransposons have evolved primarily in eukaryotic organisms though they are found across all three domains of life (Beauregard *et al.*, 2008). Retrotransposons are divided into four subgroups (Eickbush and Jamburuthugoda, 2008), the subgroup that are present within bacteria are group II iterons. Retrotransposition was first studies in *Lactococcus lactis* (Cousineau *et al.*, 2000). The sequence requirement for retrotransposition is more relaxed than it is for retrohoming. The intron targets ssDNA as opposed to dsDNA and uses the replication fork to prime the synthesis of cDNA therefore not requiring endonuclease action (Beauregard *et al.*, 2008).

1.9.2.4 Inverted Terminal Repeats

Inverted terminal repeats are located at both ends of transposons, they refer to a sequence of nucleotides that is inverted at the other end of the transposon for, an example is shown in Figure 1.2. ITRs facilitate movement as they contain transposase binding sites, transposase catalyses the movement of the transposon by binding to these sites and cleaving the DNA (Derbyshire and Grindley, 1992; Ichikawa *et al.*, 1987). Any DNA that lies between the two

ITRs is moved as shown in Figure 1.1.

Figure 1.2: Illustration representing a transposon, the inverted terminal repeats are shown in the green sections. Inverted terminal repeating sequences that are a common feature of many transposons.

ATCGCA		TGCGAT
TAGCGT		ACGCTA

1.9.2.5 Miniature Inverted-repeat Transposable Elements (MITEs)

Miniature inverted repeat transposable elements are small, generally less than 300bp and contain inverted terminal sequences (Bardaji *et al.*, 2011). The small size of MITEs results in their activity having a high impact on the prokaryotic genome allowing them to contribute to phenotypic variation via the generation of new alleles or by generating new regulatory signals for pre-existing genes (Bardaji *et al.*, 2011; Delihas, 2008). MITEs lack genes encoding transposase and are therefore dependent on autonomous DNA transposons for their mobilisation (Munoz-Lopez and Garcia-Perez, 2010).

1.9.2.6 One Ended Transposition

One ended transposition refers to replicon fusion that takes place when only one end of the transposable element is present. This mechanism was primarily studied using artificial constructs of the transposons Tn*3*, Tn*21* and Tn*1721* that retained the transposase gene yet had only one inverted terminal repeat(Liebert *et al.*, 1999). The cointegrates that are formed in one ended transposition are composed of variable lengths of the donor replicon and the complete target replicon. One ended transposition also exists in nature as well as in artificial constructs. The insertion sequence IS*102* from *Shigella*

dysenteriae mediates co-integration via one ended transposition (Machida *et al.*, 1982).

1.9.2.6.1 Composite Transposons Class I composite transposons are characterised by being flanked by two insertion sequences and transpose via a conservative cut and paste mechanism (Kleckner, 1981). These insertion sequences carry a transposase gene that encodes a protein that catalyses the transposition reaction. The DNA that is located in between the two insertion sequences is typically not involved in transposition itself although these accessory genes may be important in the maintenance of a transposable element within a bacterial population. An example of this is a transposon that contains a gene that confers resistance to antibiotics or heavy metals thereby providing a selective advantage to host bacteria that contain the transposon (Ochman *et al.*, 2000). As insertion sequences are transposable elements, it is possible for the insertion sequences at the flanking regions of composite transposons to move independently of the whole transposon (Hallet and Sherratt, 1997). This possibility has been eliminated in the case of the composite transposons Tn*5* and Tn*10* however, as these only one of their insertion sequences contains an active transposase (Ochman *et al.*, 2000).

1.10 Conjugative Transposons

Conjugative transposons are transposable DNA elements that are able to excise from the host chromosomal or plasmid DNA that they are integrated within and form a circular transfer intermediate (Salyers *et al.*, 1995; Scott, 2002). A single stranded form of this circularised DNA is able to transfer to a recipient cell via conjugation where it is integrated into recipient host DNA

after first becoming double stranded (Ochman *et al.*, 2000). Conjugative transposons are a diverse group of elements that were first identified in Gram positive cocci, conjugative transposons have since been identified in Gram negative bacteria and the proteobacteria (Ochman *et al.*, 2000). There is a diversity in the nomenclature regarding conjugative transposon classification, with some conjugative transposons being assigned a Tn number as in the case with Tn*916* with others being given a CTn label such as CTnERL identified in *Bacteroides*. Some conjugative transposons are given an ICE number with ICE referring to "integrative conjugative element".

There is a diverse range in size of conjugative transposons, for example Tn*919* is 15.4kb compared to the much larger 70kb Tn*5276* (Fitzgerald and Clewell, 1985; Salyers and Shoemaker, 1994). Overall the conjugative transposons are a diverse group of mobile elements however, there are some features that are common to the group such as the necessity to form circular intermediates for conjugative transfer. However some non conjugative transposons such as IS*911* also form circular transposition intermediates, therefore the formation of a circular transposition intermediate does not necessarily make a transposon conjugative (Ochman *et al.*, 2000). A circular intermediate is necessary for transposition because the origin of transfer *oriT* is located towards the centre of the transposon rather than at the terminus. Another feature that is shared throughout conjugative transposons is that they all integrate into the recipients DNA (both genomic and plasmid) after they transfer. This integration is characterised by the integrases which are encoded by the transposon, these integrases are distantly related to one another sharing residues in the presumed catalytic region (Hallet and Sherratt, 1997; Ochman *et al.*, 2000). Site specificity for integration varies between different conjugative transposons, for example Tn*916* integrates almost randomly into host DNA

however it has a preference for A-T rich regions (Trieu-Cuot *et al.*, 1993). Whereas the conjugative element CTnDOT from *Bacteroides* integrates selectively into approximately seven sites (Cheng *et al.*, 2001).

1.11 Non-Conjugative Transposons

Transposon nomenclature does not indicate whether a transposon is conjugative or nonconjugative, for example Tn*1545* is conjugative whereas Tn*1546* is nonconjugative (Salyers *et al.*, 1995).

1.12 Tn*916* Family of Elements

In the late 1970s, chromosomal as opposed to plasmid mediated transferable resistance was first discovered (Franke and Clewell, 1981). Experiments that mated the DS16 strain of *Enterococcus faecalis* with the plasmid free strain JH2-2, some of the tetracycline resistant transconjugants contained the conjugative transposon Tn*916* linked to pAD1, a plasmid that had also transferred from DS16. Some derivatives of DS16 that where pAD1 was absent were also able to transfer tetracycline resistance to previously sensitive strains. Further experimentation revealed that this resistance transfer was mediated by a conjugative transposon that was designated Tn*916* (Franke and Clewell, 1981). This was the first incidence of a conjugative transposon being found to confer antibiotic resistance (Franke and Clewell, 1981).

The genetic structure of many mobile genetic elements (MGEs) is organised in a modular manner (Tribble *et al.*, 1999; Caparon and Scott, 1989). These modules are involved in the excision of the element from the host DNA, conjugation and integration into the target DNA as well as regulatory and

accessory functions that are not related to mobility or conjugation (Burrus *et al.*, 2002). Since the initial discovery of Tn*916* there have been numerous MGEs discovered that have genetic similarities to Tn*916* (Roberts *et al.*, 2001). Within this family of elements the genes that encode conjugative and regulatory proteins are generally conserved whereas variation exists in the accessory genes as well as the genes that encode the excisionases and integrases or recombinases. Some of the elements within the family have also been found to contain insertions of other, smaller elements that themselves confer resistance to other antibiotics such as macrolides (Salyers *et al.*, 1995) as well as the heavy metal mercury (Scott, 2002). Some of the Tn*916* family contain group II introns and insertion sequences (Teuber *et al.*, 1999). Transposons belonging to the Tn*916* family of elements have been found within a diverse range of species (Hummel *et al.*, 2007; Devirgiliis *et al.*, 2009; Rizzotti *et al.*, 2009) meaning that this family of MGEs has an extremely diverse range of host bacteria (Herzog-Velikonja *et al.*, 1994; Sato *et al.*, 1992; Cao *et al.*, 1994).

1.12.0.1 Tn*916*

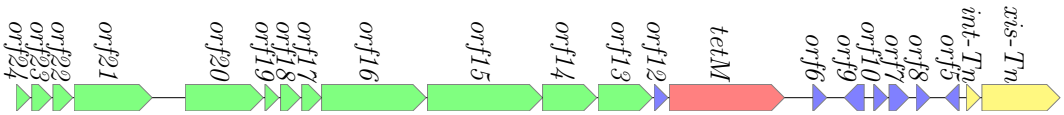
The transposon Tn*916* was initially discovered when tetracycline resistance was observed to be transferred between *Enterococcus faecalis* strains DS16 and *Enterococcus faecalis* JH2-2 without any plasmids being detected (Franke and Clewell, 1981). The element that was responsible for this resistance transfer was shown to be the conjugative transposon Tn*916*. Since this time, many transposable elements that belong to the Tn*916* family of element have been discovered in a wide range of bacterial species (Agersø *et al.*, 2006). Tn*916* is composed of four regions, each adapted to a specific function. These functions are regulation, conjugation, recombination and acces-

sory genes that are not involved in the process of conjugative transposition (Senghas *et al.*, 1988). Such accessory genes may offer selective advantages to hosts that contain the transposon such as antibiotic resistance genes. In the case of Tn*916* the accessory gene is *tetM*, a gene that confers resistance to the antibiotic tetracycline.

For an element to be considered a member of the Tn*916*-like family, specific criteria must be met, these criteria are based upon the organisational structure of the element, the core region must be similar at a DNA level to the original Tn*916* element.

The detection of *int* and *xis* along with *tetM* and in some cases *ermB* 4.10 hint at the possibility of a Tn*916*-like element being present in these isolates, however there is not enough information to confirm this with certainty, further PCRs to test for other regions of Tn*916* would be required. The Tn*916* family has already been detected in many of the species that have been identified in this study. Of the species that tested positive for *int* and *xis* suggesting that an element related to Tn*916* may be present (*S. aureus*, *S. agalactiae*, *S. dysgalactiae*, *S. hominis* and *S. pyogenes*. Transposons belonging to the Tn*916* family have been previously detected in and known to exist within populations of *S. aureus* (De Vries *et al.*, 2009), *S. agalactiae* (Rice, 1998), *S. dysgalactiae* (Haenni *et al.*, 2010), and *S. pyogenes* (Feng *et al.*, 2010). There has been no report of the *tetM* gene or any Tn*916*-like transposons being detected in *S. hominis* or *S. simulans*, *tetM* was also not detected in these isolates however the *int* and *xis* genes were detected via PCR in *S. hominis* suggesting that this species may have acquired an element related to Tn*916* however these genes were not detected in *S. simulans*. The erythromycin resistance determinant *ermC* was also detected in one isolate of *S. hominis* and *S. simulans*, the genetic support of *ermC* is

Figure 1.3: Schematic diagram of Tn*916* with the modules associated with different functions highlighted. Green: conjugative transfer, blue: transcriptional regulation, yellow: recombination.



usually plasmid mediated and this gene has not been detected on a transposon, however this does not mean that it is definitely located on a plasmid, as *ermB* was originally discovered on a plasmid pCA1 (Clewell and Franke, 1974) and has since been detected upon transposons such as Tn*917* (Perkins and Youngman, 1984).

The tetracycline resistance determinant *tetM* has been detected in *C. striatum* (Martinez-Martinez *et al.*, 1996). However transposable elements that belong to the Tn*916* family have not been reported in this species. The *tetW* gene was first reported in *C. striatum* in China (Li *et al.*, 2015). To date this is the only reported incidence of *tetW* in *C. striatum*.

1.12.1 Modular Function

Elements in the Tn*916* family contain open reading frames that are organised into functional modules (Senghas *et al.*, 1988). These modules are involved in processes such as conjugation, transcriptional regulation, recombination as well as accessory functions such as antimicrobial resistance genes (Flannagan *et al.*, 1994). The different modules of Tn*916* are shown in Figure 1.3.

1.12.2 Structural Organisation of Tn*916*

The original Tn*916* was first isolated from *Enterococcus faecalis* (Franke and Clewell, 1981). This element is the original model of a large family of conjugative transposons that reside within a diverse range of host bacteria. There are 24 open reading frames that comprise the structure of Tn*916*, these ORFS encode putative proteins that have a molecular mass that ranges between 2.9 and 93.7 kDa. The majority of elements that belong to the Tn*916* family carry the accessory gene *tetM* that confers resistance to tetracycline. An exception is Tn*5386* that was originally isolated from *Enterococcus faecium*, this element contains ORFs that with homology to genes that confer resistance to the lantibiotics class of antibiotics (Rice *et al.*, 2007).

1.12.3 Tn*916* and Other Elements

Tn*916* elements may contain insertion sequences, if two identical IS were inserted into a Tn*916* like element, then the sequence between the two IS could become incorporated into a class II transposon and move to another location on the genome (Perreten *et al.*, 1997). Tn*916* may also be associated with nonconjugative elements, for sample Tn*917*, a transposon that carries the erythromycin resistance determinant *ermB* (McDougal *et al.*, 1998). The resulting transposon, Tn*3872* confers resistance to both tetracycline and erythromycin.

1.12.4 Tn*916* and Conjugation

The conjugation of Tn*916* contributes to the spread of antibiotic resistance genes. For conjugation to be initiated the relaxase encoded by ORF20 must nick *oriT*, which is 466bp in length and lies between *orf21* and *orf20*, it

is made up of 6 sets of inverted repeats (Jaworski and Clewell, 1995) *oriT* also interacts with the Tn*916* tyrosine recombinase (Rocco and Churchward, 2006).

1.12.5 Regulation of Tn*916*

The region of Tn*916* that is associated with regulation is composed of *orf7*, *orf8*, *orf9* and *orf12* and is conserved throughout the majority of the elements in the Tn*916* family (Su *et al.*, 1992; Celli and Trieu-Cuot, 1998).
orf12 is 86bp in length, it encodes several inverted repeat sequences that can form hairpin structures in mRNA followed by uridine residues, which are speculated to be a rho factor independent transcriptional terminator (Su *et al.*, 1992). *orf12* also contains rare codons that function to couple the expression of *orf12* and the genes present downstream of it such as *tetM*.

1.12.5.1 Tn*916* in the Absence of Tetracycline

Tn*916* in typical growth conditions where tetracycline is absent, the ribosome stalls around the leader sequences of *orf12* due to a lack of tRNA modules for the codons that are present at the beginning of *orf12*. This stalling causes the ribosome to lag behind the RNA polymerase which causes a termination of transcription in the majority of cases therefore limiting the expression of TetM. A basal level of TetM is maintained within the cell by instances where the RNA polymerase breaks free of the termination site and reads through into *tetM* and transcribes it. This basal level of TetM in the absence of tetracycline provides protection to some of the cellular ribosomes should the bacteria become exposed to tetracycline (Su *et al.*, 1992).

1.12.5.2 Tn*916* in the Presence of Tetracycline

Tn*916* regulation involves a mechanism called transcriptional attenuation, this involves an interaction between tetracycline and the ribosomes that removes the transcriptional terminator (Su *et al.*, 1992). Tetracycline renders most of the ribosomes inactive via reversible binding. Due to the low level of ribosomal protection provided by basal TetM levels, protein synthesis does not cease completely however it is slowed down which results in an accumulation of charged tRNA. The increased availability of tRNA leads to an increase in the translation of *orf12* by the ribosomes that have been protected by TetM. Transcription occurs from the promoter that is present upstream of *orf12* and travels downstream through *tetM* and surrounding ORFs.

Once the majority of ribosomes have been protected by TetM, the expression of *tetM* returns to lower levels as the availability of charged tRNA molecules is reduced due to many of them having been recruited for the protected ribosomes.

1.12.5.3 Tn*916* Promoters

Tn*916* contains four promoters, these are PtetM, Porf7, Porf9 and Pxis (Celli and Trieu-Cuot, 1998). When tetracycline is absent, the expression of orf9 causes the downregulation of promoter Porf7 resulting in a reduction of downstream genes including *orf7* and *orf8*. The expression of *orf9* is reduced in the presence of tetracycline which results in the upregulation of Porf7 increasing expression of *orf7*, *orf8* and other downstream genes (Celli and Trieu-Cuot, 1998).

The linking of Tn*916* regulation to the presence of tetracycline allows the

bacteria to react to the presence or absence of tetracycline via the down-regulation or upregulation of gene expression within Tn*916* increasing the expression of *tetM* when it is necessary (Celli and Trieu-Cuot, 1998).

1.13 Aims and Objectives

The overall aim of this project was the investigation into the molecular basis of bacterial antimicrobial resistance. A method was developed allowing for the rapid screening of Tn*916* based on PCR producing two large amplicons which were subsequently digested providing a restriction profile allowing the identification of Tn*916*.

The collection of clinical isolates obtained from patients at the Queen Elizabeth hospital in Norfolk, focusing primarily on tetracycline and erythromycin resistance in *Streptococcus* and *Staphylococcus* species.

The investigation of antibiotic resistance in saliva that was collected from healthy volunteers, analysing the antimicrobial resistance genes that are present within the metagenomes of these individuals as well as using culture to investigate the different species of resistant bacteria that are present with a focus on tetracycline and erythromycin antibiotics.

The transposon-aided capture (TRACA) technique was also employed in order to characterise the plasmid population that is present within these saliva samples.

Chapter 2

Materials & Methods

All antibiotics were purchased from Sigma Alderich. All agar and media was purchased from Thermo Scientific unless otherwise stated.

2.0.1 Primer Design

Primers for PCR and DNA sequencing were designed by manually selecting a region of DNA that was 21bp in length, the DNA was analysed using IDT oligo analyser to check that the primers were of a sufficient standard. The criteria included were that the primer must have a %GC content of between 40% and 60%, the melting temperature must be between 50°C and 59°C, and the self and hetero dimer delta G score (referring to the amount of energy required to break the dimer bonds) was below -10kcal/mole. These properties were analysed using the program OligoAnalyzer (IDT).

2.0.2 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed by combining the reagents as shown in Table 2.1, the reactions were run on a thermocycler under con-

ditions that were specific for each reaction based upon the product size and primer melting temperature. Biomix Red is a ready made mastermix produced by Bioline, the details of the concentrations of Biomix Red are proprietary, however it contains reaction buffer, dNTPs and $MgCl_2$.

Table 2.1: Each polymerase chain reaction contains the reagents and volumes detailed in this table.

Reagent	Volume	Starting conc.	Final conc.
Biomix Red	12.5 µl	2x	1x
ddH_2O	9.5 µl	–	–
Forward primer	1 µl	10µM	0.4µM
Reverse primer	1 µl	10µM	0.4µM
Template DNA	1 µl	variable	variable

2.0.3 Gel Eletrophoresis

Agarose gels were prepared at a concentration of 0.75%. The agarose and tris acetate EDTA(TAE) buffer were microwaved until the agarose had melted, this solution was allowed to cool briefly for approximately 5 minutes before having GelRed (Biotium) added to it to a concentration of 1.25%. The gel was then mixed and poured into a casting tray and allowed to set over the course of 20 minutes. The set gel was transferred to an electrophoresis tank where it was submerged in TAE buffer where 2.5µl of the relevant DNA ladder was loaded into the first well followed by 2.5µl of each sample. Electrophoresis was carried out at 100 volts over the course of 1 hour. The outcome of the gel was visualised under ultra-violet light.

2.0.4 Processing of Saliva Samples

Approximately 5ml saliva samples were obtained from healthy volunteers. The samples were diluted in BHI broth to 10^{-3} and 10^{-4} before 100µl was spread on to brain heart infusion (BHI) agar that contain 20 % defibrinated horse blood as well as either 4 µg/ml tetracycline or erythromycin.

The serial dilution steps taken during sample dilution are as follows:

100µl saliva $\longrightarrow$ 900µl BHI = 1ml 10^{-1}
100µl 10^{-1} $\longrightarrow$ 900µl BHI = 1ml 10^{-2}
100µl 10^{-2} $\longrightarrow$ 900µl BHI = 1ml 10^{-3}
100µl 10^{-3} $\longrightarrow$ 900µl BHI = 1ml 10^{-4}

2.0.5 Freezing Down Isolates for Storage

Isolates were grown in 5ml BHI broth overnight at 37°C for 24 hours aerobically with shaking. Following incubation 1ml of overnight broth was mixed with 1ml BHI broth containing 20% glycerol to produce a final glycerol percentage of 10%. The isolates were immediately stored at -80°C.

2.0.6 Preparation of Media

For molecular cloning using *E. coli* lysogeny broth (LB) medium was used. For growing strains isolated from wounds brain heart infusion (BHI) medium combined with 20% difibrinated horse blood (Thermo) was used.

2.0.7 DNA Extraction using FastDNA Spin Kit for Soil (MP Bio)

Saliva samples were centrifuged at 4000g for 15 minutes to pellet bacteria. The pellet was re-suspended in 978µl sodium phosphate buffer and 122µl MT buffer before being lysed by the FastPrep Instrument (MP Bio) for 40 seconds at a speed setting of 6.0. The lysed cells were centrifuged at 14000g for 10 minutes and the supernatant transferred to a clean 2ml microcentrifuge tube and combined with 250µl protein precipitation solution then inverted 10 times. The mixture was centrifuged at 14000g to separate the precipitated protein, the supernatant was combined with 1ml binding matrix and mixed via rotation for 2 minutes before being left to settle for 3 minutes. 500µ supernatant was discarded and the remaining supernatant was passed through the SPIN Filter (MP Bio) via centrifugation at 14000g for 1 minute. The DNA was eluted in 100µl ddH_2O and stored at -20°C.

2.0.8 DNA Extraction Using Glass Beads

1µl of bacterial cells were suspended in a combination of 40µl H_2O, 160µl 0.05mM NaOH and glass beads (sizes 425 - 600µm and $\leq$106µl) and vortexed thoroughly, the mixture then was incubated at 80°C for 45 minutes, then allowed to cool to room temperature for 10 minutes. Once the mixture had cooled, 36µl of Tris-HCl (1M) is was added. It was subsequently centrifuged at 4000rpm for 10 minutes and the supernatant was retained and stored at -20°C.

2.0.9 Plasmid Extraction

E. coli bacterial hosts were incubated overnight at 37°C aerobically while shaking in 5ml LB broth containing relevant antibiotic acting as a selective marker, in the case of pUC19 or T-Eazy this was 100µg/ml ampicillin, for pCC1BAC this was 12.5µg/ml chloramphenicol. Plasmids were extracted from the overnight culture as described in the protocol supplied with the QIAprep Spin Miniprep Kit (Qiagen). Quality and quantity of plasmid yields were assessed by Qubit analysis and gel electrophoresis on a 1.5% gel for 1 hour.

2.0.10 PCR Product Clean up

PCR product was cleaned up using the QIAquick PCR Purification Kit in accordance with the manufacturers protocol (Qiagen).

2.0.11 DNA Extraction from Agarose Gels

Bands of DNA were visualised on UV transillumiator and cut from the remaining gel using a scalpel. The DNA was extracted from the gel cut out using the ISOLATE II PCR and Gel Kit (Bioline) in accordance with the protocol supplied by the manufacturer.

2.0.12 Quantification of Extracted DNA

The working solution was prepared by preparing a 1:200 dilution of Qubit reagent into Qubit buffer, 1µl of DNA was added to 199µl working solution, mixed via vortex and incubated for 2 minutes before being read using the Qubit fluorometer. This method was used to quantify all extracted DNA.

2.0.13 Restriction Digestion

Restriction digestion has been carried out using the enzymes *Hin*cII (Promega), *Hin*dIII (Promega) and *Sau*3AI (NEB) in according to the protocols supplied with these enzymes.

2.1 Minimum Inhibitory Concentration (MIC) Testing

2.1.1 Preparation of Antibiotics

The antibiotics ampicillin, kanamycin and chloramphenicol were dissolved in ddH_2O. Tetracycline was dissolved in 50% ethanol/50% H_2O, the antibiotics vancomycin and erythromycin were dissolved in 100% ethanol.

2.1.2 Preparation of Inoculum

The inoculum is prepared by growing the isolates overnight on ISO sensitest agar in accordance with Table 2.2. Approximately 4-5 of these colonies are resuspended in ISO sensitest broth. The absorbance of the resuspended bacteria is measured using a UV spectrophotometer at a wavelength of 650nm. The absorbance reading determines the amount of this broth that is added to 5ml sterile broth, the volume added based for each absorbance reading is shown on Table 2.2.

2.2 Speciation of Bacteria

The species of bacteria was determined using PCR to amplify the 16S gene, and compare it with the NCBI database as described in Section 4.2.4.

Table 2.2: Dilution of bacterial suspensions adapted from BSAC susceptibility guidelines.

	Absorbance Reading at 650nm	Volume(µl) to transfer to 5ml H_2O
Staphylococci	>0.3 – 0.6	20
Streptococci	>0.05 – 0.1	250
Enterococci	>0.05 – 0.1	125

2.3 Molecular Cloning

2.3.1 Ligation

Ligation reaction mixture was prepared as described in Table 2.3 and incubated at 22°C for 10 minutes..

Table 2.3: Reagents and volumes used in the ligation reaction, the ligation mixture was heated at 25°C for 15 minutes. The reaction buffer contains 400mM Tris-HCl, 100mM $MgCl_2$, 100mM DTT, 5mM ATP and had a pH of 7.8 at 25°C.

Reagent	Volume	Concentration
H_2O	Make up to 20µl total	–
2x Buffer	10µl	10x
Vector	Variable	1mg/ml
DNA	Variable	1:1-5:1 ratio with vector
T4 Ligase	1µl	1U/µl
Total	20µl	

2.3.2 Desalting of Ligation Reaction

Agarose cones were produced by heating 0.9g glucose, 0.5g agarose and 50ml H_2O in a microwave, cooled to 50°C and 800µl was pipetted into a 1.5ml microcentrifuge tube. A 0.5ml microcentrifuge tube was placed in this molten solution in order to act as a mould. The solution was then left to solidify and the 0.5ml tube removed. The ligation reaction was transferred to the agarose cone for 1 hour to allow any salt present in the solution to diffuse into the glucose-agarose cone.

2.3.3 Transformation using pUC19

The procedure for transforming DNA into the vector pUC19 is adapted from the protocol for α-select Gold Competent Cells (Bioline). Once the competent cells had been thawed on ice, 50µl cells and 5µl of ligated DNA were mixed together and agitated gently. The resulting mixture was then left incubating on ice for 30 minutes before being transferred to a water bath at a temperature of 42°C for 30 seconds to heat shock the cells. Following this the tubes were returned to ice for 2 minutes. 950µl of SOC media (Sigma) was added to the reaction which was then incubated while shaking at 37°C for 1 hour, 200µl of transformed was spread onto LB agar plates that contained 100µg/ml ampicillin and 4µg/ml tetracycline that were incubated at 37°C overnight.

2.3.4 Electroporation

The electroporation cuvettes and 1.5ml micro-centrifuge tubes were pre-chilled on ice. 2µl of the desalted ligation reaction that was prepared in Section 2.3.1 was combined with 50µl of Transformax EPI300 Electrocompe-

tent *E. coli* cells (Epicentre) and transferred to the electroporation cuvette. Electroporation was carried out at 1800V, 25μF and 200Ω. 950μl SOC media was added to the electroporated cells and they were transferred to a 15ml tube and incubated for 1 hour at 37°C with shaking before 100μl was plated onto LB plates that contained 12.5μg/ml chloramphenicol and incubated overnight at 37°C.

2.3.5 Strains used throughout study

Table 2.4: List of bacterial strains that were used throughout the study. A more detailed list containing the specific attributes of each isolate/clone is presented in Appendix .1

Species	Isolate IDs
Bacillus subtilus	BS34A CU2189
Staphylococcus aureus	QE01 QE02 QE03 QE04 QE05 QE06 QE07 QE08 QE09 QE10 QE11 QE12 QE13 QE14 QE15 QE16 QE17 QE18 QE19 QE20 QE21 QE22 QE23 QE24 QE25 QE26 QE27 QE28 QE29 QE30 QE31 QE32 QE33 QE34 QE35 QE36 QE47 QE50 QE55 QE56 QE69 QE70 QE82 QE83 QE84 QE85 QE86 QE94
Streptococcus agalactiae	QE40 QE45 QE48 QE49 QE89 QE96 QE98 QE102
Streptococcus dyscalactiae	QE41 QE95 QE99 QE107 QE111
Streptococcus group A	QE37 QE38 QE39 QE43 QE44 QE51 QE52 QE53 QE54 QE57 QE58 QE60 QE61 QE62 QE63 QE64 QE65 QE66 QE67 QE68 QE71 QE72 QE73 QE74 QE75 QE76 QE77 QE78 QE79 QE80 QE81 QE87 QE88
Streptococcus group B	QE103
Streptococcus group C	QE46
Streptococcus group G	QE90 QE101 QE105 QE109 QE112
Corynebacterium striatum	QE92 QE93 QE106
Enterococcus faecalis	QE113 QE114 QE115 QE116 QE117 QE118 QE119 QE120 QE121 QE122 QE123 QE124 QE125 QE126 QE127 QE128 QE129 QE130 QE131 QE132 QE133 QE134 QE135 QE136
Escherichia coli K-12	TRACA1 TRACA2 TRACA3 TRACA4 TRACA5 TRACA6 TRACA7 TRACA8 TRACA9 TRACA10 TRACA11 TRACA12 TRACA13 TRACA14 TRACA15 TRACA16 TRACA17 TRACA18 TRACA19 TRACA20 TRACA21 TRACA22 TRACA23 TRACA24 TRACA25 TRACA26 TRACA27 TRACA28 TRACA29 TRACA30 TRACA31 TRACA32 TRACA33 TRACA34 TRACA35 TRACA36 TRACA37 TRACA38 TRACA39 TRACA40 TRACA41 TRACA42 TRACA43 TRACA44 TRACA45 TRACA46 TRACA47 TRACA48 TRACA49 TRACA50 TRACA51 TRACA52 TRACA53 TRACA54 TRACA55 TRACA56 TRACA57 TRACA58 TRACA59 TRACA60 TRACA61 TRACA62 TRACA63 TRACA64 TRACA65 TRACA66 TRACA67 TRACA68

Chapter 3

Investigation into Resistance Genes and Mobile Elements in the Oral Cavity

3.1 Bacteria in the Oral Cavity

3.1.1 Introduction to the Oral Microbiome

The human microbiome has been co-evolving alongside humans throughout history developing into a symbiotic relationship conferring benefits to both humans and microbiota (Clemente *et al.*, 2012; Hooper and Gordon, 2001). The human oral microbiome is composed of bacteria, archaea, viruses fungi and protozoa (Wade, 2013). Some bacteria that reside within the oral cavity are responsible for two common diseases that are found within humans, these being dental caries and periodontal disease. The human oral microbiome is home to a large number of diverse bacterial species, approximately 1000 different species of bacteria have been found within the oral cavity (Wade,

2013). The community of bacteria that reside within the oral cavity has been shown to be the second most complex bacterial community following those in the colon (Wade, 2013).

3.1.2 The Role of Oral Bacteria in Health and Disease

The acquisition of the oral microbiome begins with vertical transmission from the mother to the child during childbirth, the composition of the oral microbiome is influenced by the method of delivery (i.e. Caesarian or vaginal), it has been observed that in 3 month old infants that were delivered via a vaginal childbirth that their oral microbiome shows a higher taxonomic diversity than those delivered by Caesarian (Dominiguez-Bello *et al.*, 2010; Zaura *et al.*, 2014). It has also been observed that infants who were delivered via Caesarian acquired the pathogenic bacteria *Streptococcus mutans* approximately a year earlier than infants born via vaginal childbirth (Li *et al.*, 2005) which suggests that it's possible that the method of childbirth could have a lasting impact upon the oral microbiome (Costello *et al.*, 2013; Zaura *et al.*, 2014). In addition to the method of childbirth, the initial development of the oral microbiome is also influenced by whether the infant is fed using breast milk or with formula milk (Romani Vestman *et al.*, 2013). In breastfed infants, *Lactobacillus* was observed to have colonised the oral cavity significantly more frequently than infants who had been fed with formula milk (Vestman *et al.*, 2013). In another study, *Lactobacillus* isolates were cultured from breast fed infants and tested for their ability to inhibit the growth of *Streptococcus mutans* and *Streptococcus sanguinis*, it was observed that the *Lactobacillus* isolates did inhibit the growth of the Streptococcus isolates, which may suggest a potential mechanism explaining the difference within the oral microbiota between infants fed with breast milk and infants

fed with formula milk in that the *Lactobacillus* bacteria indigenous to breast milk may suppress the growth of other bacterial species (Holgerson *et al.*, 2013). In addition to the vertical transfer of bacteria from the parent to the infant, the developing oral microbiome is influenced by horizontal transfer from other individuals within the same environment such as siblings (Baca *et al.*, 2012; Stahringer *et al.*, 2012; Zaura *et al.*, 2014).

3.2 Diseases of the Oral Cavity

3.2.1 Dental Caries

Dental caries (tooth decay) is a common infectious disease found in humans, there has been a reduction in the incidence of dental caries within populations where the fluoridation of public water has taken place as such the prevalence of dental caries varies in between different populations, in certain populations there is an epidemic of this disease (Scannapieco, 2013). Dental caries is considered to be a poly-microbial disease involving multiple species of bacteria, a group of bacteria that play a pivotal role in the development of dental caries is the carcinogenic streptococci. This group includes the streptococcal species *S. mutans*, *S. aureus*, *S. cricetus*, *S. macacae*, and *S. downeii*. These bacteria metabolise dietary sugar into lactic acid as well as producing polysaccharides that are involved in biofilm production, these polysaccharides slow the rate in which the lactic acid is able to diffuse away from the surface of the tooth therefore increasing tooth demineralisation. In order to acquire this disease state, both the carcinogenic bacteria and fermentable carbohydrates are required (Scannapieco, 2013). As well as the carcinogenic streptococci, the presence of acidogenic lactobacilli has been observed within carious lesions, however these lactobacilli are considered to

be primarily involved with the progression of the disease state as opposed to its initiation, it is also possible that the development of dental carries is influenced by the base producing microorganisms within the oral biofilm which may have a buffering effect upon the lactic acid that's being produced (Scannapieco, 2013).

Dental caries is a common disease found in children and can occur soon after the teeth have erupted. In less severe cases this disease leads to an impaired quality of life and is a source of pain however this disease can lead to hospitalisation and fatality in the most serious cases (Gross *et al.*, 2012). Research that has recently been carried out using molecular techniques such as 16S rDNA profiling in order to catalogue the bacterial species present within dental caries has suggested that there are a number of other species of bacteria that are over represented in carious lesions besides *S. mutans* and lactobacilli, these include *Neisseria*, *Bifidobacterum*, *Selenomonas* , *Propri-onibacteria* as well as other species of streptococci (Peterson *et al.*, 2013). Another study investigating early childhood caries used anaerobic culture methods to investigate the microbiota present in serious cases of this disease and observed that the species that were associated with severe early child-hood caries included *S. mutans* as other studies show, however alongside *S. mutans* the following species were also shown to have an association with this disease state: *Veillonella parvula*, *Acinomyces gerensceriae*, *Scardovia wiggsiae* and *Streptococcus cristatus* (Tanner *et al.*, 2011). It is speculated that increase in the numbers of *Veillonella* in caries is likely to be because within the carious lesion there is abundant environmental lactate present and *Veillonella* is able to metabolise lactate and is therefore able to survive within this environment (Scannapieco, 2013).

3.2.2 Periodontal Diseases

Periodontal diseases occur as a result of bacteria that live in poly-microbial biofilms either at or below the gingival margin, these diseases progress largely due to an inflammatory response that is initiated by subgingival species (Borgnakke *et al.*, 2013; Teles *et al.*, 2013). Bacteria are able to colonise the tooth surface within a few hours of tooth cleaning, this colonisation primarily occurs in the regions of the gingival margin as well as the inter-dental spaces. The biofilm that is developing in these regions is responsible for producing various biologically active products that include endotoxins, protein toxins, chemotactic peptides and organic acids. Such products diffuse into the gingival epithelium, initiating the host response eventually leading to gingivitis (Kornman *et al.*, 1997). The clinical description of gingivitis is recognised by a colour change in the gums from Pink to red, this colour change is accompanied by other symptoms such as bleeding and swelling, due to the fact that in most cases this disease state is not painful, it may remain untreated in individuals for a significant amount of time. Gingivitis may progress in to periodontitis, periodontitis is a more serious disease state that refers to the destruction of the supporting bone of the tooth and the periodontal ligament alongside the inflammation of the gingiva that is characteristic of gingivitis, if the condition periodontists remains untreated then it can result in the destruction of the supporting tissues of the tooth ultimately leading to tooth loss (Scannapieco, 2013). There are environmental and endogenous factors that are associated with an increased incidence of periodontist, these factors include stress, oral hygiene, obesity, smoking as well as potential genetic associations (Curtis *et al.*, 2011). The bacteria that reside within the oral cavity are necessary for both the onset and the progression of periodontal disease (Teles *et al.*, 2013). Research that is focused upon culture based

methods have indicated that different bacteria are involved with different forms of this disease, for example in the case of gingivitis, the flora shifts from what is predominantly an environment that contains facultative Gram positive bacteria that contains a high percentage of Streptococci to a flora that is associated with increased levels of Gram negative bacteria, commonly of the genera *Campylobacter*, *Capnocytophaga*, *Fusobacterium* (Haffajee and Socransky, 2005). However the condition periodontitis is associated with a shift to mostly anaerobic species, such species include *Tannerella forsythia*, *Porphyromonas gingivalis* and *Treponema denticola* (Scannapieco, 2013). In addition to the presence of pathogenic bacteria, periodontitis also occurs as a result of the inflammatory response mediated by the immune system of the host. In addition to virulence factors, pathogen associated molecular patterns are displayed by such bacteria, these structural moieties are recognised by the pathogen recognition receptors in the host, such receptors contribute to the development of inflammation by activating downstream signalling pathways in inflammatory cells which then leads to an increase in the production of inflammatory cytokines which then in turn leads to the onset of both local and systemic inflammation (Haffajee and Socransky, 2005). Based on such observations it is therefore possible to conclude that periodontal pathogenesis is a result of a complex yet subtle interactions between both the bacterial species that reside within the oral microbiome and cells that belong to the host. A community of bacteria which is potentially pathogenic may arise from a small number of bacteria, these bacteria are named "keystone pathogens" (Darveau *et al.*, 2012). These bacterial species influence the host immune response in such a manner that results in an impaired immune surveillance as well co-opting other species of bacteria that typically reside in the oral cavity to contribute to pathogenesis. Therefore it is possible that disease

expression may be a result of a limited yet changing group of bacteria, the combined actions of which promote the inflammatory response within the host and the subsequent tissue destruction (Scannapieco, 2013).

3.2.3 Endodontic Infections

An endodontic infection is most likely to occur once bacteria have penetrated into the root canal system which usually occurs as a result of a dental carious lesion. However there are other routes of entry that bacteria are able to exploit, such routes include periodontal disease that leads to access to dental tubules, tooth abrasion or erosion that exposes the pulp, fracture as a result of trauma or the microleakage or restorations (Narayanan and Vaishnavi, 2010). The usual result of an endodontic infection is apical periodontitis, this condition is characterised by the destruction of the bone and the periodontal ligament that surrounds the tooth (Scannapieco, 2013). According to results of both traditional culture dependent studies as well as modern molecular culture independent studies, the bacterial species that have been identified from samples of teeth that have been infected endodontically generally reflects the bacterial species that comprise the flora of dental plaque. The genera that have been most commonly observed in such infections include *Lactobacillus*, *Streptococcus*, *Prevotella* and *Eubacterium* (Siqueira and Rocas, 2009). As is the case in the commensal bacteria that reside in the oral microbiome, many of the bacterial species present are not currently cultivable (Narayanan and Vaishnavi, 2010). In many cases, primary endodontic infections are mixed infections that involve at least two different species of bacteria. Secondary endodontic infections occur as a result of either the failure of the treatment used to treat the initial infection or the recolonisation of the root canal system (Scannapieco, 2013). Bacterial diversity in secondary

endodontic infections has been observed to be diminished when compared to the bacterial diversity in primary endodontic infections however the bacteria *Enterococcus faecalis* has often been isolated in these cases (Narayanan and Vaishnavi, 2010).

3.3 Ethics

The collection of saliva samples was approved by the Anglia Ruskin ethics committee, project number: FST/FREP/12/349.

3.3.1 Screening Metagenomic DNA for Genes Associated with Antimicrobial Resistance

Metagenomic DNA was extracted from each of the saliva samples (processed as described in section 2.0.4) using the FastPREP homogeniser (MP Bio) as described in section 2.0.7. DNA yields are presented on Table 3.1. Polymerase chain reaction (PCR) was employed to screen each metagenomic DNA sample for the presence of different genes that confer resistance to antibiotics as well as genes that are associated with mobile elements *int* and *tndX*). This data is presented on Table 3.2, the profile of each sample appears to be similar with 25 of 27 samples containing the tetracycline resistance gene *tetM*, all samples tested positive for the presence of the tetracycline resistance gene *tet32* and the *int* gene that is associated with Tn*916*-like transposable elements. In addition to this, the erythromycin resistance gene *ermB* was identified in all samples. However the other resistance genes that were screened for (tetracycline resistance genes *tetQ*, *tetX*, *tetX-1* ; erythromycin resistance genes *ermA* and *ermC*, the vancomycin resistance gene *vanA*, oxacillinase producing genes *OXA23*, *OXA48*, *OXA58* and *OXA40* were all failed to am-

Table 3.1: Metagenomic DNA extracted from saliva samples using the FastPrep homogeniser (MP Bio).

Sample	Quantity ng/µl	Sample	Quantity ng/µl
S1	248	S16	194
S2	230	S17A	35
S3	202	S17B	29
S4	91	S18	20
S5	47	S19	111
S6	111	S20	4.9
S7	36	S21	68
S8	68	S22	111
S9	70	S24	4.9
S10	133	S25	68
S11	192	S26	47.2
S12	172	S27	82
S13	65	S28	150
S14	109	S29	176
S15	216		

plify as was the recombinase gene *tndX*. However it is not possible to conclude that these genes were absent because no positive controls were included in these reactions, results presented on Table 3.2.

3.3.2 Analysis of Bacterial Isolates Cultured from Saliva

Table 3.2: Results from PCRs carried out on the metagenomic DNA extracted from the saliva samples, various antibiotic resistance genes were tested for as well as genes associated with transposable elements.

	tetM	*tet32*	*ermA*	*ermB*	*ermC*	*int*	*tndX*	*VanA*	*tetX*	*tetX-1*	*tetQ*	*OXA23*	*OXA48*	*OXA58*	*OXA40*
S1	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S2	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S3	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S4	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S5	x	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S6	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S7	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S8	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S9	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S10	x	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S11	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S12	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S13	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S14	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S15	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S16	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S17	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S18	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S19	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S22	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S23	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S24	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S25	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S26	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S27	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S28	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x
S29	✓	✓	x	✓	x	✓	x	x	x	x	x	x	x	x	x

Table 3.3: PCR reactions carried out specific for areas of Tn*3872* corresponding with the regions shown in Fig 3.1.

ID	Morphology	A	B	C	D	E	F	G	H	I	J
S2-1	1	✓	✓	✓	✓	x	✓	✓	✓	✓	✓
S2-2	1	✓	✓	✓	✓	x	✓	✓	✓	✓	✓
S2-3	1	✓	✓	✓	✓	x	✓	✓	✓	✓	✓
S2-4	1	x	✓	✓	✓	x	x	✓	✓	✓	✓
S2-5	1	✓	✓	✓	✓	x	✓	✓	✓	✓	✓
S2-7	1	x	✓	x	✓	x	x	✓	✓	✓	✓
S2-8	2	✓	✓	✓	✓	x	✓	✓	✓	✓	✓
S2-9	2	✓	✓	✓	✓	x	✓	✓	✓	✓	✓
S2-10	2	✓	✓	✓	✓	x	✓	✓	✓	✓	✓
S2-11	1	✓	✓	✓	✓	x	✓	✓	✓	✓	✓
S2-12	1	✓	✓	✓	✓	x	✓	✓	✓	✓	✓
S2-13	1	✓	✓	✓	✓	x	✓	✓	✓	✓	✓
S2-14	1	✓	✓	✓	✓	x	✓	✓	✓	✓	✓
S2-15	1	✓	✓	✓	✓	x	✓	✓	✓	✓	✓
S2-16	1	✓	✓	✓	✓	x	✓	✓	✓	✓	✓
S2-17	1	✓	✓	✓	✓	x	✓	✓	✓	✓	✓

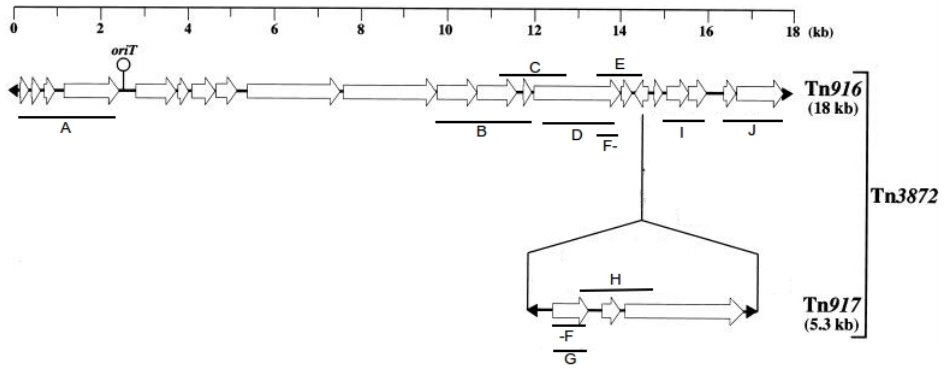


Figure 3.1: Diagram of Tn*3872* adapted to show the different regions amplified by the sets of PCR primers that corresponds with the reactions shown in Table 3.3. Adapted from (Poyart *et al.*, 2000)

Initial antibiotic resistance selection took place on BHI agar plates that contained 20% defibrinated horse blood and 2μg/ml erythromycin to select for erythromycin resistant isolates in accordance with BSAC guidelines. Any colonies that appeared on these plates following incubation at 37°C for 24-48 hours were sub cultured on to further plates that contained the same antibiotic in order to confirm whether or not the resistance was genuine. The isolates that produced full growth following subculturing were divided based upon their morphological characteristics and transferred to BHI broth containing 10% glycerol in order to allow storage at -80°C. The isolates were further tested for resistance to tetracycline at a concentration of 4μg/ml as well as an increased concentration of erythromycin at 10μg/ml. 16 isolates that were resistant to both 4μg/ml tetracycline and 10μg/ml erythromycin were taken forward for further analysis. Further analysis involved investigating what antibiotic resistance genes were present as well as mapping the mobile element that the genes were located on. This was achieved by screening for the presence of known resistance genes using PCR. The PCR showed

that in the case of all 16 isolates, the resistance genes that were present were *tetM* which confers resistance to tetracycline and *ermB* that confers resistance to erythromycin.

PCR mapping was carried out in order analyse the structure of the element. The regions that were amplified are illustrated in Fig. 3.1. The PCR results are shown on Table 3.3. Sections 'A', 'E', and 'F' failed to amplify from isolate S2-4 and sections 'A', 'C', 'E' and 'F' failed to amplify from isolate S2-7, amplicons were produced for the remaining sections. All sections with the exception of 'E' were amplified in the remaining 14 isolates, suggesting that the element could be related to Tn*916*. PCR 'F' bridges Tn*916* and Tn*917*, the amplification suggests that there may have been an insertion at this point. However because the whole transposon was not amplified and the amplification results were not verified via DNA sequencing it is not possible to conclude what these elements are.

These isolates were divided into 2 groups based upon morphology. The species identity was determined first by the sequencing of the *16S* gene which revealed that in the case of both morphologies the species was *Streptococcus salivarius*. This identification was later confirmed by *sodA* sequencing.

3.3.3 Investigation into isolates from S28

In order to characterise the different species present within the isolates obtained from S28, this sample was selected at random, and selected for using tetracycline, they have been divided into different groups based upon morphological characteristics, and screened against tetracycline in order to confirm resistance, in addition these isolates have also been screened against erythromycin (Table 3.5). To further assess the morphological characteristics of these isolates, they were cultured on *Streptococcus salivarius/mitis*

agar (Table 3.5). A morphological representative of each of these isolates was identified by sequencing the 16S gene, the bacterial species identified are displayed on Table 3.6.

Table 3.4: Minimum inhibitory concentration (MICs) of tetracycline and erythromycin for isolates from saliva sample S2.

ID	Tet (24h)	Tet (48h)	Erth (24h)	Erth (48h)
S2-1	>256	>256	16	16
S2-2	>256	>256	16	16
S2-3	>256	>256	16	16
S2-4	>256	>256	16	16
S2-5	>256	>256	16	16
S2-6	>256	>256	16	16
S2-7	>256	>256	16	16
S2-8	>256	>256	16	16
S2-9	>256	>256	16	16
S2-10	128	128	8	8
S2-11	>256	>256	16	16
S2-12	>256	>256	16	16
S2-13	>256	>256	4	8
S2-14	128	128	2	4
S2-15	64	128	2	4
S2-16	64	128	2	8
S2-17	64	>256	16	16

Table 3.5: Isolates cultured from sample S28 on a variety of different media in order to try to identify bacteria that belong to different species, they were also screened for resistance against tetracycline and erythromycin.

ID	G+ agar	MacConkeys agar	*S. mitis/Salivarius agar*	tetR	erthR
S28 1	x	x	Deep blue, mucoid	✓	✓
S28 2	x	Pink	Sky blue, mucoid	✓	✓
S28 3	x	Pink	Clear, smooth	✓	✓
S28 4	x	x	Deep blue, mucoid	✓	✓
S28 5	x	x	Clear	✓	✓
S28 6	x	x	Clear	✓	✓
S28 7	x	x	Clear, minimal growth	✓	✓
S28 8	✓	x	Sky blue, mucoid	✓	x
S28 9	✓	x	Deep blue, mucoid	✓	✓
S28 10	x	Pink	Deep blue, mucoid	✓	✓
S28 11	✓	Pink	Sky blue, mucoid	✓	✓
S28 12	x	Pink	Sky blue, mucoid	✓	✓
S28 13	x	Pink	Deep blue, mucoid	✓	✓
S28 14	x	Pink	Deep blue, mucoid	✓	✓

ID	G+ agar	MacConkeys agar	*S. mitis/Salivarius agar*	tetR	erthR
S28 15	x	Pink	Sky blue, mucoid	✓	✓
S28 16	x	x	Clear	x	✓
S28 17	x	x	Sky blue, mucoid	✓	✓
S28 18	x	Pink	Sky blue, mucoid	✓	✓
S28 19		Pink	Sky blue, mucoid	x	x
S28 20	x	x	Sky blue, mucoid	✓	✓
S28 21	x	Pink	Sky blue, containing deep blue ring	✓	✓
S28 22	✓	Pink	Minimal growth	✓	x
S28 23	✓	x	Minimal growth	✓	x
S28 24	x	x	Minimal growth	✓	x
S28 25	x	x	Minimal growth	✓	✓
S28 26	✓	x	Minimal growth	x	x
S28 27	✓	x	Minimal growth	✓	x
S28 28	✓	x	Minimal growth	✓	x
S28 29	✓	✓ Pink	Deep blue centre, sky blue edge	x	x
S28 30	✓	✓ Yellow	Grey blue mucoid	✓	x

ID	G+ agar	MacConkeys agar	*S. mitis/Salivarius agar*	tetR	erthR
S28 31	✓	✓ Yellow	Grey blue mucoid	✓	x
S28 32	x	✓ Yellow	Grey blue mucoid	✓	x
S28 33	x	✓ Yellow	Grey blue mucoid	✓	x
S28 34	x	✓ Yellow	Grey blue mucoid	✓	x
S28 35	x	✓ Yellow	Grey blue mucoid	✓	x
S28 36	x	✓ Yellow	Grey blue mucoid	✓	x
S28 37	x	✓ Yellow	Grey blue mucoid	✓	x
S28 38	x	✓ Yellow	Grey blue mucoid	✓	x
S28 39	✓	✓ Pink	Clear	✓	✓
S28 40	✓	✓ Pink	Clear	✓	✓
S28 41	✓	x	Clear	✓	✓
S28 42	x	✓ Pink	Pale blue mucoid	✓	✓
S28 43	x	✓ Pink	Pale blue mucoid	✓	✓
S28 44	✓	✓ Pink	Pale blue mucoid	✓	✓
S28 45	✓	✓ Pink	Pale blue mucoid	✓	✓
S28 46	x	✓ Pink	Pale blue mucoid	✓	✓

ID	G+ agar	MacConkeys agar	*S. mitis/Salivarius agar*	tetR	erthR
S28 47	x	✓ Pink	Clear	✓	✓
S28 48	x	✓ Pink	Blue, partial growth	✓	x
S28 49	x	x	Clear	✓	✓
S28 50	x	x	Clear	✓	x
S28 51	x	x	Clear	✓	x
S28 52	✓	✓ Pink	Clear	✓	✓
S28 53	x	✓ Pink	x	x	x

Table 3.6: The isolates were divided based upon the morphological factors that were observed based upon culturing on different agar types. A morphological representative from each of these types underwent PCR of the 16S gene which was sequenced in order to determine the bacterial species.

ID	Species (*16S*)
S28 1	*Rothia dentocariosa*
S28 2	*Rothia dentocariosa*
S28 6	*Streptococcus sanguinis*
S28 21	*Streptococcus haemolyticus*
S28 30	*Neisseria subflava*
S28 42	*Streptococcus salivarius*

3.4 Procedure Outline

The transposon aided capture of plasmids (TRACA) is a method for isolating plasmids that are present in bacteria without the need to first culture the bacteria. Therefore this technique allows the capture of plasmids that are located in bacteria that are not presently able to be cultured successfully under laboratory conditions. TRACA was initially used by Jones and Marchesi (Jones and Marchesi, 2007) to capture plasmids from human gut microbiota. In this instance TRACA is being used to identify and characterise plasmids that are present within the oral cavity of healthy human individuals.

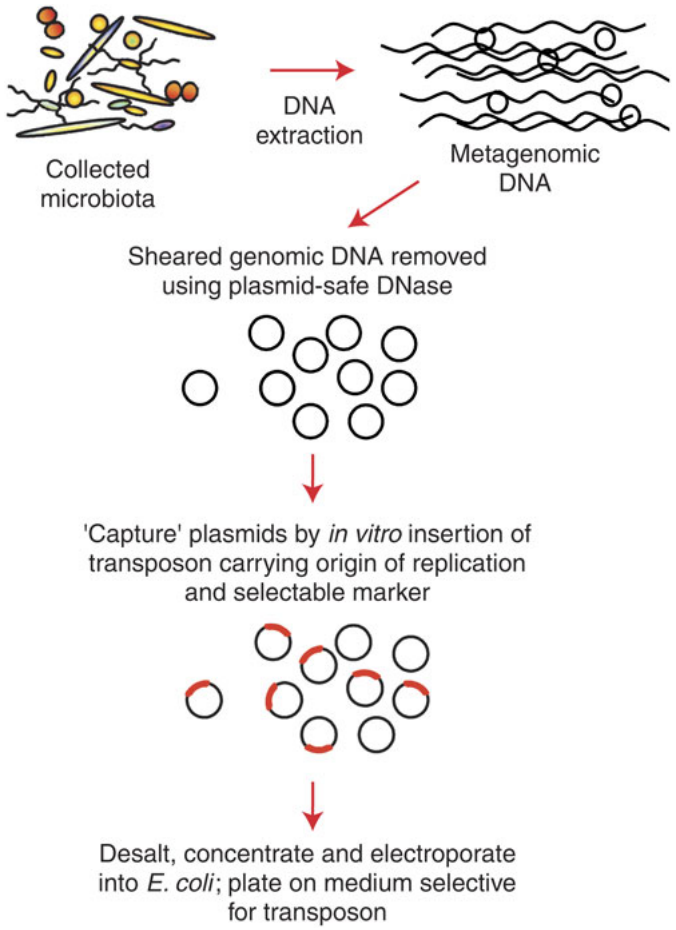


Figure 3.2: Diagram outlining the TRACA method from (Jones and Marchesi, 2007).

3.5 Method

20µl of DNA was pooled from each saliva sample from S1 to S10 producing a final volume of 200µl. The metagenomic DNA digested is shown in Table 3.7. Approximately 600ng of pooled DNA was subsequently digested for 16 hours using a plasmid safe DNase in order to remove linear DNA. The restriction digest reaction is shown in table 3.8. 12.36µg was digested. Following digestion the product was run through the QiaQuick Miniprep Kit (Qiagen) and the remaining DNA was quantified using the Qubit (Invitrogen). The final yield of DNA was 1.72ng/µl.

Table 3.7: Metagenomic DNA digested for TRACA procedure.

Sample ID	ng DNA in 20ul	Volume (µl)
S1	4960	20
S2	4600	20
S3	4040	20
S4	1820	20
S5	940	20
S6	2220	20
S7	720	20
S8	1360	20
S9	1400	20
S10	2660	20
TOTAL	24720	200
DNA ng/µl	123.6	

Table 3.8: Plasmid safe restriction digestion.

Reagent	Concentration	Negative control vol.	Reaction volume
DNA	123.6ng/µl	5µl	100µl
ATP	25mM	0.5µl	10µl
Buffer	10x	1.25µl	25µl
Enzyme	10U/µl	0µl	5µl
ddH_2O	-	5.75µl	110µl
Total		12.5µl	250µl

Table 3.9: Reagents and volumes required for the TRACA reaction.

Reagent	Concentration	Volume
DNA	1.72ng/µl	10µl
Transposon	0.1pmol/µl	1µl
Transposase	1U/µl	2µl
ddH_2O	-	32µl
Buffer	10x	5µl
Total		50µl

3.5.1 TRACA Reaction

The TRACA reaction was set up as detailed in Table 3.9 and incubated at 37°C for 2 hours then stopped via the addition of 5µl of EzTn5 stop buffer and incubated at 70°C for 15 minutes.

The plasmid is then purified using the QIAquick miniprep kit (Qiagen) in accordance with the protocol supplied. The purified plasmid is then trans-

formed into *E. coli* via electroporation by combining 4µl DNA with 50µl *E. coli* EPI300 cells (Epicentre) and electroporated as described in Section 2.3.4. Finally 100µl cells were spread onto an LB agar plate containing 50µg/ml kanamycin.

3.6 Results

68 plasmids were captured in total. Restriction digest profiling using *Sau*3AI revealed 32 different plasmid profiles, these were given a clone ID in the form of "TRACAX". 24 of these plasmids were taken forward for further analysis (Table 3.10).

Table 3.10: The plasmids that were captured using TRACA were digested using *Sau*3AI, profiles were established based upon the fragment sizes.

ID	Fragment sizes	Group	Plasmid ID
TRACA1	2700, 1500, 800, 600	1	pSPW01
TRACA2	1800, 1500, 400, 200	11	pSPW11
TRACA3	1000, 700, 400, 200	15	
TRACA4	1300, 700, 250	12	pSPW12
TRACA5	3000, 2250, 900, 850, 550, 500, 300, 250	17	
TRACA6	1200, 550, 300, 250	2	pSPW02
TRACA7	1300, 700, 250	15	
TRACA8	2250, 1500, 700, 500, 400, 350, 300	5	
TRACA9	2000, 1300, 900	3	pSPW03
TRACA10	2250, 1500, 700, 500, 400, 350, 300	5	
TRACA11	2000, 1400, 700, 550	19	pSPW20
TRACA12	2700, 1500, 800, 600	1	

ID	Fragment sizes	Group	Plasmid ID
TRACA13	1400, 400, 200	25	
TRACA14	600, 550, 400, 200	24	
TRACA15	2500, 1000, 900, 600, 550, 350, 250	9	
TRACA16	1000, 800, 200	27	
TRACA17	1900,	20	pSPW21
TRACA18	2250, 1400, 600	13	pSPW14
TRACA19	2500, 1000, 900, 600, 550, 350, 250	9	pSPW09
TRACA20	1200, 600, 200	29	
TRACA21	1000, 700, 400, 200	14	pSPW15
TRACA22	2000, 1400, 700, 550	19	
TRACA23	1250,1200, 600, 300	15	
TRACA24	1000, 900, 600, 350, 250	10	pSPW10
TRACA25	2500, 1000, 900, 600, 550, 350, 250	9	
TRACA26	1450, 1400, 400, 250	18	
TRACA27	1000, 600	28	
TRACA28	1250,1200, 600, 300	15	
TRACA29	1000, 700, 400, 200	14	
TRACA30	1000, 700, 400, 200	14	
TRACA31	1400, 300	26	
TRACA32	1250,1200, 600, 300	15	pSPW16
TRACA33	2250, 1500, 700, 500, 400, 350, 300	5	
TRACA34	1200, 600, 300	21	pSPW22
TRACA35	2250, 1400, 600	13	
TRACA36	1000, 700, 400, 200	14	
TRACA37	4000	4	pSPW04
TRACA38	2250, 1500, 700, 500, 400, 350, 300	5	pSPW05

ID	Fragment sizes	Group	Plasmid ID
TRACA39	3000, 2250, 900, 850, 550, 500, 300, 250	17	
TRACA40	1200, 600, 300	21	
TRACA41	2250, 1500, 700, 500, 400, 350, 300	5	
TRACA42	2250, 1500, 700, 500, 400, 350, 300	5	
TRACA43	1000, 600	28	
TRACA44	700, 500, 400	30	
TRACA45	3000, 2250, 900, 850, 550, 500, 300, 250	17	
TRACA46	2250, 1500, 700, 500, 400, 350, 300	5	
TRACA47	900, 800, 250	31	
TRACA48	1100,1000, 600, 250	16	pSPW17
TRACA49	3000, 2250, 900, 850, 550, 500, 300, 250	17	pSPW18
TRACA50	900, 650, 250	32	
TRACA51	1450, 1400, 400, 250	18	pSPW19
TRACA52	3000, 2250, 900, 850, 550, 500, 300, 250	17	
TRACA53	1200, 600, 300	21	
TRACA54	2250, 1500, 700, 500, 400, 350, 300	5	pSPW07
TRACA55	3200, 2000, 1200, 550, 500, 250	8	pSPW08
TRACA56	3000, 2250, 900, 850, 550, 500, 300, 250	17	
TRACA57	250, 400, 600, 1000, 1500, 3500, 4500	22	
TRACA58	2000, 1400, 700, 550	19	
TRACA59	250, 400, 600, 1000, 1500, 3500, 4500	22	
TRACA60	3000, 2250, 900, 850, 550, 500, 300, 250	17	
TRACA61	2250, 1500, 700, 500, 400, 350, 300	5	
TRACA62	1400, 300	26	
TRACA63	1450, 1400, 400, 250	18	
TRACA64	1000, 700, 400, 200	14	

ID	Fragment sizes	Group	Plasmid ID
TRACA65	1000, 700, 400, 200	14	
TRACA66	250, 400, 600, 1000, 1500, 3500, 4500	22	pSPW23
TRACA67	2250, 1500, 700, 500, 400, 350, 300	5	
TRACA68	1200, 1500	23	pSPW24

There were 19 plasmids that were successfully sequenced, these are presented in Figures 3.3 to 3.18 and Tables 3.13 to 3.28, a summary of the plasmids is presented in Table 3.11. These plasmids ranged from 780 to 5579 bp in size. The bacterial species that each plasmid originated from was determined by comparing the sequence of the origin of replication with the NCBI database. Based on this the plasmids appear to have originated from *Streptococcus agalactiae* (n = 8), *Streptococcus pneumoniae* (n = 2), *Staphylococcus aureus* (n = 2), *Neisseria gonorrhoeae* (n = 1) with 6 being unidentified. Antibiotic resistance genes were identified on 11 of the plasmids, the most common being β-lactamase belonging to the TEM family (n = 8). Other antibiotic resistance genes identified included chloramphenicol acetyltransferase (n = 3) and aminoglycoside phosphotransferase (n = 3). The activity of these genes was assessed via MIC testing shown below. The MIC data (Table 3.12) suggests that the β-lactamase and chloramphenicol acetyltransferase genes that are being carried by these plasmids are active. It was not possible to determine the functionality of the aminoglycoside phosphotransferase gene due to the presence of the *kanR* gene on the Ez-Tn*5* transposon present in the plasmid.

In addition to antibiotic resistance genes, a number of ORFs were identified, comparison with the NCBI database revealed that the majority of these ORFs were hypothetical proteins the function of which is currently unknown.

Table 3.11: Summary of the plasmids that were captured using TRACA, including their IDs, the species that they most closely resemble similarity to, their GC content and any resistance genes detected.

Plasmid ID	Closest Homologue	QC %	ID %	Acc.	GC%	Length (bp)	Resistance Gene	*OriR* Species
pSPW01	puCLIB	58	99	EU918604	41.5	4431	TEM-116	*S. pneumoniae*
pSPW02	pLM006	90	100	KX077950.1	49.7	2139	TEM-116	None identified
pSPW05	pRAB11	99	99	JN635500	39.58	5579	TEM-116, CAT	*S. aureus*
pSPW06	pRAB11	99	99	JN635500	39.52	5592	TEM-116, CAT	*S. aureus*
pSPW08	pUCLIB	63	99	EY918604.1	44.05	4154	TEM-116	None identified
pSPW09	pRAB11	99	99	JN635500	39.62	5561	TEM-116, CAT	*S. pneumoniae*
pSPW10	No match						None identified	*S. agalactiae*
pSPW11	pTRACA42	98	99	HM560025.1	51.42	1430	None identified	*S. agalactiae*
pSPW12	pTRACA42	99	99	HM560025.1	51.01	1392	None identified	None identified
pSPW15	pTetHS016	50	79	HQ622101.1	34.73	3049	None identified	*N. gonorrhoeae*
pSPW18	pCAG,I-Scel	74	100	JF714900.1	44.77	3511	TEM-116	None identified
pSPW19	pTRACA42	99	99	HM560025.1			None identified	*S. agalactiae*
pSPW21	pTRACA42	99	98	HM560025.1	51.78	1491	None identified	*S. agalactiae*
pSPW22	pTRACA42	100	99	HM560025.1	51.86	1425	None identified	*S. agalactiae*
pSPW23	pSV009	88	99	KT373982.1	49.53	2427	TEM-116	None identified
pSPW24	pTRACA42	100	99	HM560025.1	53.21	780	None identified	None identified

3.6.1 Similarity with Cloning Vectors

Nine of the plasmids sequenced appeared to share features with a variety of cloning vectors, including antibiotic resistance genes and β-galactosidase.

3.7 Antibiotic Resistance Activity

Table 3.12: The plasmids captured via TRACA underwent MIC testing with Ampicillin and chloramphenicol. All had an MIC greater than 512 μg/ml with the exception of pSPW08 which was 32 μg/ml. The dashes refer to clones that were sensitive to the antibiotic showing no signs of resistance. The negative control strain used was *Bacillus subtilis* CU2189 that is known to be sensitive to ampicillin and chloramphenicol.

Plasmid ID	Ampicillin μg/ml	Chloramphenicol μg/ml
pSPW01	>512	-
pSPW02	>512	-
pSPW05	>512	16
pSPW06	>512	16
pSPW08	32	-
pSPW09	>512	8
pSPW18	>512	-
pSPW23	>512	-

3.8 Annotated Plasmids

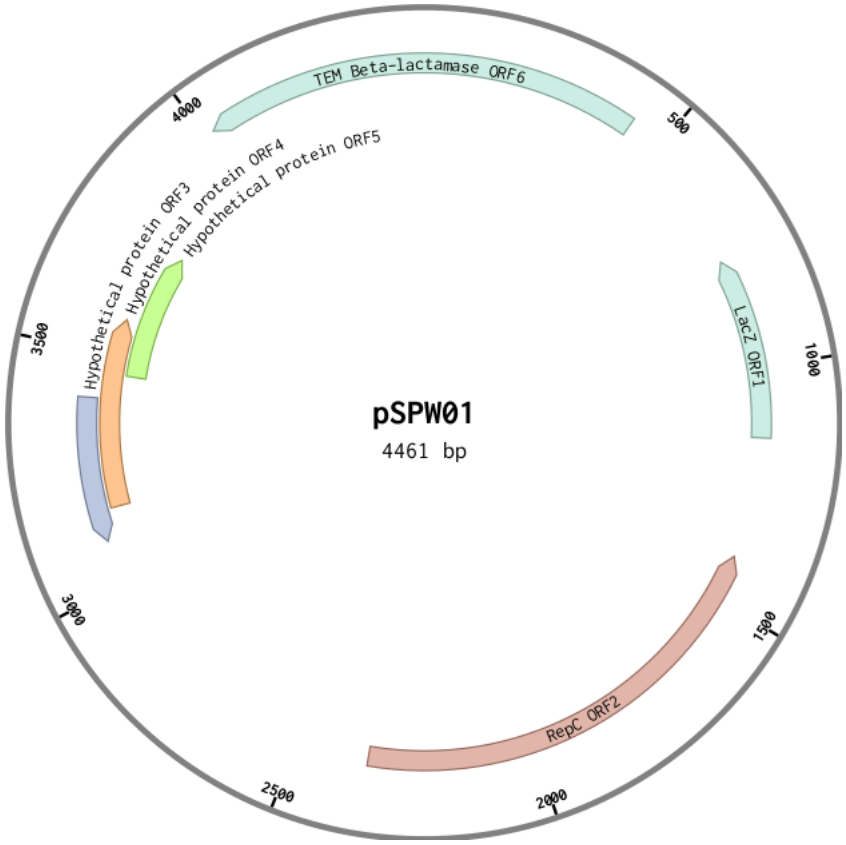


Figure 3.3: pSPW01 contains ORFs that match with a TEM family β-lactamse, however the functionality of this could not be assessed because the transposon appears to have inserted within this sequence.

Table 3.13: The ORFs identified on pSPW01 as shown in Fig. 3.3, their closest homologues and the species that the ORF originated from where applicable.

ORF Location	Closest homologue	QC%	ID%	Species
3988 - 399 c	TEM Class A β-lactamase	99	99	
733 – 1116 c	LacZ	82	96	
1374 – 2318 c	RepC	99	88	*S. pneumoniae*
3063 – 3371 c	Hypothetical protein	85	93	
3129 – 3554	Hypothetical protein	98	100	
3428 – 3736	Hypothetical protein	91	99	

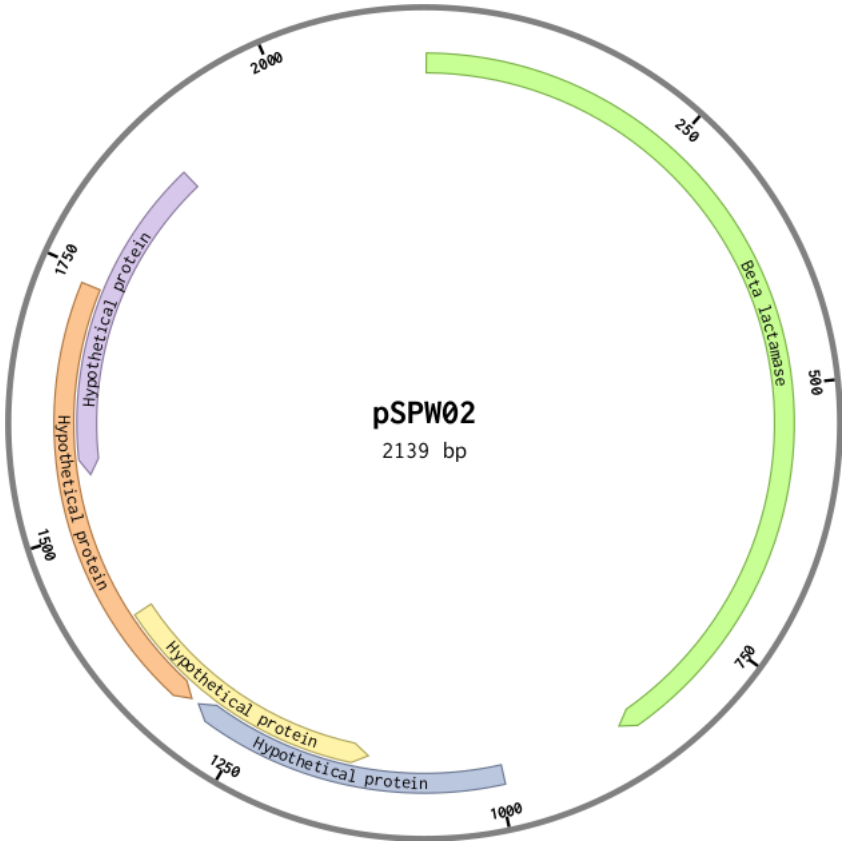


Figure 3.4: This plasmid contains a β-lactamase gene from the TEM family, this gene has 98% query cover of TEM-1. Other ORFs partially match hypothetical proteins, one of which is originally from *Bacillus cereus*, there was no origin of replication detected on this plasmid.

Table 3.14: The closest homologues to the ORFs identified in pSPW02 as shown in Fig. 3.4, their query cover and identity match % and the species that they originated from where applicable.

ORF Location	Closest homologue	QC %	ID %	Species
3-875	TEM β-lactamase	98	100	
479-790 c	Unknown (CV)	85	100	
995 -1300	Hypothetical protein	89	100	
1127-1405 c	Hypothetical protein	91	99	
1309-1737 c	Hypothetical protein	78	100	
1553-1879 c	Hypothetical protein	67	100	*Bacillus cereus*

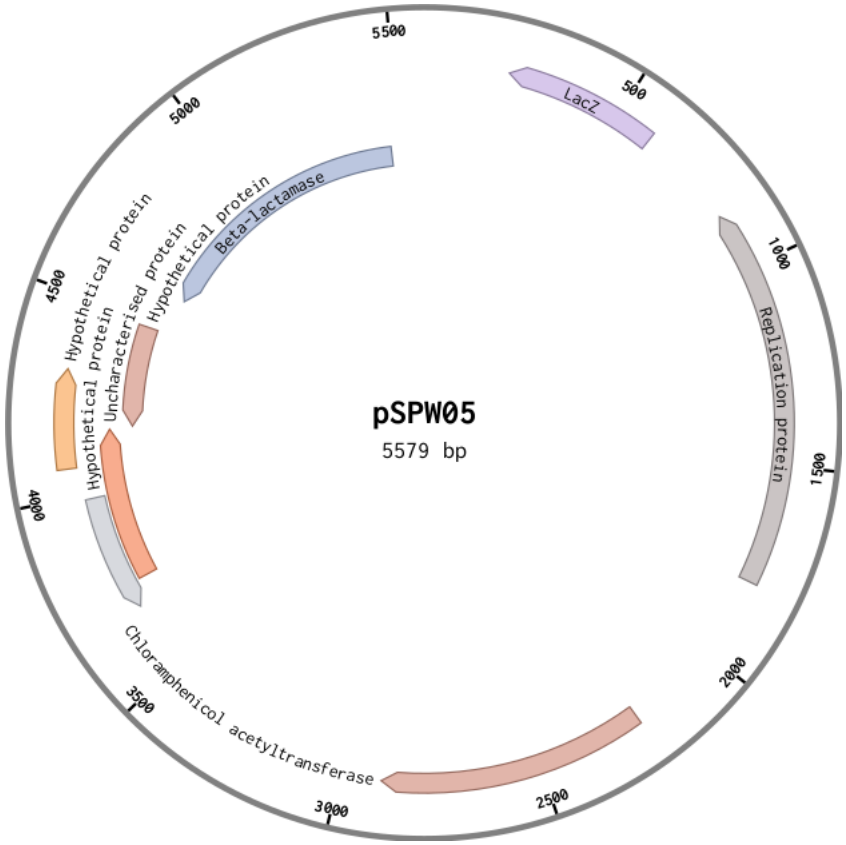


Figure 3.5: Plasmid pSPW05 contains two antibiotic resistance genes, a β-lactamase from the TEM family and chloramphenicol acetyltransferase, both of these genes were shown to confer resistance in the clonal host. ORFs also matched hypothetical proteins from a range of species and an origin of replication from *S. aureus* suggesting that the plasmid originated from this species.

Table 3.15: The closest homologues to the ORFs identified in pSPW05 as shown in Fig. 3.5, their query cover and identity match % and the species that they originated from where applicable.

ORF Location	Closest homologue	QC %	ID %	Species
213 - 596 c	LacZ	82	96	
854 - 1798 c	Rep initiation protein	99	100	*S. aureus*
1694 - 2026	Hypothetical protein	94	96	*S. aureus*
2234 - 2896	Chloramphenicol acetyltransferase	98	100	Multispecies
3676 - 3984 c	Hypothetical protein	99	83	*E. coli*
3742 - 4167	Uncharacterised protein	94	97	*Y. pseudoterberculorosis*
4071 - 4349	Hypothetical protein	91	99	Multispecies
4176 - 4481 c	Hypothetical protein	89	100	
4601 - 5473 c	TEM β-lactamase	98	100	

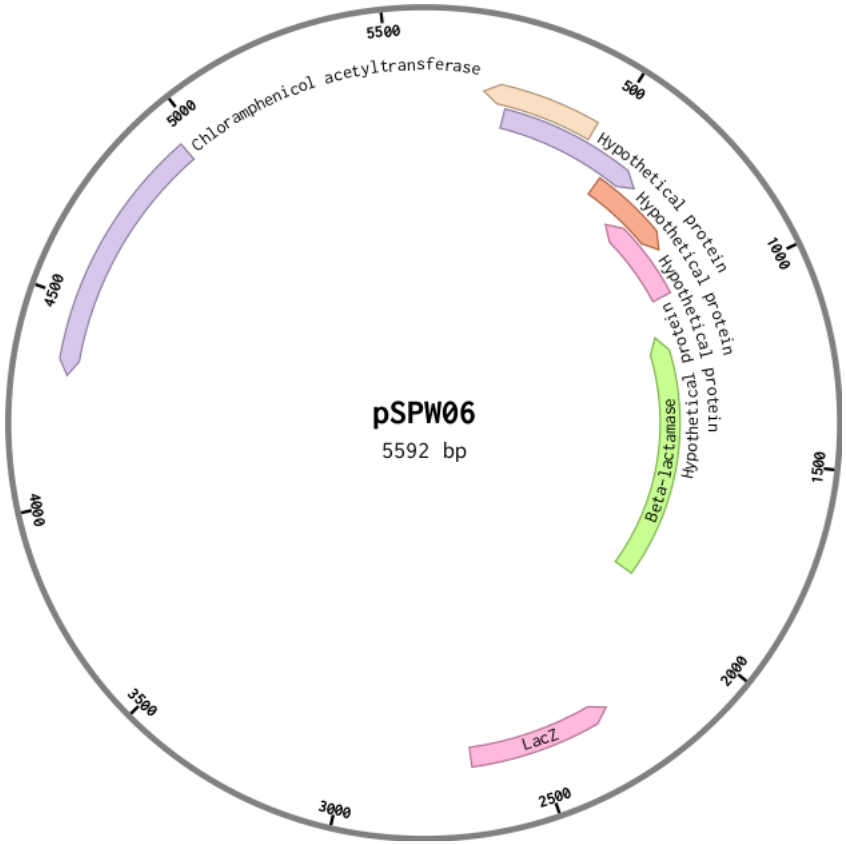


Figure 3.6: A TEM family β-lactamase and chloramphenicol acetyltransferase were identified on pSPW06, both of these genes conferred resistance in their hosts. An origin of replication originating from *S. aureus* was identified.

Table 3.16: The closest homologues to the ORFs identified in pSPW06 as shown in Fig. 3.6, their query cover and identity match % and the species that they originated from where applicable.

ORF Location	Closest homologue	QC%	ID%	Species
159-467 c	Hypothetical protein	99	82	*E. coli*
225-650	Hypothetical protein	98	99	
554-832	Hypothetical protein	91	99	Enterobacteriaceae
659-964 c	Hypothetical protein	89	100	
1084-1956 c	TEM β-lactamase	98	100	
2290-2673 c	LacZ	82	96	
3010-3876 c	Replication protein	98	99	*S. aureus*
4313-4954	Chloramphenicol acetyltransferase	97	99	

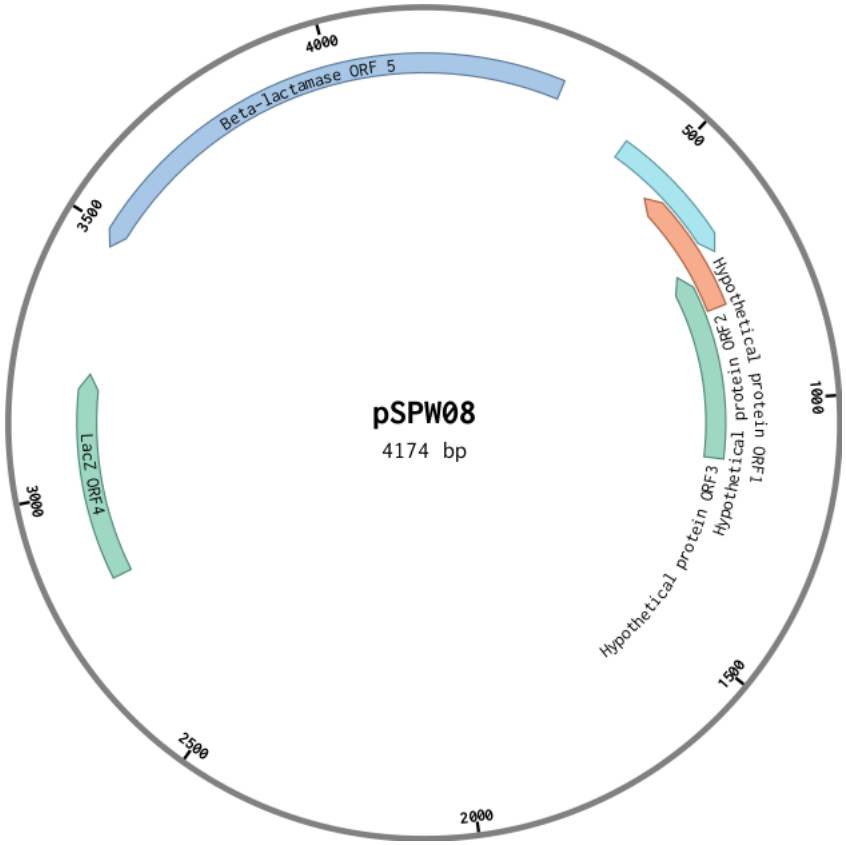


Figure 3.7: The ORFs on pSPW08 matched a TEM family β-lactamase as well as ORFs predicted to encode hypothetical proteins that have been detected in a variety of species. A *lacZ* gene was also detected. There was no origin of replication detected on this plasmid. Growth on CLED agar and revealed that the ORF identified as *lacZ* was not functional.

Table 3.17: The closest homologues to the ORFs identified in pSPW08 as shown in Fig. 3.7, their query cover and identity match % and the species that they originated from where applicable.

ORF Location	Closest homologue	QC %	ID %	Species
394-669	Hypothetical protein	82	100	
496-774 c	Hypothetical protein	91	99	Multispecies
678-1103 c	Hypothetical protein	98	100	
861-1169	Hypothetical protein	99	83	*E. coli*
2824-3207	LacZ	82	96	
3451-4154 c	TEM β-lactamase	98	100	

Table 3.18: The closest homologues to the ORFs identified in pSPW09 as shown in Fig. 3.8, their query cover and identity match % and the species that they originated from where applicable.

ORF Location	Closest homologue	QC%	ID%	Species
225 – 650	Hypothetical protein	98	100	*Neospora caninum*
554 – 880	Hypothetical protein	94	83	Enterobacteriae
707 – 1012 c	Hypothetical protein	89	100	*Parasitella parasitica*
1158 – 1994 c	β-lactamase TEM-1	91	94	
2219 – 2653	LacZ	86	70	
2931-3875 c	Replication protein	100	97	*S. pneumoniae*
4312 -4974	Chloramphenicol acetyltransferase	98	100	

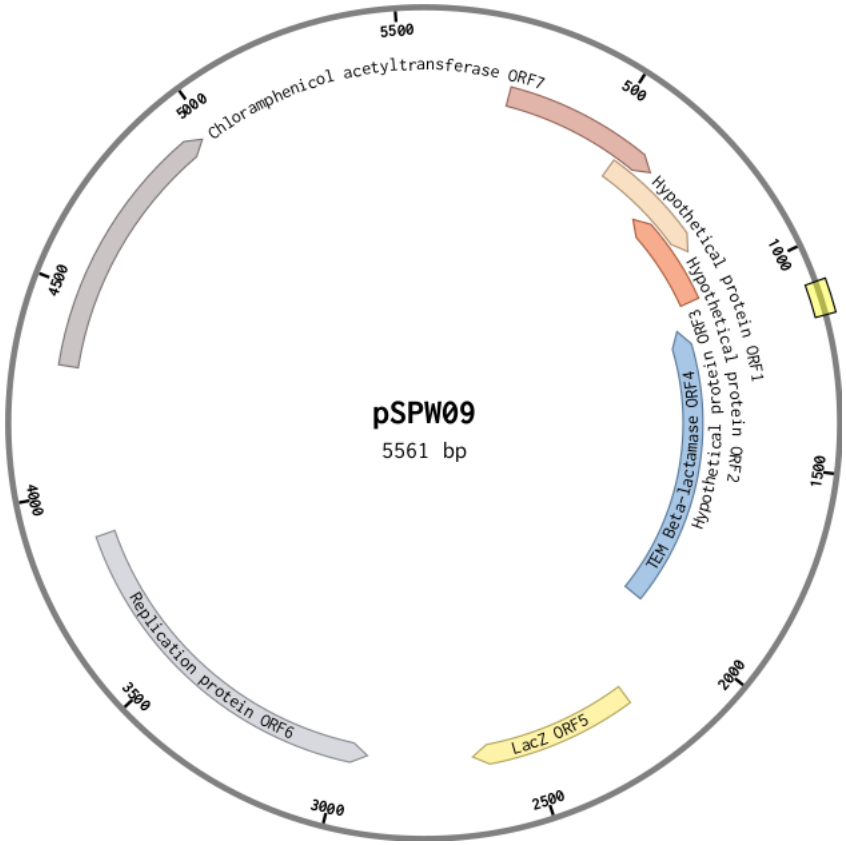


Figure 3.8: The closest homologues to the ORFs on pSPW09 were predicted to encode hypothetical proteins from a variety of species and a β-lactamase gene from the TEM family that conferred resistance to the host bacteria. In addition to this a *lacZ* gene was identified though the sequence homology was only 70% with a query cover of 86%. Growth on CLED agar revealed that the ORF identified as *lacZ* was not functional.

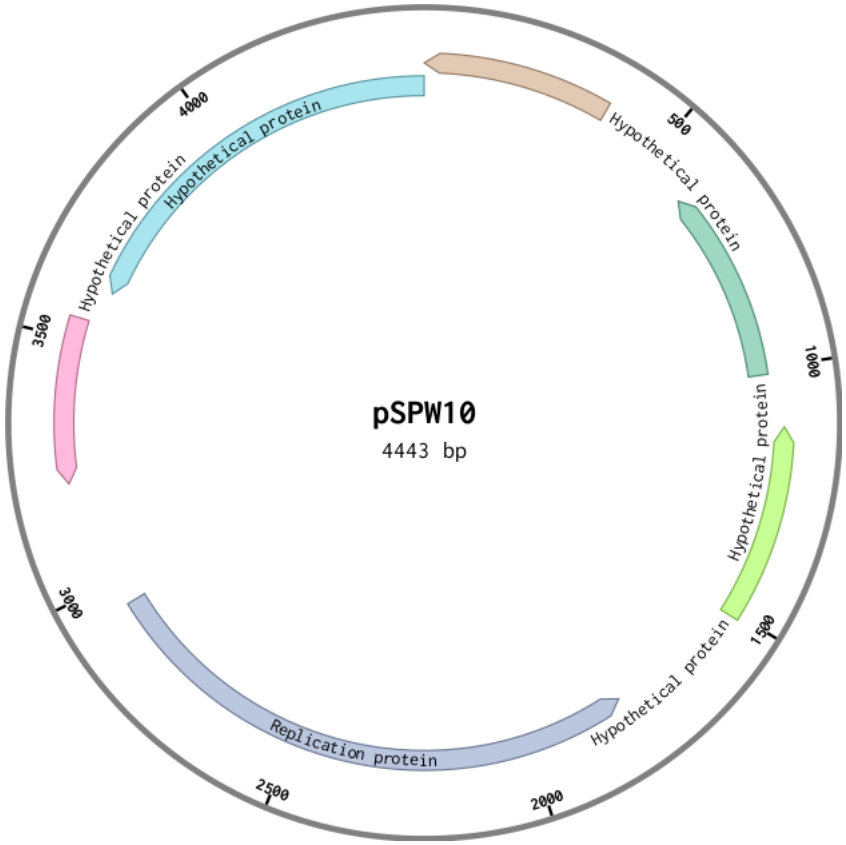


Figure 3.9: The ORFs on pSPW10 that most closely matched ORFs encoding hypothetical proteins originated from *Streptococcus* species. A potential origin of replication from *Streptococcus agalactiae* was detected, however the % ID was only 51%.

Table 3.19: The closest homologues to the ORFs identified in pSPW10 as shown in Fig. 3.9, their query cover and identity match % and the species that they originated from where applicable.

OEF Location	Closest homologue	QC%	ID%	Species
1- 373 c	Hypothetical protein	58	36	*Streptococcus gallolyticus*
604 – 1011 c	Hypothetical protein	69	33	*Streptococcus suis*
1119 – 1508 c	Hypothetical protein	93	61	*Streptococcus parasanguinis*
1788 – 2945 c	Replication protein	98	51	*Streptococcus agalactiae*
3213 – 3542 c	Hypothetical protein	85	29	
3611 – 4443 c	Hypothetical protein	94	38	

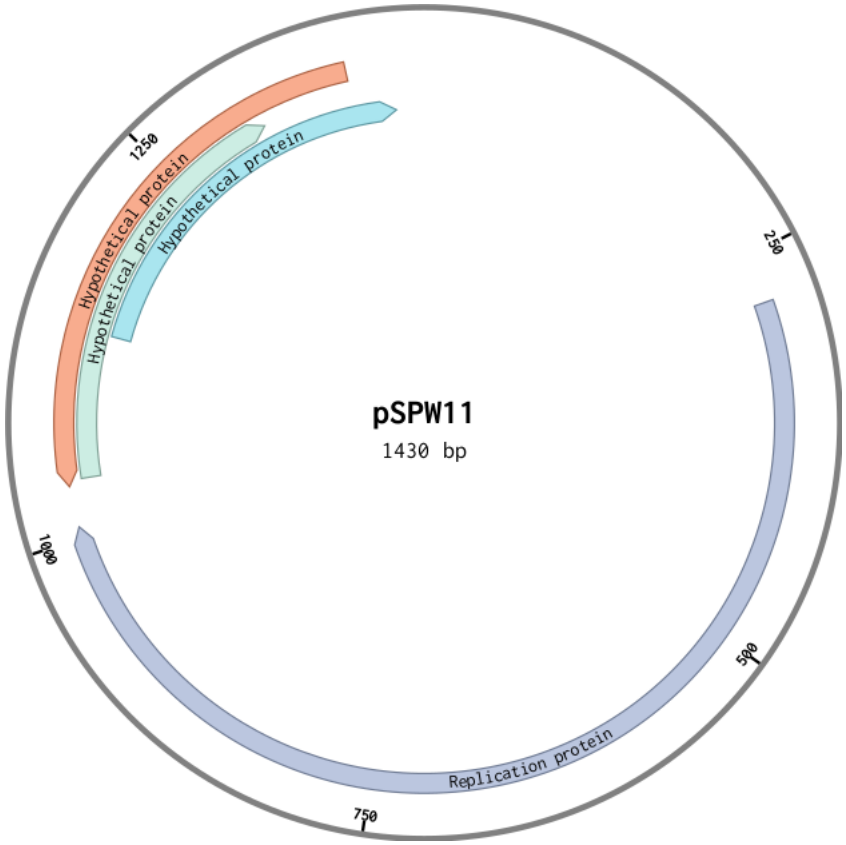


Figure 3.10: An origin of replication that most closely resembled an ori from *Streptococcus agalactiae* was detected along with ORFs that matched sequences predicted to encode hypothetical proteins.

Table 3.20: The closest homologues to the ORFs identified in pSPW11 as shown in Fig. 3.10, their query cover and identity match % and the species that they originated from where applicable.

ORF Location	Closest homologue	QC %	ID %	Species
227-625 c	Hypothetical protein	85	98	
281-1006	Replication protein	96	99	*S. agalactiae*
1033-1380 c	Lysine decarboxylase	53	40	*Comamonas* spp.
1037 – 1318	Hypothetical protein	50	98	
1135-1410	Hypothetical protein	45	93	

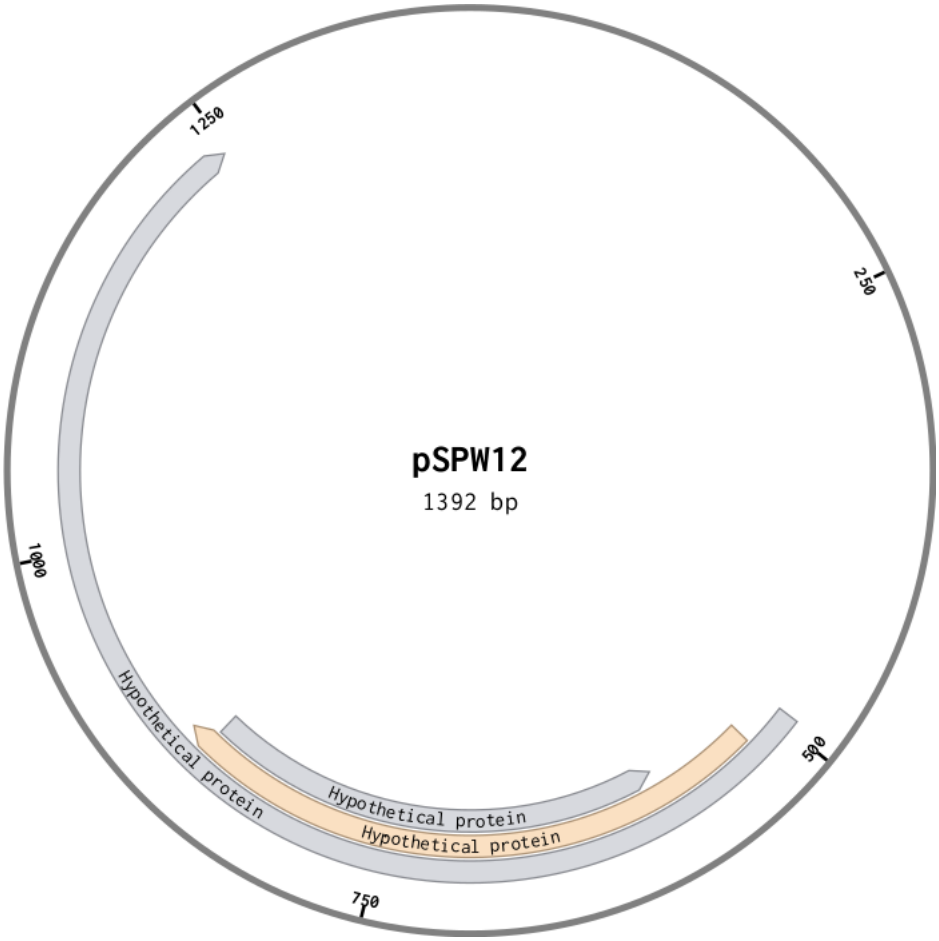


Figure 3.11: pSPW12 contains ORFs that most closely match sequences that are predicted to encode hypothetical proteins, however the sequence homology for all three of these proteins is relatively low. Two of the ORFs most closely match sequences from *S. agalactiae* suggesting that this plasmid may have originated in this species.

Table 3.21: The closest homologues to the ORFs identified in pSPW12 as shown in Fig. 3.11, their query cover and identity match % and the species that they originated from where applicable.

ORF Location	Closest homologue	QC %	ID %	Species
494-1246	Hypothetical protein	84	98	*S. agalactiae*
520-879	Hypothetical protein	21	73	*S. agalactiae*
578-865 c	Hypothetical protein	68	91	

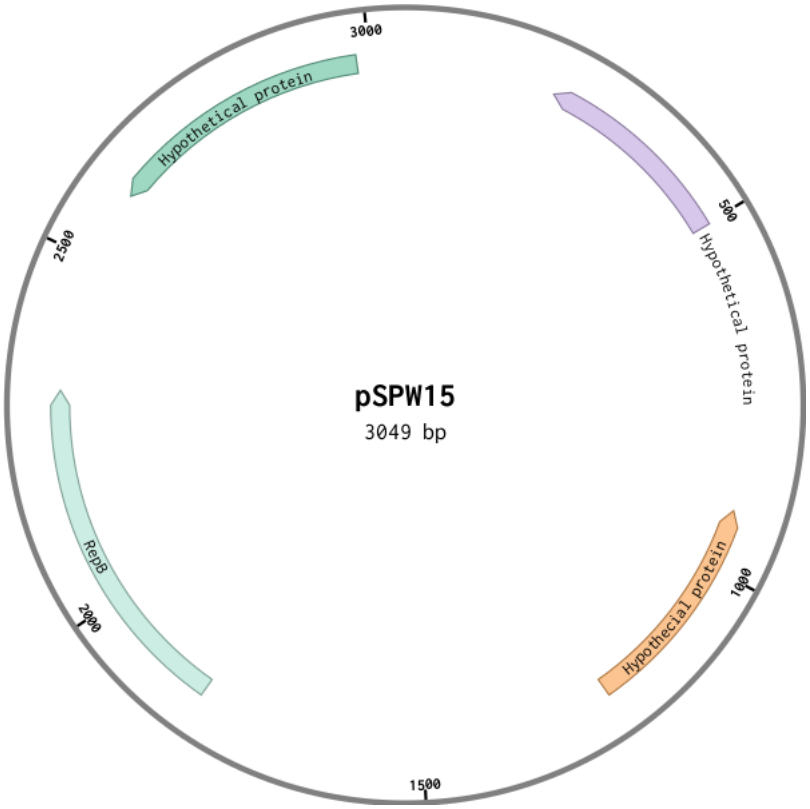


Figure 3.12: pSPW15 contained open reading frames that most closely matched sequences that are predicted to encode hypothetical proteins.

Table 3.22: The closest homologues to the ORFs identified in pSPW15 as shown in Fig. 3.12, their query cover and identity match % and the species that they originated from where applicable.

ORF Location	Closest homologue	QC%	ID%	Species
217 – 501 c	Hypothetical protein	90	100	
934 – 1227 c	Hypothetical protein	67	58	
1823 – 2308	Replication protein	86	98	*Neisseria gonorrhoeae*
2604 – 2981 c	Hypothetical protein	29	65	

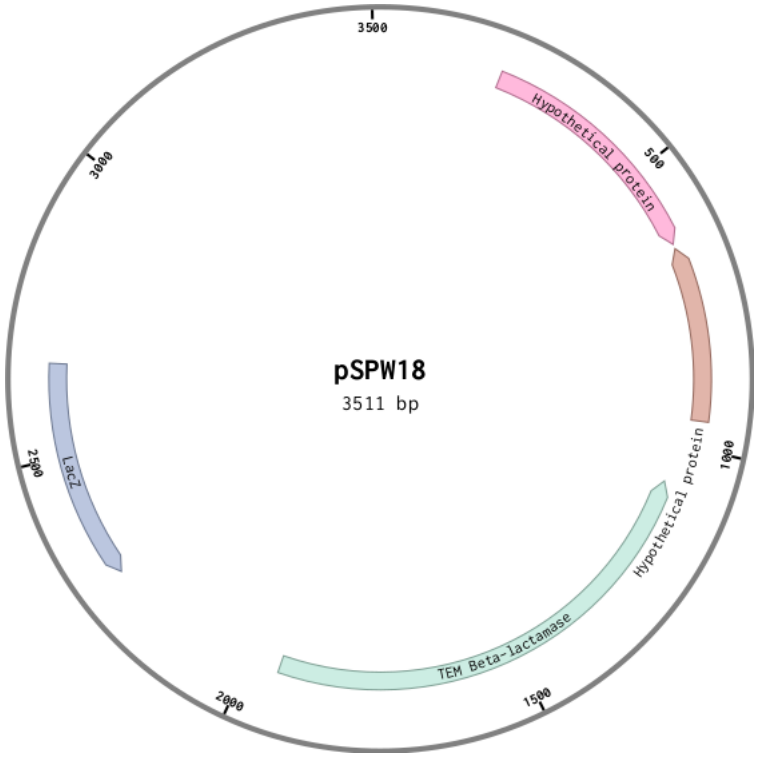


Figure 3.13: The ORFs on pSPW18 most closely resembled sequences that were predicted to encode hypothetical proteins and a TEM family β-lactamase that was shown to confer resistance to the clones. Growth on CLED agar revealed that the ORF identified as *lacZ* was not functional.

Table 3.23: The closest homologues to the ORFs identified in pSPW18 as shown in Fig. 3.13, their query cover and identity match % and the species that they originated from where applicable.

ORF Location	Closest homologue	QC%	ID%	Species
212 – 637	Hypothetical protein	98	100	*Thermoanaerobacter*
646 - 951 c	Hypothetical protein	89	100	
1071 – 1943 c	TEM β-lactamase	98	100	
2277 – 2660 c	LacZ	82	96	

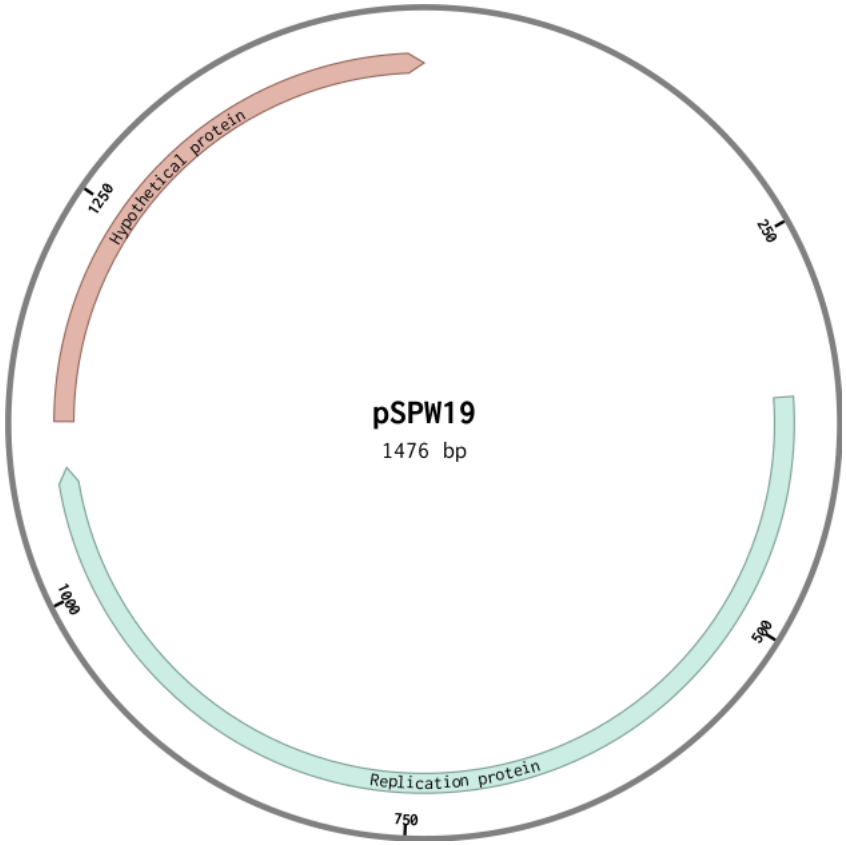


Figure 3.14: Three ORFs were detected on pSPW19, the closest homologues were sequences predicted to encode hypothetical proteins, and an origin of replication from *S. agalactiae*.

Table 3.24: The closest homologues to the ORFs identified in pSPW19 as shown in Fig. 3.14, their query cover and identity match % and the species that they originated from where applicable.

ORF Location	Closest homologue	QC %	ID %	Species
299-697 c	Hypothetical protein	85	98	
353-1078	Replication protein	96	99	*S. agalactiae*
1109-1476	Hypothetical protein	38	77	

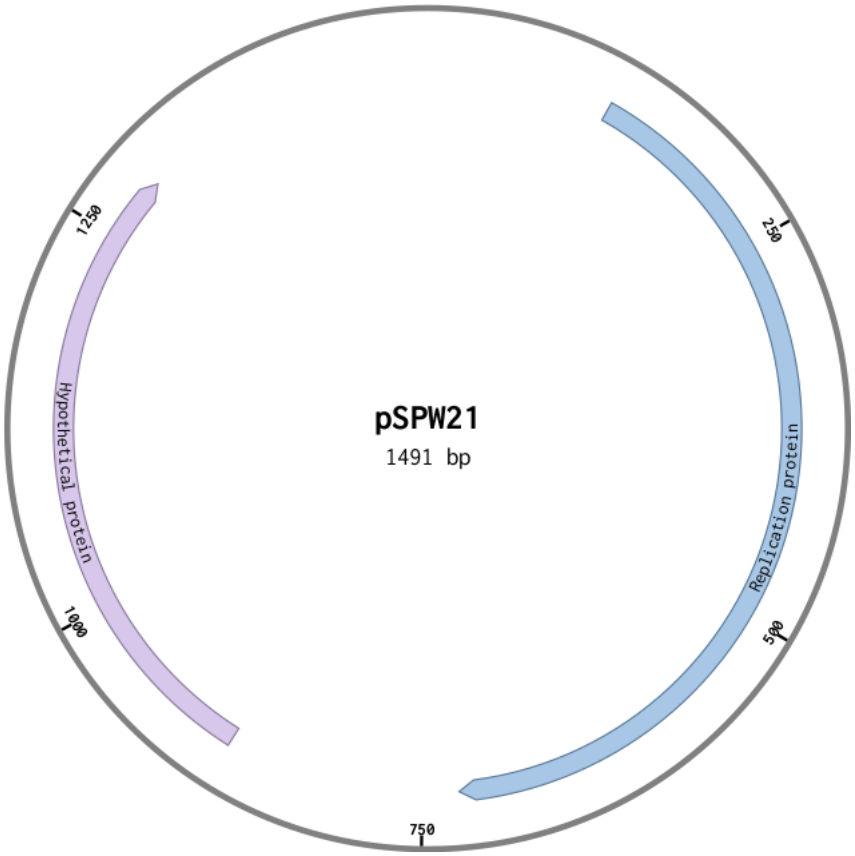


Figure 3.15: Two open reading frames were identified on pSPW21, these included an origin of replication from *S. agalactiae*. The closest match to the other ORF was a sequence predicted to encode a hypothetical protein however both the query cover and maximum identity percentages were low.

Table 3.25: The closest homologues to the ORFs identified in pSPW21 as shown in Fig. 3.15, their query cover and identity match % and the species that they originated from where applicable.

ORF Location	Closest homologue	QC%	ID%	Species
123 – 725	Replication protein	93	98	*Streptococcus agalactiae*
880 – 1293	Hypothetical protein	42	79	

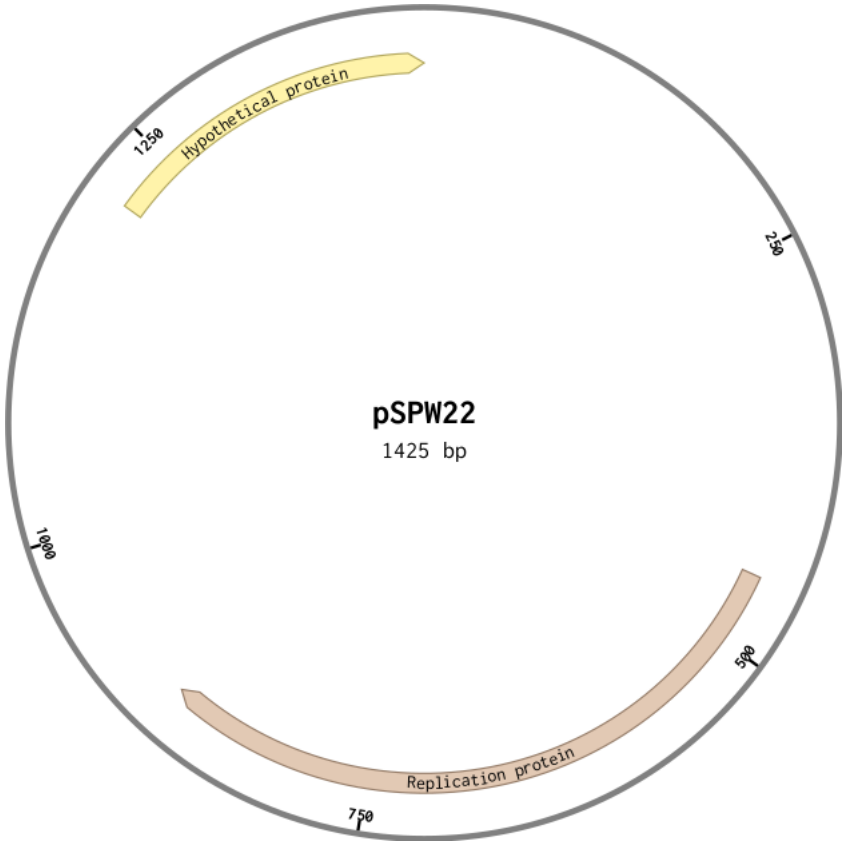


Figure 3.16: pSPW22 contained ORFs that most closely matched an origin of replication from *S. agalactiae* however the query cover is relatively low. The other closest matches were sequences predicted to encode hypothetical proteins, one of which from *S. agalactiae*. No origins of replication were detected in this sequence.

Table 3.26: The closest homologues to the ORFs identified in pSPW22 as shown in Fig. 3.16, their query cover and identity match % and the species that they originated from where applicable.

ORF Location	Closest homologue	QC%	ID%	Species
455 – 880	Replication protein	91	96	*Streptococcus agalactiae*
714 – 1181	Hypothetical protein	72	97	*Streptococcus agalactiae*
1212 – 1502	Hypothetical protein	27	96	

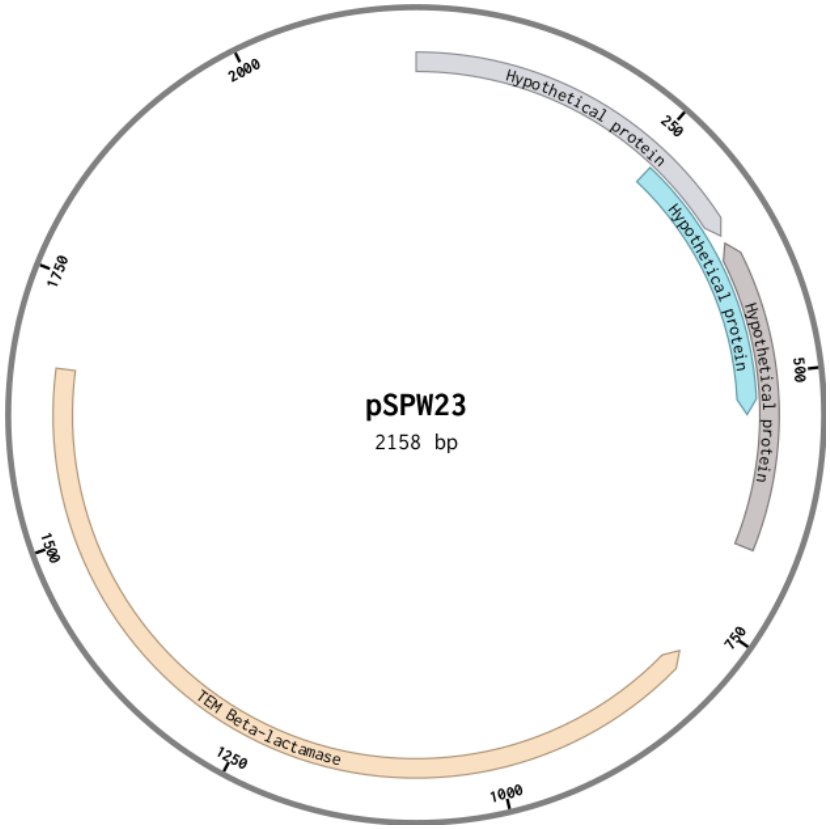


Figure 3.17: The ORFs on pSPW23 were most closely matched to sequences predicted to encode hypothetical proteins from *Erwinia amylovora* and *Escherichia coli*. A TEM familyβ-lactamase was also detected that conferred resistance to the host clone. No origin of replication was detected on this sequence.

Table 3.27: The closest homologues to the ORFs identified in pSPW23 as shown in Fig. 3.17, their query cover and identity match % and the species that they originated from where applicable.

ORF Location	Closest homologue	QC%	ID%	Species
1 – 357	Hypothetical protein	100	100	*Erwinia amylovora*
261 – 539	Hypothetical protein	91	99	*Escherichia coli*
366 – 671 c	Hypothetical protein	89	100	
791 – 1663 c	TEM β-lactamase	100	99	

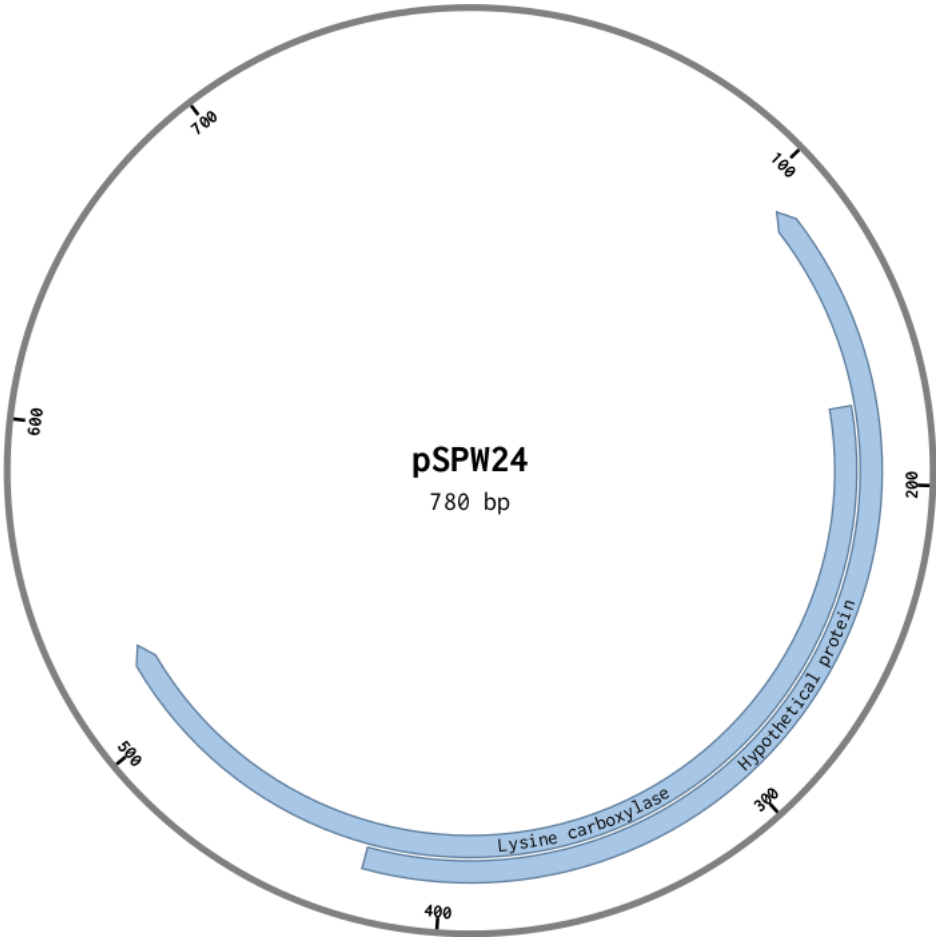


Figure 3.18: The two open reading frames on pSPW24 were most closely related to sequences predicted to encode a hypothetical protein, and lysine carboxylase. No origins of replication were detected in this sequence.

Table 3.28: The closest homologues to the ORFs identified in pSPW24 as shown in Fig. 3.18, their query cover and identity match % and the species that they originated from where applicable.

ORF Location	Closest homologue	QC%	ID%	Species
109-423 c	Hypothetical protein	56	98	
175-525	Lysine carboxylase	53	40	*Comamonas* spp.

3.8.1 Comparison of TEM β-lactamase Genes

The TEM class β-lactamase genes were compared using Clustal Omega alignment. The alignment shows a high degree of similarity between the resistance genes. The β-lactamase gene belongs to the transpeptidase superfamily and is identified as TEM-116, a variant of TEM-1. Clustal Omega analysis revealed that there are single nucleotide polymorphisms (SNPs) in this gene compared with TEM-1. These substitutions include a T − C substitution at position 18, a T − substitution at position 228, a G − A substitution at position 244 a T − G substitution at position 396 and a C − T substitution at position 545. The majority of these substitutions are silent having no impact upon the amino acid sequence of the encoded protein with the exception of a A - V (alanine to valine) substitution which occurs at position 182 on the protein.

3.8.2 *LacZ* Genes

Five of the plasmids captured contained ORFs of which the closest homologue in the NCBI database was *lacZ*. The functionality of this gene was determine by culturing the clones on Cystine-lactose-electrolyte-deficient agar (CLED)

agar. In all cases the *lacZ* gene appeared to be non-functional.

3.8.3 Comparison of Origins of Replication

BLAST analysis revealed known origins of replication on 4 plasmids 3 of these were identified as RepC and one as RepB, in addition putative origins of replication were identified on 5 plasmids showing similarity to the Rep 2 superfamily.

3.9 Discussion

3.9.1 TEM β-lactamase

TEM β-lactamase is a Class A β-lactamase (Cooksey *et al.*, 1990), that confers resistance to penicillins and early cephlosporins by hydrolysing the β-lactam ring (Salverda *et al.*, 2010). It is commonly observed in Gram negative bacteria. This enzyme was first observed in *E. coli* in 1965 (Datta and Kontomichalou, 1965). The presence of this antibiotic resistance gene on plasmids in oral bacteria may have an impact on treatment as penicillin antibiotics are widely used as a first line of treatment in oral infections. Over 100 unique variants of TEM *upbeta*-lactamase have been identified (Zeil *et al.*, 2016).

3.9.1.1 TEM-116

TEM-116 was first identified in the 1990s and has been described as occurring in a variety of Gram negative isolates and is present on conjugative plasmids that vary in size (Lahlaoui *et al.*, 2011; Maravić *et al.*, 2016). The defining mutations in TEM-116 are V82I and A184V (Hu *et al.*, 2007). These muta-

tions lie in chains separate from the active site. In the 1980s, $bla_{\text{TEM-1}}$ was engineered to contain these mutations in order to remove *Pst*I and *Hin*cII restriction sites (Jacoby and Bush, 2016). The sequences of some reported $bla_{\text{TEM-116}}$ genes, are identical to the synthetically constructed pUC vectors (Jacoby and Bush, 2016). This is the case with the sequences identified in this study. A potential problem arises here, as there have been commercial Taq polymerase PCR kits that were shown to have been contaminated with exogenous DNA containing $bla_{\text{TEM-116}}$ which has lead to the erroneous reporting of $bla_{\text{TEM-116}}$ in *S. pneumoniae* (Koncan *et al.*, 2007). A recent study investigating TEM β-lactamases identified over 50 derivatives from TEM-116 (Zeil *et al.*, 2016). The majority of these derivatives contain both of mutations associated with TEM-116 with the exception of TEM-171 which contains only the V82I mutation and TEM-181 which contains the A184V mutation. These two genes may be intermediate evolutionary steps between TEM-1 and TEM-116. Therefore despite the concern of erroneous reporting of TEM-116 due to contaminated reagents the centrality of TEM-116 within the TEM family network, as well as its wide geographical distribution and establishment on multiple plasmids suggest that TEM-116 is now a naturally occurring enzyme (Jacoby and Bush, 2016).

3.9.2 Chloramphenicol acetyltransferase

This is an enzyme that confers resistance to the antibiotic chloramphenicol. The gene that encodes this enzyme is often present on R-plasmids (Shaw and Cabelli, 1980). A diverse range of bacteria have been observed producing this enzyme as well as yeasts such as *Streptomyces* spp. Resistance to chloramphenicol is clinically important as chloramphenicol is listed as "essential" by the World Health Organisation (WHO). The antibiotic chloramphenicol is

able to cross the blood brain barrier effectively as such, chloramphenicol is used in the treatment of bacterial meningitis. The presence of plasmids in the oral cavity carrying this resistance gene could have serious consequences for individuals who develop bacterial meningitis, especially bacteria that typically reside in the oral cavity have the potential to cause this disease. For example, *Streptococcus tigurinus* a novel species of oral bacteria identified in 2012 is associated with invasive infections such as meningitis (Zbinden *et al.*, 2012, 2015).

3.9.3 Species of Origin

3.9.3.1 *Staphylococcus aureus*

The plasmids where an origin of replication had been identified appear to have originated in bacteria that are typically resident in the oral cavity. *Staphylococcus aureus* has long been known to reside in the oral cavity however it is often overlooked as a causal agent of oral infection (McCormack *et al.*, 2015). *Staphylococcus aureus* isolation rates vary between different populations and have been reported to range from 24% to 84% in the healthy population (Jackson *et al.*, 1999; Ohara-Nemoto *et al.*, 2008) *Staphylococcus aureus* is associated with mucosal disease that may be caused by endotoxins that are secreted by various strains (McCormack *et al.*, 2015). The presence of a variant of TEM-1 on plasmids suggests that there is a plasmid mediated reservoir of β-lactam resistance in the oral cavity. TEM-1 provides resistance to amoxicillin. Amoxicillin is the often the first treatment prescribed following dental infection, as such an amoxycillin resistant infection could effect the treatment that is prescribed. For example treatment with the later generation penicillin methicillin may be required, which would create a positive

selection pressure favouring resistance to methicillin.

Between 1998 and 2007 the prevalence of MRSA within the oral cavity was investigated, the authors found that for the period ranging between 1998-2007, across 11,312 samples. The results showed that 18% of the infections were caused by *S. aureus*, 10% of which were identified as being methicillin resistant. The disease state most commonly reported in the case of *S. aureus* was angular cheilitis, erythmea and swelling were reported in both MRSA and MSSA (McCormack *et al.*, 2015). The recovery of MRSA from oral samples taken from patients in a community rather than hospital setting reinforces that MRSA is not restricted to the typically cited body niches of the preineum, throat and nose and highlights the importance of infection control procedures for all patients (McCormack *et al.*, 2015).

The data generated in this study reveals that there is a plasmid containing a TEM-1 β-lactamase gene, however in the absence of transferability studies it is not possible to say whether this plasmid is capable of transferring between different bacteria. Rep protein analysis of pSPW05 indicates that this plasmid originated from *S. aureus*, in addition to the TEM-1 gene conferring resistance to β-lactam antibiotics, this plasmid also contains a gene encoding chloramphenicol acetyltransferase. This is of interest because a study was carried out in 2013 assessing the effectiveness of chloramphenicol in the treatment of *S. aureus* (Fayyaz *et al.*, 2013). This study tested 174 MRSA isolates for chloramphenicol susceptibility, revealing that 132 (75.86%) of the isolates were susceptible (Fayyaz *et al.*, 2013). The authors conclude that chloramphenicol has demonstrated good *in vitro* against *S. aureus* and is likely to contribute to the treatment of *S. aureus* by providing an effective alternative

to expensive antimicrobials in countries where resources are limited (Fayyaz *et al.*, 2013). The presence of a plasmid carrying genes that confer resistance to β-lactam antibiotics and chloramphenicol could present problems in such a situation by limiting the effectiveness of chloramphenicol as an alternative treatment.

3.9.3.2 *Streptococcus pneumoniae*

Analysis of the origin of replication from pSPW09 revealed that this plasmid likely originated from the species *Streptococcus pneumoniae*. *Streptococcus pneumoniae* is an opportunistic pathogen that typically resides in the upper respiratory tract such as the nasopharynx and oral cavity. *Streptococcus pneumoniae* is an opportunistic pathogen, infection is asymptomatic in healthy carriers however it can cause a variety of serious disease states in individuals who are immunocompromised. The most serious conditions that are caused by this bacteria are pneumonia, meningitis, sepsis, pericarditis, endocarditis and brain abscess (Deutscher *et al.*, 2011).

The presence of pSPW09, a plasmid from *Streptococcus pneumoniae* that confers resistance to β-lactam antibiotics and chloramphenicol is of concern because of the virulence of this species of bacteria as well as the fact that this plasmid was found in metagenomic DNA from individuals who have not taken any antibiotic treatment recently therefore suggesting that this plasmid is present within the healthy population in the absence of any obvious selection pressure thereby suggesting that this plasmid is part of the stable reservoir of mobile elements that carry antibiotic resistance genes.

3.9.3.3 *Streptococcus agalactiae*

Streptococcus agalactiae are typically resident in the oral cavity as well as the gastrointestinal tract. In the majority of cases this species is commensal however in immunocompromised individuals *Streptococcus agalactiae* can become an opportunistic pathogen.

Of the plasmids where an origin of replication was identified, the majority originated from *Streptococcus agalactiae* (n = 5). No antibiotic resistance genes were identified on the plasmids that originated from *Streptococcus agalactiae*. However there were a number of ORFs predicted to encode hypothetical proteins of unknown function, it is possible that these proteins confer a selective advantage to the host bacteria containing these plasmids.

3.9.3.4 *Neisseria gonorrhoeae*

The origin of replication of plasmid pSPW15 appears to have originated in *Neisseria gonorrheae*. *Neisseria gonorrhoeae* is a pathogenic bacteria, it is not typically resident within the oral cavity however it has been observed to cause oral infections. Other species of *Neisseria* are typical inhabitants of the oral cavity so it is possible that this plasmid was transferred to one of these species from *Neisseria gonorrhoeae*. It is also possible that the plasmid pSPW15 did not originate from *Neisseria gonorrhoeae* as the query cover in BLAST analysis was only 86%.

3.9.4 Plasmids Captured in Comparison to other TRACA Studies

3.9.4.1 Gut Metagenome

The plasmids that were captured from the human gut metagenome (Jones and Marchesi, 2007) appeared to be quite different in character to the plasmids that have been identified in this study. The plasmids identified from the gut metagenome were speculated to be from *Bacillus cereus*, *Bacillus licheniformis*, *Lactobacillus acidophilus*, *Selenomonas ruminatium*, *Bacillus thuringenesis* and *Campylobacter jejuni*.

3.9.4.2 Dental Plaque from Periodontal Patients

Another study (Warburton *et al.*, 2011), used TRACA to capture plasmids from patients who were suffering from periodontal disease. Dental plaque was sampled as opposed to saliva as in this study. The authors found a variety of plasmids that contained putative origins of replication that are likely to come from multiple species. The species that the plasmids originated within is different to those that were captured during the course of this research with the exception of *Neisseria gonorrhoeae*. Interestingly, none of the plasmids that were identified in (Warburton *et al.*, 2011) were predicted to be of Streptococcal origin as *Streptococcus* spp. in particular *Streptococcus mutans* is known to be an important participant in the development of dental plaque. It seems unusual that plasmids of streptococcal origin would be captured from saliva but not from plaque, this could be due to the difference in the DNA extraction techniques that were employed by the two studies, in this method the FastPrep (MP Bio) homogenizer is considerably more violent than the enzyme based DNA extraction that was employed on the

dental plaque samples (Warburton *et al.*, 2011). *Streptococcus* spp. have a thick cell wall that is difficult to lyse.

Of the plasmids captured, 6 belong to the pTRACA42 group that was first identified in dental plaque (Warburton *et al.*, 2011). This group of plasmids appears to be common within the oral cavity and were the most common group of plasmids captured from both dental plaque from patients (Warburton *et al.*, 2011) and saliva samples from healthy individuals. No antibiotic resistance genes have been identified on any of the plasmids that belong to this group on either study.

3.10 Conclusions

This research demonstrates that the TRACA system may be used to capture plasmids from bacteria in the oral metagenome. The presence of plasmids that contain antibiotic resistance genes in the absence of antibiotic selection pressure suggests that it is possible that there is another advantage that these plasmids are offering their bacterial hosts in addition to antibiotic resistance. It is likely that such an advantage is conferred by one of the ORFs identified. These results demonstrate that there is plasmid involvement in the maintenance of a reservoir of antibiotic resistance genes in commensal bacteria. The plasmids captured from saliva samples during this study had a greater homology to the plasmids that were captured via TRACA from the plaque from periodontal patients than those that were captured from the gut metagenome, this is not surprising due to the proximal relationship between where the samples were gathered from.

The plasmid group pTRACA42 appears to be common within the oral environment, to date this group of plasmids has not been observed in any other

setting. The pTRACA42 group appears to be both common in healthy and infected individuals, to date the pTRACA42 group have not been shown to harbour any genes that confer resistance to antibiotics.

Chapter 4

Bacteria Isolated from Swabs of Infected Wounds

4.1 Antibiotic Resistance in a Clinical Setting

4.1.1 Rationale

This section of research investigates the presence of antibiotic resistance genes and genes that are associated with mobile genetic elements in samples that have been obtained from within a clinical setting. The focus of this study is on tetracycline, erythromycin and vancomycin resistance. Additional work was carried out on methicillin regarding the isolates that were identified as *Staphylococcus aureus*.

Transposons are modular, the modules that they contain can be interchanged and replaced with other modules, therefore as the modules within the transposon are acquired and/or lost, they can acquire resistance to additional antibiotics, potentially resulting in multiple resistance genes being located

upon one mobile element.

4.1.2 Bacteria in Wounds

Bacteria that compose the skin flora often colonise wounds. Limitations in what bacterial species are culturable in the laboratory have historically led to a misrepresentation of what species are present on the skin flora with *S. aureus* and *S. epidermis* thought to be the most dominant species due to their being relatively easy to culture. Analysis of 16S rRNA has revealed that the diversity of bacterial species that inhabit the skin is greater than originally thought (Cogen *et al.*, 2008; Grice *et al.*, 2008). Wounds support communities of both anaerobic and aerobic bacteria, these bacteria may initiate infections or establish a less invasive relationship with the host (Cooper *et al.*, 2009). The development of infection in wounds typically results from either an increased host susceptibility or an increase in bacterial load or virulence (Cooper, 2005). Bacteria that frequently reside within the skin flora and their pathogenicity are shown in Table 4.1.

Should a break in the skin occur forming a wound, the bacteria belonging to the skin flora may enter the wound. The quantity of bacteria that enter the wound impacts upon infection and the healing rate of the wound, the effect of the microbial load on wound healing was first identified when healing in decubitus ulcers only progressed when the bacterial load was less than 10^6 CFU/ml of wound fluid (Bendy and Landman, 1964). As well as microbial load wound healing is also affected by the differences between the specific species present (Bowler *et al.*, 2001). Species that have been reported to have the most detrimental effect on wound healing are *S. aureus*, *P. aeuriginosa* and β-haemolytic streptococci (Bowler *et al.*, 2001; Danielsen *et al.*, 1998).

Table 4.1: Frequently studied bacteria that reside as part of the skin flora and their pathogenicity.

Species	Pathogenicity
Staphylococcus aureus	Typically pathogenic
Staphylococcus epidermis	Occasionally pathogenic
Staphylococcus warneri	Occasionally pathogenic
Streptococcus mitis	Occasionally pathogenic
Streptococcus pyogenes	Typically pathogenic
Propionibacterium acnes	Occasionally pathogenic
Pseudomonas aeuriginosa	Occasionally pathogenic
Acdenitobacter johnsonii	Occasionally pathogenic
Corynebacterium spp.	Occasionally pathogenic

4.1.2.0.1 Acute vs Chronic Wounds The bacterial species that colonise wounds tends to differ based on the length of time that the wound has been present, the early colonisers tend to be Gram positive bacteria that are able to grow in aerobic conditions such as *Staphylococcus* and *Streptococcus*. As time progresses anaerobic bacteria also colonise the wound, these are typically acquired from exogenous sources such as bathwater (Dow *et al.*, 1999). Chronic wounds, such as ulcers provide a polymicrobial environment where the exchange of genetic information is able to take place between bacteria. This creates conditions whereby the transfer of a resistance gene to a new species is more favourable, for example the first two cases of *S. aureus* resistant to vancomycin were isolated from chronic wound patients (Howell-Jones *et al.*, 2005). A recent study investigating the bacteria that are found in wounds observed that the most common species present is *S. aureus*, fol-

lowed by *P. aeruginosa* (Wong *et al.*, 2015).

4.1.3 Overview of Study

136 bacterial isolates have been received from different anatomical sites (Table 4.4 and 4.5), the isolates that were suspected to belong to the streptococcal or staphylococcal species were initially screened for resistance to the antibiotics tetracycline and erythromycin at concentrations of 4μg/ml, those isolates that were resistant to at least one of these antibiotics were taken forward for further analysis. These isolates are presented in Table 4.6. The isolates that were suspected of being enterococci were screened for resistance to the antibiotic vancomycin at a concentration of 4μg/ml. Vancomycin resistant isolates were taken forward for further analysis, these isolates are also presented in Table 4.6.

4.2 Methods

4.2.1 Inverse PCR

The DNA was digested with the restriction enzyme *Bam*HI, this enzyme was selected because it does not cut the *tetW* gene (Table 4.2). Following digestion The fragments were ligated (Table 4.3) to produce circular DNA. Primers facing out off the end of the *tetW* gene were used to amplify the regions which flank the gene, the primer sequences were tetW Fx: CTC TTA CAC CAA CGG GCA GAG and tetW Rx: TGG CAT ATA GCA GGC TCT CCG (this study).

Table 4.2: *Bam*HI digestion reaction, the reaction mix was incubated at 37°C for 15 minutes.

Reagent	Volume (µl)	Stock Conc.	Final Conc.
Buffer	1.5	10x	1x
BamHI	1	10U/µl	0.67U/µl
DNA	10	variable	variable
ddH_2O	2.5	–	–
Total	15	–	–

Table 4.3: Ligation reaction for the circularisation of digested DNA, the mix was incubated at 25°C for 15 minutes.

Reagent	Volume (µl)	Stock Conc.	Final Conc.
ddH_2O	8	–	–
Buffer	10	2x	1x
DNA	1	variable	variable
T4 ligase	1	3U/µl	0.15U/µl
Total	20	–	–

4.2.2 Species Received from Queen Elizabeth Hospital

The wounds of patients being treated at the Queen Elizabeth hospital were swabbed and the bacteria from the swab cultured. These cultures were transferred to agar slopes and transported to the laboratory for analysis.

Table 4.4: Anatomical site of isolation and strain species information obtained via biochemical testing carried out by the Queen Elizabeth hospital.

Number	Species	Date collected	Site collected
QE01	*Staphylococcus aureus*	07/01/13	Groin swab
QE02	*Staphylococcus aureus*	07/01/13	Leg swab
QE03	*Staphylococcus aureus*	07/01/13	Thumb swab
QE04	*Staphylococcus aureus*	07/01/13	Toe swab
QE05	*Staphylococcus aureus*	07/01/13	Genital swab
QE06	*Staphylococcus aureus*	07/01/13	Wound swab - site not stated
QE07	*Staphylococcus aureus*	07/01/13	Wound swab left thigh
QE08	*Staphylococcus aureus*	07/01/13	Ulcer swab
QE09	*Staphylococcus aureus*	07/01/13	Wound swab foot
QE10	*Staphylococcus aureus*	07/01/13	Wound swab leg
QE11	*Staphylococcus aureus*	07/01/13	Wound swab arm
QE12	*Staphylococcus aureus*	08/01/13	Swab ear
QE13	*Staphylococcus aureus*	08/01/13	Wound swab knee
QE14	*Staphylococcus aureus*	08/01/13	Swab shin
QE15	*Staphylococcus aureus*	09/01/13	Swab umbilical cord
QE16	*Staphylococcus aureus*	09/01/13	Nephrostomy
QE17	*Staphylococcus aureus*	09/01/13	Wound swab line site
QE18	*Staphylococcus aureus*	09/01/13	Wound swab pilonidal
QE19	*Staphylococcus aureus*	09/01/13	Wound swab forearm
QE20	*Staphylococcus aureus*	09/01/13	Wound swab abdomen
QE21	*Staphylococcus aureus*	09/01/13	Ulcer swab foot
QE22	*Staphylococcus aureus*	08/01/13	Wound swab toe
QE23	*Staphylococcus aureus*	08/01/13	Wound swab finger

Number	Species	Date collected	Site collected
QE24	*Staphylococcus aureus*	08/01/13	Ulcer swab
QE25	*Staphylococcus aureus*	08/01/13	Genital swab penis
QE26	*Staphylococcus aureus*	10/01/13	Wound swab - site not stated
QE27	*Staphylococcus aureus*	09/01/13	Throat swab
QE28	*Staphylococcus aureus*	09/01/13	Head
QE29	*Staphylococcus aureus*	10/01/13	Ear swab
QE30	*Staphylococcus aureus*	10/01/13	Sputum
QE31	*Staphylococcus aureus*	10/01/13	Genital swab high vaginal
QE32	*Staphylococcus aureus*	10/01/13	Ulcer swab ankle
QE33	*Staphylococcus aureus*	10/01/13	Ulcer swab shin
QE34	*Staphylococcus aureus*	10/01/13	Swab arm
QE35	*Staphylococcus aureus*	10/01/13	Leg
QE36	*Staphylococcus aureus*	12/11/12	Urine
QE37	*Streptococcus* group A	13/11/12	Swab throat
QE38	*Streptococcus* group A	16/11/12	Wound swab
QE39	*Streptococcus* group A	11/01/13	Throat swab
QE40	*Streptococcus* group B	11/01/13	Genital swab high vaginal
QE41	*Streptococcus* group G	12/01/13	Pressure sore swab
QE42	*Streptococcus* group A	08/01/13	Genital swab high vaginal
QE43	*Streptococcus* group A	07/01/13	Throat swab
QE44	*Streptococcus* group A	07/01/13	Genital swab vulval
QE45	*Streptococcus* group B	11/11/12	Blood culture
QE46	*Streptococcus* group C	18/11/12	Blood culture
QE47	*Staphylococcus aureus*	28/11/12	Blood culture
QE48	*Streptococcus* group B	08/12/12	Blood culture
QE49	*Streptococcus* group B	11/12/12	Blood culture

Number	Species	Date collected	Site collected
QE50	*Staphylococcus aureus*	09/01/13	Nose swab
QE51	*Streptococcus* group A	07/01/13	Throat swab
QE52	*Streptococcus* group A	07/01/13	Ear swab
QE53	*Streptococcus* group A	07/01/13	Wound swab
QE54	*Streptococcus* group A	03/01/13	Genital swab high vaginal
QE55	*Staphylococcus aureus*	12/01/13	Would swab sacrum
QE56	*Staphylococcus aureus*	12/01/13	Wound swab Groshong line site
QE57	*Streptococcus* group A	19/12/12	Wound swab perianal
QE58	*Streptococcus* group A	27/12/12	Wound swab left arm
QE59	*Streptococcus* group A	17/12/12	Throat swab
QE60	*Streptococcus* group A	17/12/12	Ear swab
QE61	*Streptococcus* group A	18/12/12	Ear swab
QE62	*Streptococcus* group A	18/12/12	Throat swab
QE63	*Streptococcus* group A	11/12/12	Throat swab
QE64	*Streptococcus* group A	13/12/12	Throat swab
QE65	*Streptococcus* group A	20/12/12	Gastrostomy site swab
QE66	*Streptococcus* group A	20/12/12	Throat swab
QE67	*Streptococcus* group A	06/12/12	Foreskin swab
QE68	*Streptococcus* group A	07/12/12	Genital swab penis
QE69	*Staphylococcus aureus*	07/12/12	Sputum
QE70	*Staphylococcus aureus*	07/12/12	Nose swab
QE71	*Streptococcus* group A	28/11/12	Nose swab
QE72	*Streptococcus* group A	30/11/12	Ear swab
QE73	*Streptococcus* group A	29/11/12	Throat swab
QE74	*Streptococcus* group A	04/12/12	Sub-phrenic swab
QE75	*Streptococcus* group A	26-/11/12	Ear swab

Number	Species	Date collected	Site collected
QE76	*Streptococcus* group A	26/11/12	Throat swab
QE77	*Streptococcus* group A	27/11/12	Pus swab
QE78	*Streptococcus* group A	26/11/12	Throat swab
QE79	*Streptococcus* group A	22/11/12	Wound swab
QE80	*Streptococcus* group A	22/11/12	Nose swab
QE81	*Streptococcus* group A	23/11/12	Ear swab
QE82	*Staphylococcus aureus*	14/01/13	Breast aspirate
QE83	*Staphylococcus aureus*	14/01/13	Wound swab
QE84	*Staphylococcus aureus*	14/01/13	Ulcer swab
QE85	*Staphylococcus aureus*	14/01/13	Wound swab leg
QE86	*Staphylococcus aureus*	14/01/13	Ulcer swab toe
QE87	*Streptococcus* group A	14/01/13	Throat swab
QE88	*Streptococcus* group A	14/01/13	Ear swab
QE89	*Streptococcus* group B	14/01/13	PEG site swab
QE90	*Streptococcus* group G	14/01/13	Sputum
QE91	*Streptococcus* group G	14/01/13	Wound swab leg
QE92	*Streptococcus* Group B	16/05/2013	Swab leg
QE93	*Streptococcus* Group G	20/05/2013	Wound swab leg
QE94	*Streptococcus* Group G	09/05/2013	Wound swab leg
QE95	*Streptococcus* Group G	10/05/2013	Wound swab leg
QE96	*Streptococcus* Group B	08/05/2013	Genital swab high vaginal
QE97	*Streptococcus* Group G	20/05/2013	Wound swab
QE98	*Streptococcus* Group B	07/05/2013	Genital swab high vaginal
QE99	*Streptococcus* Group G	16/05/2013	Ulcer swab left leg
QE100	*Streptococcus mitis/oralis*	13/05/2013	Blood culture
QE101	*Streptococcus* Group G	09/05/2013	Ulcer swab right hallux

Number	Species	Date collected	Site collected
QE102	*Streptococcus* Group B	08/05/2013	Swab ear
QE103	*Streptococcus* Group B	14/05/2013	Genital swab high vaginal
QE104	*Streptococcus* Group G	17/05/2013	Blister swab ankle
QE105	*Streptococcus* Group G	13/05/2013	Ulcer swab leg
QE106	*Streptococcus* Group G	07/05/2013	Ulcer swab ankle
QE107	*Streptococcus* Group G	07/05/2013	Ulcer swab toe
QE108	*Streptococcus milleri*	13/05/2013	Genital swab
QE109	*Streptococcus* Group G	13/05/2013	Genital swab high vaginal
QE110	*Streptococcus* Group A	13/05/2013	Wound swab
QE111	*Streptococcus* Group A	15/05/2013	Wound swab ankle
QE112	*Streptococcus* Group G	17/05/2013	Ulcer swab ankle
QE113	*Enterococcus* sp.	08/01/2013	Faeces
QE114	*Enterococcus* sp.	12/12/2012	Faeces
QE115	*Enterococcus* sp.	06/12/2012	Faeces
QE116	*Enterococcus* sp.	17/03/2013	Faeces
QE117	*Enterococcus* sp.	06/02/2013	Faeces
QE118	*Enterococcus* sp.	19/01/2013	Faeces
QE119	*Enterococcus* sp.	07/01/2013	Faeces
QE120	*Enterococcus* sp.	12/12/2012	Faeces
QE121	*Enterococcus* sp.	16/03/2013	Faeces
QE122	*Enterococcus faecium*	27/09/2012	Mid stream urine
QE123	*Enterococcus* sp.	30/01/2013	Faeces
QE124	*Enterococcus* sp.	17/02/2013	Faeces
QE125	*Enterococcus* sp.	02/11/2012	Faeces
QE126	*Enterococcus* sp.	13/05/2013	Faeces
QE127	*Enterococcus* sp.	18/05/2013	Faeces

Number	Species	Date collected	Site collected
QE128	*Enterococcus* sp.	03/04/2013	Faeces
QE129	*Enterococcus* sp.	03/04/2013	Faeces
QE130	*Enterococcus* sp.	11/05/2013	Faeces
QE131	*Enterococcus* sp.	13/05/2013	Faeces
QE132	*Enterococcus* sp.	06/05/2013	Faeces
QE133	*Enterococcus* sp.	06/05/2013	Faeces
QE134	*Enterococcus* sp.	04/02/2013	Faeces
QE135	*Enterococcus* sp.	22/03/2013	Faeces
QE136	*Enterococcus* sp.	25/09/2012	Faeces

4.2.3 Antibiotic Susceptibility Testing

The 136 isolates were received from Queen Elizabeth hospital (Table 4.4) in Norfolk were first screened for resistance to the antibiotics tetracycline, erythromycin and vancomycin at a concentration of 4μg/ml, as this concentration is higher than the breakpoint required by the British Society for Antimicrobial Chemotherapy (BSAC) for the bacteria to be considered clinically resistant (Table 4.6). Those isolates that were resistant to any of these antibiotics were taken forward for further analysis.

4.2.4 Determination of Species

Information regarding the species of bacteria was provided by the Queen Elizabeth hospital upon receipt of the isolates. However this information was obtained using biochemical speciation methods that are known to be less accurate than molecular methods. Therefore the isolates that were identified

as resistant were speciated by amplifying the *16S* gene by PCR,(primers: 27F AGRGTTYGATYMTGGCTCAG, 1391R GACGGGCGGTGWGTRCA, 94°*C* for 5 minutes, then 34 cycles of 94°*C* for 30 seconds, 52°C for 30 minutes, 72°C for 30 seconds followed by a final extension of 72°C for 5 minutes) then sequencing this gene and comparing it to the BLAST nucleotide database. Those isolates that were identified as belonging to the genus *Streptococcus* were also identified via sequence analysis of the superoxide dismutase A gene (*sodA*), as this gene provides more accurate species data than *16S* in the case of *Streptococcus* (Poyart *et al.*, 1997). The species data is represented in Table 4.6.

4.2.5 Gene Detection

Polymerase chain reaction (PCR) was employed to detect the presence of genes linked to antibiotic resistance or mobile genetic elements. PCRs for the following genes were carried out: *tetM*, *tetO*, *tetK*, *tetL*, *tet32*, *ermA*, *ermB*, *ermC*, *mefA*, *mphA*, *ereA*, *ereB*. Representative isolates from positive PCRs were sequenced and the sequence data subsequently compared to the NCBI database in order to confirm that the PCR reactions have amplified the correct sequence.

4.3 Results

4.3.1 Anatomical Sites where Isolates were Obtained

Table 4.5: This table shows the anatomical sites where the strains were isolated from, it also shows the number of isolates that are resistant to tetracycline, erythromycin and vancomycin.

Site Identified	Number of Isolates	Tet R	Erth R	Tet & Erth R	Van R	Total R
Leg	11	2	1	3	–	6
Groin	1	–	–	–	–	–
Thumb	1	–	–	–	–	–
Toe	4	1	–	–	–	1
Genital (total)	12	–	–	–	–	–
vaginal	8	3	1	1	–	5
penis	2	–	1	–	–	1
not specified	2	–	–	–	–	–
Foot	2	–	–	–	–	–
Wound (not specified)	5	–	–	–	–	–
Cough	1	–	–	–	–	–
Head	1	–	–	–	–	–
Ear	10	1	–	–	–	1

Site Identified	Number of Isolates	Tet R	Erth R	Tet & Erth R	Van R	Total R
Arm	4	–	–	–	–	–
Ankle	5	1	1	1	–	3
Shin	2	–	–	–	–	–
Throat	11	1	1	–	–	1
Blood culture	6	3	1	–	–	4
Nose	2	–	–	1	–	1
Sputum	3	–	1	–	–	1
Pressure sore	1	–	–	1	–	1
Sacrum	1	–	–	–	–	–
Groshong line site	1	–	–	–	–	–
Gastrostomy site	1	–	–	–	–	–
Nostril	1	–	–	–	–	–
Nephrostomy	1	–	–	1	–	1
Hallux	1	–	–	–	–	–
Faeces	23	–	–	–	20	20
Urine	2	–	–	–	1	1

Site Identified	Number of Isolates	Tet R	Erth R	Tet & Erth R	Van R	Total R
Breast	1	–	1	–	–	1
PEG site	1	1	–	–	–	1

4.3.1.1 Anatomical Sites and Antibiotic Resistance

Antibiotic resistance testing revealed that 25 isolates (18%) were resistant to tetracycline, 19 isolates (14%) were resistant to erythromycin and 21 isolates (15%) were resistant to vancomycin.

Strains that displayed resistance to the antibiotics that they were tested against in this study were most commonly isolated from the legs (n = 11), vagina (n = 5) and faeces (n = 20). The resistant bacteria that were isolated from the legs included a diverse range in bacterial species composed of *Streptococcus pyogenes*, *Corynebacterium striatum*, *Streptococcus dysgalactiae*, *Staphylococcus simulans* and *Staphylococcus aureus*.

4.3.2 Isolate Data

Table 4.6: Table showing *16S* and *sodA* DNA sequencing speciation results, QC = query cover, ID = maximum identity, resistance to antibiotics tetracycline (tet) erythromycin (erth) and vancomycin (van) and positive PCR results. "✓" = positive result, "-" = negative result (not resistant/PCR did not amplify).

Sample ID	Closest Homologue	QC%	ID%	Tet resistant	Erth resistant	Van resistant	Positive PCR Reactions
QE16	*Staphylococcus aureus*	100%	100%	✓	✓	-	*int*, *xis*, *tetM*, *ermA*
QE25	*Staphylococcus aureus*	99%	99%	-	✓	-	*int*, *xis*, *ermC*
QE28	*Staphylococcus aureus*	100%	100%	✓	✓	-	*int*, *xis*, *tetM*, *ermA*
QE40	*Streptococcus agalactiae*	99%	99%	✓	✓	-	*ermB*, *tetM*, tetO, RPP
QE41	*Streptococcus dysgalactiae*	99%	99%	✓	✓	-	RPP
QE42	*Staphylococcus hominis*	99%	99%	-	✓	-	*int*, *xis*
QE45	*Streptococcus agalactiae*	100%	100%	✓	-	-	*tetM*, *int*, *xis*
QE47	*Staphylococcus aureus*	98%	97%	-	✓	-	*ermC*
QE48	*Streptococcus agalactiae*	100%	100%	✓	-	-	*int*, *xis*, *tetM*
QE49	*Streptococcus agalactiae*	97%	93%	✓	✓	-	*ermB*, *tetM*, *ermC*, *int*, *xis*
QE50	*Staphylococcus aureus*	98%	99%	-	✓	-	*ermC*, *int*, *xis*
QE59	*Staphylococcus hominis*	100%	100%	-	✓	-	*ermC*, *int*, *xis*

Sample ID	Closest Homologue	QC%	ID%	Tet resistant	Erth resistant	Van resistant	Positive PCR Reactions
QE62	-	-	-	✓	-	-	
QE69	*Staphylococcus aureus*	100%	100%	-	✓	-	*ermC*, *int*, *xis*
QE82	*Staphylococcus aureus*	99%	98%	-	✓	-	*ermA*, *int*, *xis*
QE89	*Streptococcus agalactiae*	100%	100%	✓	-	-	*int*, *xis*, *tetM*
QE91	*Streptococcus pyogenes*	100%	100%	✓	-	-	*tetM*, *int*, *xis*
QE92	*Corynebacterium striatum*	100%	99%	✓	✓	-	RPP, *tetW*
QE93	*Corynebacterium striatum*	100%	100%	✓	✓	-	RPP *tetW*
QE94	*Staphylococcus aureus*	95%	84%	-	✓	-	*ermC*
QE95	*Streptococcus dysgalactiae*	100%	100%	✓	✓	-	
QE96	*Streptococcus agalactiae*	100%	100%	✓	-	-	*tetM*, *int*, *xis*
QE97	*Streptococcus pyogenes*	100%	100%	✓	-	-	*tetM*, *int*, *xis*
QE98	*Streptococcus agalactiae*	100%	100%	✓	-	-	*tetM*, *int*, *xis*
QE99	*Streptococcus dysgalactiae*	100%	100%	✓	-	-	*tetM*, *int*, *xis*
QE102	*Streptococcus agalactiae*	100%	100%	✓	-	-	RPP, *tetM*
QE103	-	-	-	✓	-	-	RPP, *tetM*
QE104a	*Streptococcus pyogenes*	100%	99%	✓	✓	-	*tetM*, *int*, *xis*

Sample ID	Closest Homologue	QC%	ID%	Tet resistant	Erth resistant	Van resistant	Positive PCR Reactions
QE104b	*Staphylococcus simulans*	100%	100%	-	✓	-	*ermC*
QE105		-	-	✓	-	-	RPP
QE106	*Corynebacterium striatum*	99%	99%	✓	✓	-	RPP
QE107	*Streptococcus dysgalactiae*	100%	100%	✓	-	-	*tetM*, *int*, *xis*
QE110	*Streptococcus pyogenes*	100%	100%	✓	-	-	*tetM*, *int*, *xis*
QE111	*Streptococcus dysgalactiae*	100%	100%	✓	-	-	*tetM*, *int*, *xis*
QE113	*Enterococcus faecalis*	100%	100%	-	-	✓	*vanA*
QE114	*Enterococcus faecalis*	100%	100%	-	-	✓	*vanA*
QE115	*Enterococcus faecalis*	99%	99%	-	-	✓	*vanA*
QE116	*Enterococcus faecalis*	99%	99%	-	-	✓	*vanA*
QE117	*Enterococcus faecalis*	100%	100%	-	-	✓	*vanA*
QE118	*Enterococcus faecalis*	100%	100%	-	-	✓	*vanA*
QE119	*Enterococcus faecalis*	99%	99%	-	-	✓	*vanA*
QE120	*Enterococcus faecalis*	100%	100%	-	-	✓	*vanA*
QE121	*Enterococcus faecalis*	99%	99%	-	-	✓	*vanA*
QE122	*Enterococcus faecalis*	99%	99%	-	-	✓	*vanA*

Sample ID	Closest Homologue	QC%	ID%	Tet resistant	Erth resistant	Van resistant	Positive PCR Reactions
QE123	*Enterococcus faecalis*	99%	99%	-	-	✓	*vanA*
QE124	*Enterococcus faecalis*	100%	100%	-	-	✓	*vanA*
QE125	*Enterococcus faecalis*	100%	100%	-	-	✓	*vanA*
QE126	*Enterococcus faecalis*	99%	99%	-	-	✓	*vanA*
QE127	*Enterococcus faecalis*	100%	100%	-	-	✓	*vanA*
QE128	*Enterococcus faecalis*	99%	99%	-	-	✓	*vanA*
QE129	*Enterococcus faecalis*	99%	99%	-	-	-	
QE130	*Enterococcus faecalis*	100%	100%	-	-	✓	*vanA*
QE131	*Enterococcus faecalis*	98%	99%	-	-	✓	*vanA*
QE132	*Enterococcus faecalis*	99%	99%	-	-	✓	*vanA*
QE133	*Enterococcus faecalis*	100%	100%	-	-	✓	*vanA*
QE134	*Enterococcus faecalis*	100%	100%	-	-	✓	*vanA*
QE135	*Enterococcus faecalis*	100%	100%	-	-	✓	*vanA*
QE136	*Enterococcus faecalis*	98%	98%	-	-		

Table 4.7: Distribution of species by anatomical site.

	Penis	Head	Blood	Sputum	Nose	Breast	Leg	Kidney
S.aureus	1	1	1	1	1	1	1	1
S. agalactiae	Vaginal	PEG Site	Ear	Blood				
	3	1	1	3				
S. dysgalactiae	Pressure sore	Leg	Ankle					
	1	3	1					
S. pyogenes	Leg	Wound	Ankle	Vaginal				
	1	1	1	1				
C. striatum	Calf	Leg	Ankle					
	1	1	1					
S. hominis	Throat	Vaginal						
	1	1						
S. simulans	Ankle							
	1							

4.3.2.1 Confirmation of PCR Results via Sequencing

Sanger sequencing was performed on PCR product from representative isolates that were positive for the different genes in order to confirm that the PCR results were genuine. The isolates selected as representatives and the DNA sequencing data are shown in Table 4.8. Following sequencing, these isolates were used as positive controls in PCRs specific for their respective genes. No template controls (NTCs) were used as negative controls.

Table 4.8: Sanger sequencing results of PCR product from representative isolates where amplification had taken place using PCR primers that are specific for resistance genes. These results confirm that PCR product amplified was the target gene. QC refers to query cover, ID refers to the percentage identity match of the sequence to the closest homologue in the NCBI database.

ID	Closest Homologue	QC%	ID%
QE16	*ermA*	100	99
QE40	*ermB*	99	100
QE25	*ermC*	100	98
QE16	*int* and *xis*	100	100
QE40	*tetO*	99	100
QE113	*vanA*	99	100

4.3.3 Distribution of genes

The *int* (integrase) and *xis* (excisionase) genes were detected in 11 isolates, in 100 % of cases these isolates also contained antibiotic resistance genes 9 tetracycline (*tetM*), 2 tetracycline and erythromycin, (*tetM* and *ermA*). Phenotypic resistance was observed in 100 % of the isolates where resistance determinants were detected.

tetM was the most abundant tetracycline resistance gene, detected in 2 (25%) of *Staphylococcus aureus* isolates, 1 (33%) *Corynebacterium striatum* isolate, 7 (87.5%) *Streptococcus agalactiae* isolates 5 (100%) *Streptococcus dysgalactiae* and 4 (100%) *Streptococcus pyogenes* isolates.

4.3.4 Tetracycline Resistance Genes

4.3.4.1 *tetM*

The gene *tetM* was the most commonly observed tetracycline resistance gene that was observed in the tetracycline resistant isolates (76%). This gene was observed in a wide variety of species. In 84% of the isolates containing *tetM*, the *int* and *xis* genes were also identified, revealing the possibility that in these cases, the *tetM* gene may have been acquired on an element that is related to Tn*916*.

4.3.4.2 *tetO*

tetO was observed in a single isolate of *Streptococcus dysgalactiae* that was isolated from a vaginal swab. This isolate tested positive for both *tetO* and *tetM*. The MIC for this isolate was 32μg/ml, as this isolate contains both *tetO* and *tetM* it is not possible to determine from the MIC which (if not both) of these tetracycline resistance genes are active and responsible for conferring resistance to tetracycline in the case of this isolate.

4.3.5 Speciation

4.3.5.1 Accuracy of Biochemical Speciation Test vs *16S* or *sodA* Sequencing

The pathology laboratory at the Queen Elizabeth hospital carried out biochemical testing in order to determine the species of the strains that were received. DNA sequencing of the *16S* or *sodA* genes in the case of streptococci was carried out in order to verify any bacterial species data. The data that was supplied regarding the biochemical testing has been compared with the data obtained via DNA sequence analysis in order to determine the ac-

curacy of the biochemical testing. Strains identified as *S. aureus* were agreed by both the biochemical and molecular testing methods in 100% of the cases. Group B Streptococci (*Streptococcus agalactiae* identification agreement was achieved in 100% of the cases).

4.3.6 MIC Data

Table 4.9: Minimum inhibitory concentrations of tetracycline and erythromycin required to prevent growth of the bacterial isolates from wounds. The dashes in this table refer to isolates that were sensitive to the antibiotic and displayed no evidence of resistance.

ID	Tetracycline (μg/ml)	Erythromycin (μg/ml)
QE16	34	>256
QE25	-	>256
QE28	64	>256
QE40	32	>256
QE41	4	-
QE42	-	8
QE45	16	-
QE47	-	>256
QE48	16	-
QE49	32	>256
QE50	-	>256
QE59	-	2
QE62	32	-
QE69	-	>256
QE82	-	>256
QE89	16	-
QE91	4	-
QE92	32	-
QE93	32	-
QE94	-	>256

ID	Tetracycline (μg/ml)	Erythromycin (μg/ml)
QE96	32	-
QE97	4	-
QE98	8	-
QE99	8	-
QE102	8	-
QE103	8	-
QE104a	4	>256
QE104b	-	8
QE105	4	-
QE106	16	>256
QE107	8	-
QE110	4	-
QE111	4	-

4.3.7 Antibiotic Resistance Genes

The following antibiotic resistance genes were identified via PCR *ermA* (n = 3) *ermB* (n = 2), *ermC* (n = 6). *ermC* was the most commonly observed erythromycin resistance determinant, it was observed in *Staphylococcus aureus* (n = 5), *Staphylococcus simulans* (n = 1) and *Streptococcus hominis* (n = 1). Erythromycin resistance determinants *ermA* and *ermB* were less common, with *ermA* being observed in *Staphylococcus aureus* (n = 3) and *Steptococcus agalactiae*.In the case of 6 erythromycin resistant isolates, all of the erythromycin resistance genes that were tested for failed to amplify. The PCR reactions were negative for the resistance genes *tetK*, *tetL*, *tet32*, *mphA*, *mefA*, *ermA*, *ermB*. The species and resistance genes are presented in

Table 4.10. A representative of each gene was analysed by Sanger sequencing (Table 4.8) and the sequence compared to the NCBI database in order to confirm whether the PCR data was accurate. These representatives were used as positive controls in the PCRs, NTCs were used as negative controls.

4.3.8 Evidence of Transposable Elements

As a preliminary test for the presence of mobile genetic elements, PCR was carried out amplifying the integrase and excisionase genes that are are associated with a variety of different transposons, the data is shown on Table 4.10. The integrase and excisionase genes were widespread amongst the isolates. The *int&xis* PCR reaction was positive in isolates of *Staphylococcus aureus* (n = 6), *Staphylococcus aureus* (n = 2), *Streptococcus dysgalactiae* (n = 5), *Streptococcus agalactiae* (n = 8) and *Streptococcus pyogenes* (n = 2).

4.3.8.1 Integrase and Excisionase

The PCRs that were carried out amplifying the *int* and *xis* genes that were originally detected on the conjugative transposon Tn*916*. The integrase and excisionase enzymes are involved with the mobility of the element. The excisionase protein is encoded by the *xis* gene, the integrase protein is encoded by the *int* gene. Integrase is a heterobivalent recombinase that is regulated by excisionase.

Excisionase has a modest stimulating effect upon excision compared with the $^{\lambda}$,Xis from bacteriophage λ (Abbani *et al.*, 2005). The integrase protein is able to bridge the core-type and DR2-type sites on the left arm of the transposon. The binding of excisionase does not alter the stability of the DR2-integrase-core bridges or the ability of integrase therefore the regulation effect of excisionase on integrase is loose (Abbani *et al.*, 2005).

Table 4.10: The distribution of tetracycline and erythromycin resistance determinants and integrase/excisionase genes that were detected by species.

Species	*tetM* (n)	*tetO* (n)	*tetO* (n)	*tetW* (n)	*ermA* (n)	*ermB* (n)	*ermC* (n)	*int* and *xis* (n)
Staphylococcus aureus	2			-	3	-	5	6
Staphylococcus hominis	-	-	-	-	-	-	1	2
Staphylococcus simulans	-	-	-	-	-	-	1	-
Corynebacterium striatum	1	-	-	2	-	-	-	-
Streptococcus agalactiae	8	1	1	-	-	1	-	5
Streptococcus dysgalactiae	5	-	-	-	-	-	-	1
Streptococcus pyogenes	4	-	-	-	-	-	-	4
Species	*tetM* (%)	*tetO* (%)	*tetW* (%)	*ermA* (%)	*ermB* (%)	*tetW* (%)	*ermC* (%)	*int* and *xis* (%)
Staphylococcus aureus	25	-	-	37.5	-	-	62.5	75
Staphylococcus hominis	-	-	-	-	-	-	50	100
Staphylococcus simulans	-	-	-	-	-	-	100	-
Corynebacterium striatum	33	-	66	-	-	66	-	-
Streptococcus agalactiae	100	12.5	-	-	12.5	-	-	62.5
Streptococcus dysgalactiae	100	-	-	-	-	-	-	20
Streptococcus pyogenes	100	-	-	-	-	-	-	100

4.3.9 Species Detected

4.3.9.1 *Staphylococcus hominis*

S. hominis was only detected in two of the resistant wound isolates. This is as expected, as this species is commensal only causing infection in individuals who are immunocompromised. In both cases these isolates are resistant to erythromycin and sensitive to tetracycline. MICs were lower in the case of these two isolates than most of the other erythromycin resistant isolates. 50% (n = 1) of the erythromycin resistant isolates contained the erythromycin resistant determinant *ermC*, this determinant has been detected in this species previously and is frequently present (Aslantaş *et al.*, 2013).

4.3.9.2 *Streptococcus agalactiae*

Streptococcus agalactiae is a group B streptococci. This species is an opportunistic pathogen that is usually carried by asymptomatic individuals. Of the strains that were received were identified as Group B streptococci, 100% (n = 8) were resistant to tetracycline and 25% (n = 2) were resistant to erythromycin. All tetracycline resistant *S. agalactiae* isolates contained the determinant *tetM* which is consistent with the common occurrence of *tetM* in this species according to the literature (Fischer *et al.*, 2013), one of these isolates also contained *tetO*. All erythromycin resistant isolates contained *ermB*.These findings are consistent with other reports investigating tetracycline and erythromycin resistance determinants as *tetM*, *tetO* and *ermB* have all been frequently detected in *S. agalactiae*, the frequency of distribution between *tetM* and *tetO* that has been observed in this study is also consistent with previous reports where although both detected, *tetM* is far more common (Dutra *et al.*, 2014; Dogan *et al.*, 2005; Gherardi *et al.*, 2007;

Bergal *et al.*, 2015). The gene *ermB* is also a common resistance gene found in *S. agalactiae* (Lee and Lai, 2015).

4.3.9.3 *Streptococcus dysgalactiae*

S. dysgalactiae is a group C streptococci. Of the strains received were identified as Group C streptococci,5 were resistant to tetracycline and 2 were resistant to erythromycin. The determinant *tetM* was detected in 100% of the tetracycline resistant isolates, *tetM* is a commonly observed resistance gene in *S. dysgalactiae* (Abdelsalam *et al.*, 2013; Hashikawa *et al.*, 2004; Burdett *et al.*, 1982). The determinant that was responsible for conferring resistance to erythromycin in two of the isolates was not detected.

4.3.9.4 *Streptococcus pyogenes*

S. pyogenes is a pathogenic species of bacteria that is the cause of Group A streptococcal infections. *S. pyogenes* may cause a variety of different disease conditions, these range from skin and throat infections to systemic diseases such as endocarditis or meningitis. In this study, *S. pyogenes* / Group A Streptococci was identified in 4 isolates, all 4 of which were resistant to tetracycline and 1 of which were resistant to erythromycin. Isolates showing phenotypic resistance to tetracycline all tested positive for the resistance determinant *tetM*, a gene that frequently present in tetracycline resistant *S. pyogenes* (Perez-Trallero *et al.*, 2007).

4.3.9.5 *Enterococcus faecalis*

The species *E. faecalis* was originally classified as *Streptococcus faecalis* or "Group D Streptococci". All but two of the isolates were observed to be resistant to vancomycin (Results in Table 4.6 the negative control *B. subtilis*

CU2189 was used, no positive control was available however the presence of the *vanA* gene was confirmed by DNA sequencing of product from QE113 (Table 4.8)). These because bacteria were not screened for resistance to tetracycline or erythromycin because resistance to these antibiotics is so common that they are rarely considered for treatment of enterococcal infections. Enterococci resistant to vancomycin first emerged in the mid 1980's.

4.3.9.6 *Staphylococcus aureus*

The following QE isolates were identified as being *S. aureus*: QE16, QE25, QE28, QE47, QE69 and QE82 (Table 4.6). These isolates were screened for methicillin resistance in accordance with the British Society for Antimicrobial Chemotherapy (BSAC) guidelines by being cultured on ISO-sensitest agar containing 4μg/ml cefoxitin. Isolates QE16, QE28, QE47 and QE69 grew indicating that they were methicillin resistant (Table 4.11). The resistant isolates were tested for the presence of the *mecA* gene via PCR associated with the staphylococcal chromosomal cassette *SCCmec*. All four isolates tested positive for *mecA* suggesting that these isolates may be carrying *SCCmec* however this cannot be confirmed due to the lack of a positive control (Table 4.11).

Table 4.11: Cefoxitin susceptibility screening and *SCCmec* PCR results. *Bacillus subtlis* strain CU2189 and a no template control (NTC) were used as negative controls. A positive control was not available.

ID	Growth on Cefoxitin (4µg/ml)	*SCCmec* PCR
QE16	✓	positive
QE28	✓	positive
QE47	✓	positive
QE69	✓	positive
QE82	–	negative
CU2189	–	negative
NTC	–	negative

4.4 Development of a PCR Based Method Facilitating the Detection of Tn*916*

A method allowing the rapid screening of the transposon Tn*916* in bacterial isolates has been developed. This method is based upon two PCR reactions, that amplify two regions encompassing the whole transposon, the amplicon was then digested using the restriction enzyme *Hin*cII, run on an 0.75% agarose gel for 1 hour and the resulting digestion profile was then used to identify whether or not the transposon is Tn*916*. The control strains were CU2189 and BS34A, information regarding these controls is shown on Table 4.13.

Initially, the PCR was performed using two primers, one specific for each end of Tn*916* using the primers (LongA-01F GGACTTATCACACTTTAT-

CAAGG, LongB-PW01R CTGTAGGAAGATACTTCACG) provided by Dr Phillip Warburton, using reaction conditions of 95°C for 4 minutes, followed by 30 cycles of 95°C for 30 seconds, 53°C for 30 seconds 68°C for 15 minutes followed by a final extension of 68°C for 15 minutes. LongAmp Taq (Promega, USA), a taq polymerase that is specialised for the amplification of long DNA fragments was used. The amplification was unsuccessful, leading to the further attempt to amplify the Tn*916* transposon (Fig. 4.1) by dividing the transposon into two target amplicons (Fig. 4.2). The PCR on the first section was performed using the primers (LongA-01F GGACTTATCACACTTTATCAAGG, LongA-CT25R AAACAGAAGCAGTGAGAAGA) and the PCR on the second section was performed using the primers LongB-CTn9824F GAAAACTTTAGTGATTGGTGG, LongB-PW01R CTGTAGGAAGATACTTCACG) provided by Dr Phillip Warburton. The following reaction conditions were used: 95°C for 4 minutes, followed by 30 cycles of 95°C for 30 seconds, 50°C for 30 seconds, 68°C for 10 minutes, with a final extension of 68°C for 10 minutes. These conditions proved effective (Fig. 4.3), a single strong band in the positive control BS34A (details regarding this strain shown on Table 4.13) was produced from the Long A primers, however the reaction using the Long B primers produced a "smear" suggesting the presence of non specific binding, and the possibility that the annealing temperature tested may be too low for these primers. To investigate this, a temperature gradient PCR was performed.

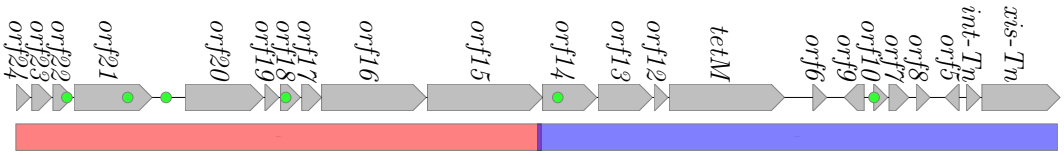


Figure 4.1: Schematic diagram of Tn*916* detailing the two sections that were amplified via PCR to produce two separate amplicons that cover the majority of the transposons structure. Amplicon A ranges covers positions 38 to 9844 and Amplicon B covers positions 9824 to 17947. The red rectangle represents the area covered by primer set 'A' and the blue rectangle represents the area covered by primer set 'B'. The green circles show the restriction target sites for *Hin*cII, these sites are detailed in Table 4.12

Table 4.12: Fragment sizes of each amplicon following digestion with *Hin*cII and the location of where upon the amplicon that each fragment originated from.

Amplicon A			**Amplicon B**		
Start	End	Length	Start	End	Length
1	916	916	1	202	202
917	2127	1211	203	5113	4911
2128	2589	462	5114	8124	3011
2590	5364	1775			
4365	9807	5443			

4.4.1 Temperature Gradient PCR

The first temperature gradient PCR was performed between a temperature range of 51°C and 54.5°C on both sets of primers however significant non specific binding was still observed within the reaction using the "Long B"

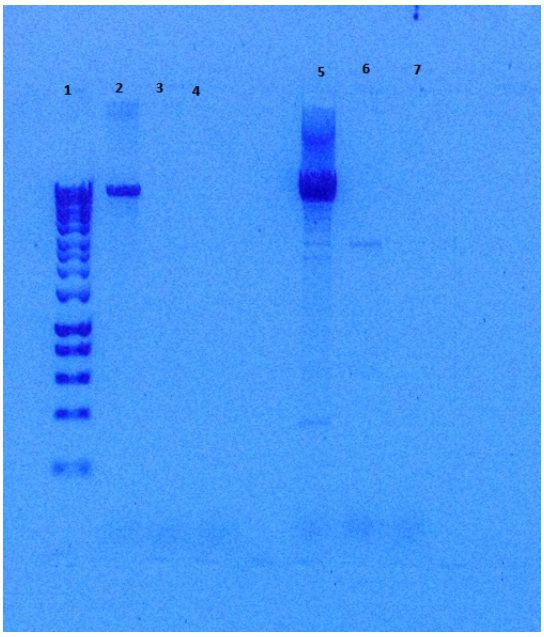


Figure 4.2: "Long A" and "Long B" PCR reactions run at 95°C for 4 minutes, followed by 30 cycles of 95°C for 30 seconds, 50°C for 30 seconds, 68°C for 10 minutes, with a final extension of 68°C for 10 minutes. Information regarding the control strains BS34A and CU2189 is presented in Appendix .1

0.75% agarose gel run at 100V for 1 hour, lanes from left to right:

1) Hyperladder 1kb,

2) BS34A with primer set A (Tn*916* positive)

3) CU2189 with primer set A (Tn*916* negative)

4) No template control with primer set A

5) BS34A with primer set B (Tn*916* positive)

6) CU2189 with primer set B (Tn*916* negative)

7) No template control with primer set B

primers (Fig. 4.3). To address this two approaches were attempted, another heat gradient was carried out only using the "Long B" primers, the temperature range of the gradient was expanded to run from 51°C to 58°C, in addition to this, reactions were carried out using half the primer concentration (5µl). An annealing temperature of 58°C and primers at a concentration of 10µM (Fig. 4.4).

4.4.2 Restriction Digest with *Hin*cII

A restriction digest was carried out on section A and section B of Tn*916*, with *Hin*cII (Fig 4.5).

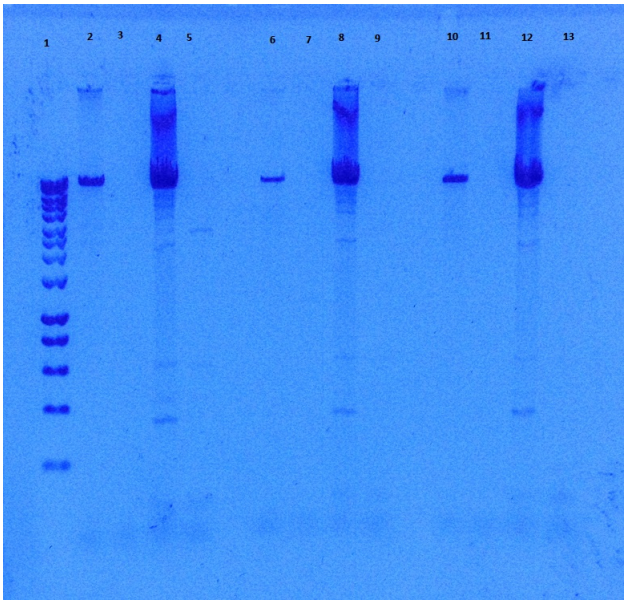


Figure 4.3: Primer sets "A" and "B" run on a temperature gradient PCR. The temperature gradient travels from left to right and ranges between 51°C and 54.5°C. 0.75% agarose gel run at 100V for 1 hour, lanes from left to right:

1) Hyperladder 1kb,

2) BS34A with primer set A (Tn*916* positive)

3) CU2189 with primer set A (Tn*916* negative)

4) BS34A with primer set B (Tn*916* positive)

5) CU2189 with primer set B (Tn*916* negative)

6) BS34A with primer set A (Tn*916* positive)

7) CU2189 with primer set A (Tn*916* negative)

8) BS34A with primer set B (Tn*916* positive)

9) CU2189 with primer set B (Tn*916* negative)

10) BS34A with primer set A (Tn*916* positive)

11) CU2189 with primer set B (Tn*916* negative)

12) BS34A with primer set B (Tn*916* positive)

13) CU2189 with primer set B (Tn*916* negative)

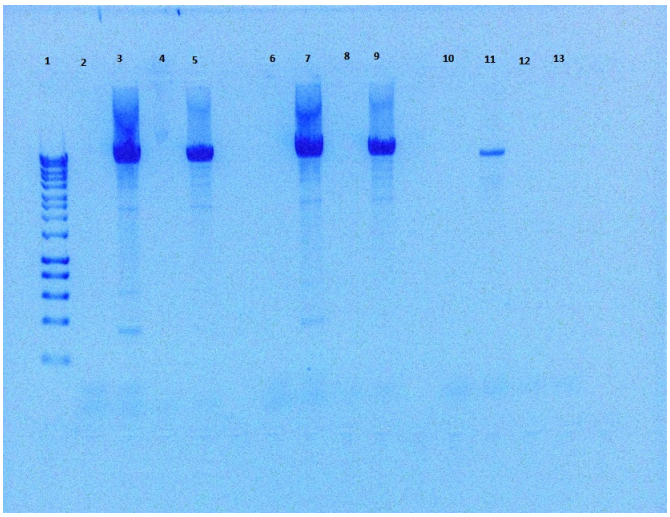


Figure 4.4: Primer sets "A" and "B" run on a temperature gradient PCR. The temperature gradient travels from left to right and ranges between 51°C to 58°C. 0.75% agarose gel run at 100V for 1 hour, lanes from left to right:

1) Hyperladder 1kb,

2) CU2189 10µM primer, ≈51°C (Tn*916* negative)

3) BS34A 10µM primer, ≈51°C (Tn*916* positive)

4) CU2189 5µM primer, ≈51°C (Tn*916* negative)

5) BS34A 5µM primer, ≈51°C (Tn*916* positive)

6) CU2189 10µM primer ≈55°C (Tn*916* negative)

7) BS34A 10µM primer ≈55°C (Tn*916* positive)

8) CU2189 5µM primer ≈55°C (Tn*916* negative)

9) BS34A 5µM primer ≈55°C (Tn*916* positive)

10) CU2189 10µM primer ≈58°C (Tn*916* negative)

11) BS34A 10µM primer ≈58°C (Tn*916* positive)

12) CU2189 5µM primer ≈58°C (Tn*916* negative)

13) BS34A 5µM primer ≈58°C (Tn*916* positive)

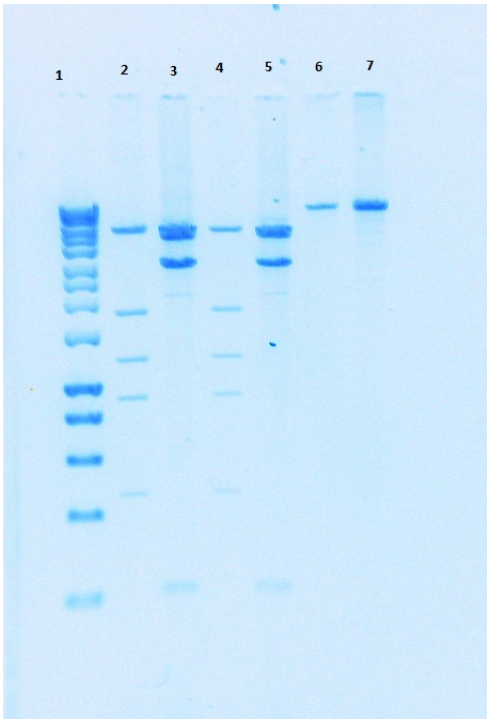


Figure 4.5: *Hinc*II digestion carried out in duplicate on the two amplicons from, they were ran on 0.75% agarose gel, run for 1 hour at 100V. Lanes from left to right,

1) Hyperladder 1kb

2) Amplicon A from strain BS34A digested with *Hinc*II

3) Amplicon B from strain BS34A digested with *Hinc*II

4) Amplicon A from strain BS34A digested with *Hinc*II

5) Amplicon B from strain BS34A digested with *Hinc*II

6) Amplicon A no enzyme (negative control)

7) Amplicon B no enzyme (negative control)

Table 4.13: The control strains, CU2189 is a laboratory generated strain of *Bacillus subtilis* that does not contain Tn*916*, BS34A is derived from CU2189 and contains a single copy of Tn*916*. CU2189 was used as a negative control and BS34A was used as a positive control.

Strain	Species	Transposon content	Reference
CU2189	*B. subtilis*	None	(Christie *et al.*, 1987)
BS34A	*B. subtilis*	Tn*916*	(Roberts *et al.*, 2003)

4.4.3 Long PCR and *Hin*cII Digest on *tetM* Positive Isolate DNA

The PCR technique described above was employed on the isolates where the tetracycline resistance determinant *tetM* had been detected (Table 4.10). The isolates that had a restriction profile that matched Tn*916* were QE16, QE28, QE45. Isolates QE107, QE110 and QE111 matched section B of Tn*916* but not section A suggesting that the latter three isolates may contain an element that is related to Tn*916* (Figure 4.6).

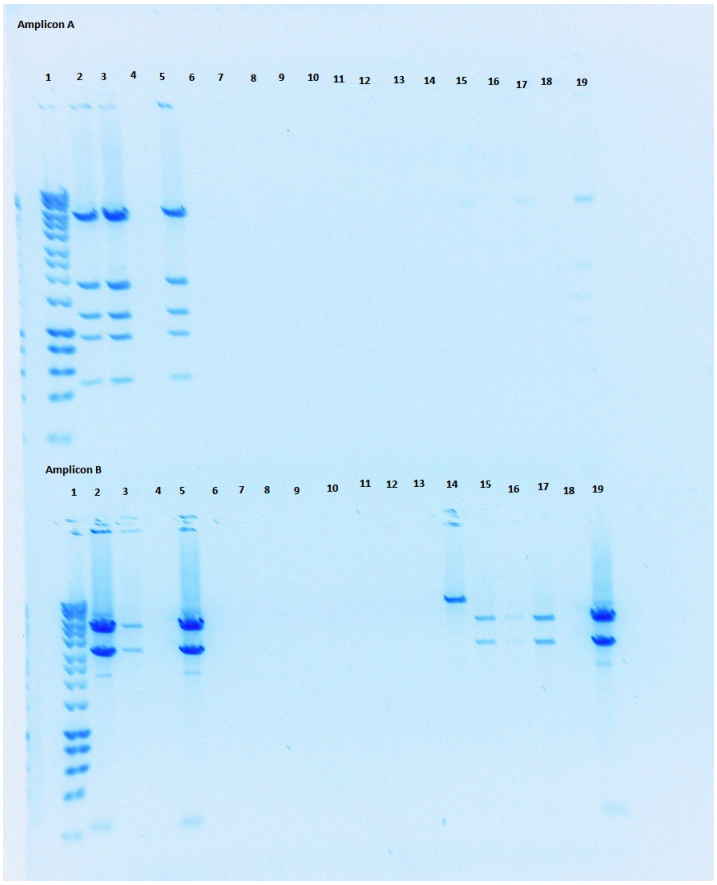


Figure 4.6: Digest of long PCR amplicons from QE isolates with *Hin*cII. Lanes from left to right:

1) Hyperladder 1kb
2) QE16
3) QE28
4) QE40
5) QE45
6) QE48
7) QE89
8) QE91
9) QE97
10) QE98
11) QE99
12) QE102
13) QE103
14) QE104A
15) QE107
16) QE110
17) QE111
18) CU2189 (negative control)
19) BS34A (positive control)

4.5 Investigation into *tetW* gene in *Corynebacterium striatum*

4.5.1 *tetW* in *Corynebacterium striatum*

Two *Corynebacterium striatum* isolates, tested positive using degenerate primers that amplify multiple genes that encode ribosomal protection proteins (RPP), and negative using the PCR primers for the common antibiotic resistance gene *tetM*. Sanger sequence analysis of the PCR product that was produced using the RPP primers revealed that the tetracycline resistance gene that the *Corynebacterium striatum* isolates were carrying was *tetW*. There is only one such case in the literature of this gene being found in this species of bacteria. However tetracycline resistance in *Corynebacterium striatum* itself is not a new observation. Tetracycline resistance determinants previously reported in this species include *tetM* (Roberts *et al.*, 1992) and the plasmid mediated *tetAB* (Tauch *et al.*, 1999).

Previous research has investigated the association between *tetW* and mobile genetic elements, and it was revealed that this resistance determinant was harboured by Tn*B1230* originally from *Butyrivbrio fibrisolvens* (Melville *et al.*, 2014). The flanking regions of *tetW* from *Corynebacterium striatum* in this study do not match Tn*B1230* (Fig. 4.7, 4.8) suggesting that *C. striatum* acquired this gene via different means. ClustalW analysis was carried out comparing Tn*B1230* with Tn*5253*, Tn*1311* and the plasmid pRAB11 and no matching areas were found. There is currently no record in the literature of *tetW* being associated with the Tn*5253* family.

With this gene having only been observed in *C. striatum* on one previous occasion within China, and the regions that immediately flank this gene containing a replication protein that originates from *S. pneumoniae* or *S. aureus*.

This gene suggests that the section of DNA next to *tetW* was originally from either *S. pneumoniae* or *S.aureus*. Presently there is no record of *tetW* being detected in *S. pneumoniae* however this gene has been detected in *S. aureus* (Spanu *et al.*, 2011).

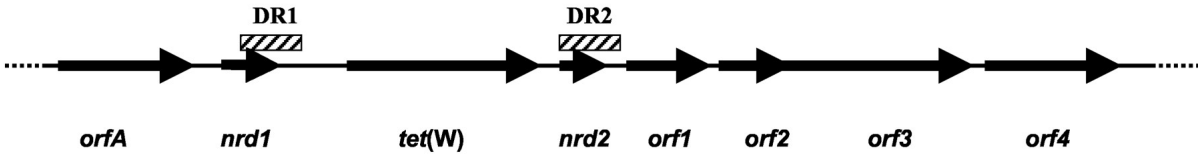


Figure 4.7: Schematic of Tn*B1230*, adapted from (Melville *et al.*, 2014)

Figure 4.8: Diagram representing the DNA amplified via inverse PCR in this study, the black line represents noncoding DNA, the red section represents an ORF, of which the translated amino acid sequence matches a replication protein from *S. pneumoniae* (Acc. WP_079118347.1).

Figure 4.9: The blue represents the ORF identified on the common region of DNA between pRAB11 and Tn*5253*, the red represents the ORF identified on the segment of DNA flanking *tetW* that was amplified via inverse PCR.

4.5.1.1 Tn*1311*

Tn*1311* was first characterised in 2011 (Mingoia *et al.*, 2011). This transposon belongs to the Tn*5253* family. This family of transposons are large (>50 kb). The prototype transposon for this family Tn*5253* is composed of

two smaller independent conjugative transposons Tn*5251* (almost identical to Tn*916* and Tn*5252*. Analysis revealed that the flanking regions matched with the conjugative transposons Tn*5253* and Tn*1311* originally from *Streptococcus pneumoniae* as well as the plasmid pRAB11, these regions are shown on Fig. 4.10).

4.5.1.2 Tn*5253* and pRAB11

ClustalW analysis (Bioedit) of Tn*5253* and pRAB11 revealed that there is a 14560bp region that is common to Tn*5253* and pRAB11, with the exception of a T−G substitution at position 53808 on Tn*5253*. Analysis with Artemis revealed two potential open reading frames (ORFs). Nucleotide BLAST analysis showed that these two potential ORFs revealed a variety of homologues, mostly originating from *Streptococcus* and *Staphylococcus* species, these are shown in Table 4.14. A schematic of Tn*5253* is shown in Fig.4.11.

An ORF was detected upon the region of DNA found in both Tn*5253* and pRAB11 at position 177 to 1121. The closest homologue to the translated protein of this ORF is a replication protein originating from *S. aureus* (Acc. WP_012386790.1) (99/100). The sequence flanking the *tetW* gene in *C. striatum* contained an ORF that when compared to the NCBI database, matched (querey cover 100%/ ID 100%) a partial replication protein from *S. pneumoniae* (Acc. WP_079118347.1). The sequence of DNA amplified from the regions flanking *tetW* in *C. striatum* was 720bp in length, and contained one ORF located from position 718 to 371. When compared against the NCBI database, the closest homologue to this ORF (matching 100% query cover and 100% ID) was a partial replication protein originating from *S. pneumoniae*, however this amino acid sequence also matches part of the replication

protein translated from the ORF present on the region of DNA common to Tn*5253* and pRAB11 (Fig. 4.9).

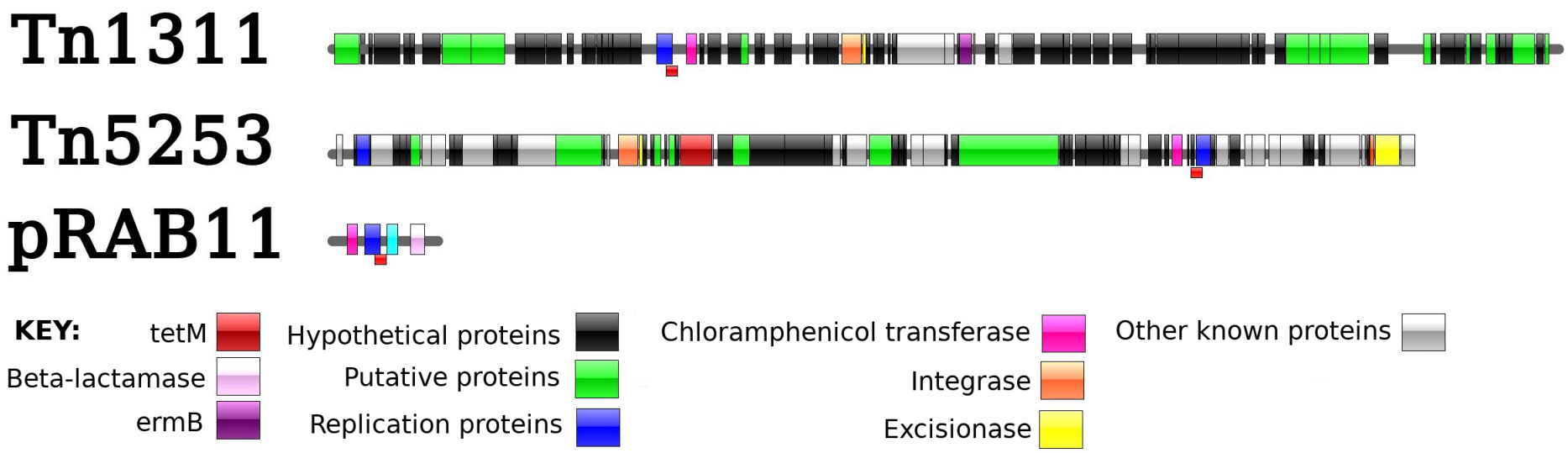


Figure 4.10: The red line represents the sequence amplified by inverse PCR, it's location shows where the sequence identified from the inverse PCR coming off *tetW* is situated on Tn*5253*, Tn*1311* and pRAB11. The properties of the ORFS on these elements is displayed in the key.

Table 4.14: Closest matches on the BLAST nucleotide database to the two potential open reading frames that were identified using Artemis on the section of DNA common to both Tn*5253* and pRAB11.

Region 281− 1	QC%	ID%	Accession number
pRAB11	100	100	JN635500.1
pRIT23	100	99	LC257602.1
pRIT22	100	99	LC257599.1
S. pneumoniae strain Hu15	100	99	CP020551.1
S. pneumoniae strain Hu17	100	99	CP020549.1
S. aureus knockdown vector pSD1	100	99	KX685167.1
S. mitis strain SVGS 061	100	99	CP014326.1
pRIT21	100	99	LC121520.1
Region 117 − 1121	QC%	ID%	Accession number
S. pneumoniae strain Hu15 genome	100	100	CP020551.1
S. pneumoniae strain Hu17 genome	100	100	CP020549.1
S. aureus strain Gv88 plasmid pGv88	100	100	CP012017.1
S. aureus strain Gv51 pGv51	100	100	CP012016.1
S. aureus replication protein	100	100	AB982227.1
S. pneumoniae ICE6BST90	100	100	HG799499.1
S. pneumoniae ICESpcIc1	100	100	HG799494.1
S. aureus plasmid pBmb9393	100	100	CP005289.1

4.6 Discussion

4.6.1 The Antibiotic Resistance Genes that were Detected

4.6.1.1 Erythromycin

Over the course of this study, the most commonly observed erythromycin resistance determinant was *ermC*, followed by *ermA* and *ermB*. These genes are rRNA methylase enzymes. These rRNA methylases confer resistance to erythromycin via the methylation of the 23S ribosomal rRNA. Of the erythromycin resistance genes that were identified belonged to the rRNA methylase group. The PCRs for genes that confer resistance to erythromycin via alternative mechanisms (efflux, the production of inactivating enzymes) were negative in all cases. There were erythromycin resistant isolates that did not amplify with any of the primers that were tested.

4.6.1.2 Tetracycline

tetM was the most commonly detected tetracycline resistance gene that was detected in these isolates followed by *tetO*. These findings are consistent with the published literature whereby *tetM* is the most frequently occurring tetracycline resistance gene, and it is often found within tetracycline resistant isolates that have been obtained from wounds (Emaneini *et al.*, 2013; Schmitz *et al.*, 2001).

4.6.1.3 Vancomycin

Throughout this study, vancomycin resistance was only observed in enterococci. All vancomycin resistant enterococci tested positive for the presence

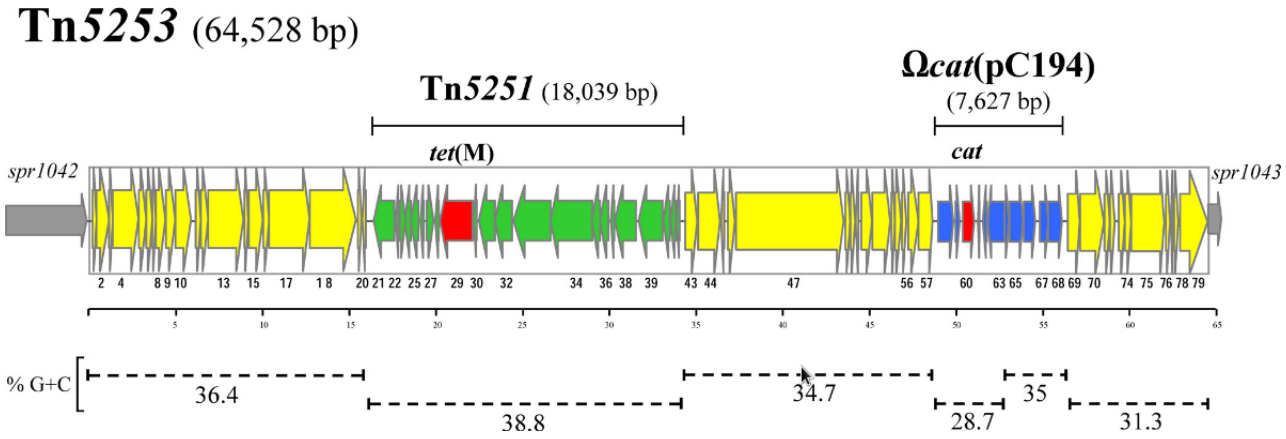


Figure 4.11: Diagram showing the structure of Tn*5253* from (Iannelli *et al.*, 2013)

of the *vanA* gene.

4.6.2 Species Identified Compared with the Literature

4.6.2.1 *Staphylococcus aureus*

In total, 47 isolates of *S. aureus* were received. Of the resistant isolates, 7 (15%) were identified as resistant to tetracycline or erythromycin.

The bacteria *S. aureus* was fist isolated from a leg abscess and identified in the 1880's (Ogston, 1984). This species is typically resident upon the skin and around the nose and respiratory tract of healthy individuals. *S. aureus* is a clinically important species of bacteria, as while it is not pathogenic under normal conditions, it is an opportunistic pathogen that can cause soft skin infections leading to abscess development, respiratory infections and food poisoning if it enters the digestive tract.

The most frequently detected erythromycin resistant determinants in the *S. aureus* isolates were *ermA* (37.5%) and *ermC* (62.5%), according to the literature these genes are commonly found to confer resistance in erythromycin resistant *S. aureus* (Shahsavan *et al.*, 2012).

tetM was the only tetracycline resistant determinant that was detected in *S. aureus*, the presence of this gene in tetracycline resistant *S. aureus* is not surprising as this gene has been frequently detected in tetracycline resistant *S. aureus* in previous studies (Emaneini *et al.*, 2013).

4.6.2.2 *Staphylococcus hominis*

S. hominis was only identified in two of the resistant wound isolates. This lower incidence is as expected, as this species is usually commensal predominantly causing infection in individuals who are immunocompromised. In both cases these isolates are resistant to erythromycin and sensitive to tetracycline. MICs were lower in the case of these two isolates than most of the other erythromycin resistant isolates. 50% (n = 1) of the erythromycin resistant isolates contained the erythromycin resistant determinant *ermC*, this determinant has been detected in this species previously and is frequently present (Aslantaş *et al.*, 2013). In recent years the emergence of *S. hominis* infections has been noted in nosocomial infections (Voineagu *et al.*, 2013), nosocomial infections involving *S. hominis* appear most often in patients who have invasive treatment, such as a catheter inserted (Doneli and Francolini, 2013) or surgery (Kleeff *et al.*, 2015). In the case of this study, one *S. hominis* isolate was taken from a vaginal swab and the other from the throat, though this species is not often detected at these sites it has previously been observed causing infection at both the vagina (Hoist *et al.*, 1987) and the throat (Xiao *et al.*, 2012). While *S. hominis* may usually be associated with immunocompromised patients, it has been observed as the cause of serious infection in immunocompetent individuals, for example an apparently immunocompetent female who developed mastitis where only *S. hominis* was detected within the aspirate (Datta *et al.*, 2015).

4.6.2.3 *Streptococcus* spp.

The streptococcal species detected were either opportunistic pathogens in the cases of *S. agalactiae* and *S. dysgalactiae* or pathogenic (*S. pyogenes*) therefore the presence of these species in wound sites is not unusual. The detection of *S. agalactiae* was predominantly in blood culture and vaginal swabs, this is consistent with previous reports where there have been periodic reports of *S. agalactiae* sepsis (Lerner *et al.*, 1977) in some cases in patients with no risk factors (Warner *et al.*, 2016). Vaginal infections caused by *S. agalactiae* are observed (Maniatis *et al.*, 1996) *S. agalactiae* is still not uniformly acknowledged as a vaginal pathogen in the literature (Savini *et al.*, 2013), the findings of this study add further evidence supporting the case that *S. agalactiae* is a vaginal pathogen. Tetracycline resistance is common in this species, most frequently conferred by *tetM* (Fischer *et al.*, 2013), the results generated by this study are consistent with the literature as *tetM* is present in all tetracycline resistant isolates of *S. agalactiae*. The determinant *tetM* is also commonly observed in *S. dysgalactiae*, and this determinant was detected in all *S. dysgalactiae* isolates. *S. dysgalactiae* and *S. pyogenes* were isolated from wounds and blisters, these species have commonly been reported as the causes of such infections (Brandt and Spellerberg, 2009). As with the previous streptococcal species mentioned, *tetM* has been reported previously and its detection amongst all tetracycline resistant *S. pyogenes* isolates is consistent with reports in the literature (Soriano *et al.*, 2014).

4.7 Conclusion

The most frequently detected tetracycline resistance gene was *tetM*, this gene was present in a diverse range of unrelated bacterial species, it was also often

associated with the genes *int* and *tn* which suggests that the gene may be located upon a transposable element belonging to the Tn*916* family.

The method of detecting Tn*916* by a two stage PCR followed by the analysis of a profile produced by restriction digest appears to be an effective method for the cheap and efficient detection of this element. There is a potential for this method to be developed further to include other elements allowing for an inexpensive way to screen for the presence of different elements which could perhaps be a useful tool for screening bacterial isolates for common elements to maintain a surveillance detailing the frequency of these elements, and how they may shift over time. The close similarity between the simulated restriction digest with the actual profile that was generated with *Hinc*II indicates that should the development of such a database of profiles be undertaken, it may be useful to first generate carry out such simulations for a variety of different restriction enzymes in order to generate profiles that sufficiently differ between different related elements therefore allowing distinctions to be made with a reduced risk of error.

The detection of a phenotypically active *tetW* in *Corynebacterium striatum* for the first time outside of China suggests that this gene is moving between species, whether *tetW* is located upon a mobile element is uncertain, as analysis of the DNA regions that flank the gene did match known mobile elements such as the Tn*5253* family and pRAB11, it is not possible to determine exactly what element this gene is located upon, only that it is possibly located upon an element that belongs to the Tn*5253* family. As there is currently no record of *tetW* being associated with the Tn*5253* family, it is possible that this gene is being harboured by a transposon belonging to this family that

is yet to be characterised. That the flanking region contained part of a gene that encodes a replication protein originating from *Streptococcus pneumoniae* suggests that the gene may have originated in this species.To date, there has been no reports of *tetW* in *S. pneumoniae*.

Chapter 5

Discussion

5.1 Difference Between the Sample Sources

The samples that were used in this study were sourced from two different areas, one aspect focusing upon the antibiotic resistance genes and their genetic support in the oral cavity of healthy volunteers and the other investigating bacteria that have been isolated from swabs of wounds where there may be infection present. The reason for doing this was to assess the difference between the resistant species that are present as well as the types of antibiotic resistance gene and mobile elements.

5.1.1 Antibiotic Resistance

Antibiotic resistance to tetracycline and erythromycin was widely detected in both isolates that were cultured from saliva and those that were cultured from wounds. The resistance genes that were discovered in the isolates from wounds have all been previously reported in their respective species as shown on Table 5.1.

PCR analysis revealed that the genes responsible for resistance in the isolates

from wounds were often also present in the metagenomic DNA extracted from saliva. For example, *tetM* was the most common resistance gene detected in the wound isolates, this gene was also detected in almost all of the metagenomic DNA, these observations are consistent with literature reports as *tetM* is frequently detected.

The distribution of erythromycin genes however seems to be different. The gene *ermB* was detected in every metagenomic saliva sample indicating that it is abundant in the resistome in the absence of selection pressure. However despite this *ermB* was only detected in $\approx 10\%$ of the erythromycin resistant isolates that were obtained from the wound cultures. However other studies have previously reported *ermB* in each of the erythromycin resistant isolates.

5.2 Tn*916*-like Elements

Evidence suggesting the presence of elements that belong to the Tn*916* family was detected in both the isolates from wounds and the saliva samples. The *int* and *xis* genes were present in all of the metagenomic DNA samples that were taken from saliva. These findings are consistent with reports that the Tn*916* family are widely disseminated.

The presence of *int* from Tn*916* in all of the metagenomic DNA samples taken from saliva are also consistent with previous reports demonstrating that this gene is ubiquitous in the oral metagenome (Seville *et al.*, 2009).

5.3 The Role of Plasmids

The TRACA section of this study detected plasmids from the saliva samples from healthy individuals indicating the presence of a plasmid population har-

Table 5.1: The antibiotic resistance genes that were detected in the species that were identified from the wound swabs compared with whether each gene has been previously reported within the literature. In each case, the genes that have been detected throughout this study have been reported previously.

	tetM	Reported	Reference	*tetO*	Reported	Reference	*tetW*	Reported	Reference
S. aureus	✓	✓	(Schmitz *et al.*, 2001)	–	–	No report	–	–	No report
S. agalactiae	✓	✓	(Dogan *et al.*, 2005)	✓	✓	(Dogan *et al.*, 2005)	–	–	No report
S. dysgalactiae	✓	✓	(Abdelsalam *et al.*, 2013)	–	✓	(Traverso *et al.*, 2016)	–	–	No report
S. hominis	–	✓	(Murugesan, 2015)	–	–	No report	–	–	No report
S. pyogenes	✓	✓	(Wong and Yuen, 2012)	–	✓	(Perez-Trallero *et al.*, 2007)	–	✓	(Palmieri *et al.*, 2011)
C. striatum	✓	✓	(Martinez-Martinez *et al.*, 1996)	–	–	No report	✓	✓	(Li *et al.*, 2015)
S. simulans	–	✓	(Chajecka-Wierzchowska *et al.*, 2014)	–	–	No report	–	–	No report
	ermA	Reported	Reference	*ermB*	Reported	Reference	*ermC*	Reported	Reference
S. aureus	✓	✓	(Schmitz *et al.*, 2000)	–	✓	(Steward *et al.*, 2005)	✓	✓	(Schmitz *et al.*, 2000)
S. agalactiae	–	✓	(Dogan *et al.*, 2005)	✓	✓	(Dogan *et al.*, 2005)	–	✓	(Dogan *et al.*, 2005)
S. dysgalactiae	–	✓	(Silva *et al.*, 2015)	–	✓	(Prabu *et al.*, 2013)	–	✓	(Lo *et al.*, 2015)
S. hominis	–	✓	(Khan *et al.*, 2002)	–	✓	(Szczuka *et al.*, 2016)	✓	✓	(Khan *et al.*, 2002)
S. pyogenes	–	✓	(Richter *et al.*, 2005)	–	✓	(Palmieri *et al.*, 2011)	–	–	No report
C. striatum	–	–	No report	–	–	No report	–	–	No report
S. simulans	–	✓	(Di Modugno *et al.*, 2002)	–	✓	(Simeoni *et al.*, 2008)	✓	✓	(Simeoni *et al.*, 2008)

bouring antibiotic resistance genes in the absence of antimicrobial selection pressures. The antibiotic resistance genes that were detected conferred resistance to β-lactam antibiotics and aminoglycosides. Therefore the findings are clinically relevant as β-lactam antibiotics are frequently prescribed as the first course of antimicrobial treatment following an infection.

5.4 The Resistome

The results generated by this study add to the body of evidence concerning the resistance reservoir of antibiotic resistance genes. The genes that were detected were common causes of antimicrobial resistance such as *tetM* and *ermB*.

5.5 Targets for Antimicrobial Therapy

All of the saliva samples that were taken showed evidence indicating the presence of Tn*916*-like elements within their metagenomic DNA in the form of integrase and excisionase genes. That the Tn*916* family of elements frequently harbour antibiotic resistance genes that confer resistance to a range of unrelated antibiotics, their ubiquitous presence within the oral cavity is a cause for concern. This is furthered by that they often carry genes that confer resistance to tetracycline such as *tetM* as tetracycline is frequently used in the treatment of oral infections because it is able to penetrate further into the gum than other classes of antibiotics. Therefore, these integrase and excisionase genes could be potential targets for therapy, for a compound that inhibits either one of these genes could prevent the transposon, and therefore its accessory genes such as *tetM* from transferring between different bacte-

ria thereby limiting the problem of a reservoir of Tn*916*-like elements in the commensal flora as the ability of Tn*916*-like elements to transfer to the pathogenic bacteria would be reduced.

Chapter 6

Conclusions

The metagenomic study investigating the prevalence of various antibiotic resistance determinants in the oral cavity of healthy volunteers has highlighted that there appears to be a general consensus in the antibiotic resistance genes that comprise the oral resistome in the case of tetracycline and macrolide antibiotics. The tetracycline resistance determinant *tetM* was frequently detected in both the oral metagenome and the isolates that were cultured from wounds highlighting the importance of this resistance determinant in tetracycline therapy. In addition to this, evidence of the transposon Tn*916* and related elements was frequently detected in both the saliva and wound samples. In addition to the presence of elements that are related to Tn*916*, there was no evidence for the presence of any elements that belong to the Tn*5397*-like family, suggesting that elements that are related to Tn*916* are more prevalent. Therefore in future research that may take place aiming to prevent the transfer of mobile elements that contain resistance determinants, the Tn*916* family are a reasonable target.

The species that were identified in the wound isolates were not unusual, in that they were mostly bacterial species that typically reside on the skin and

have been previously associated with opportunistic nosocomial infections. The earliest detection of *tetW* in *C. striatium* was observed in this study. The isolates containing the gene were obtained in 2013, approximately two years before the first and only report that exists to date. This detection as well as the subsequent detection of *tetW* in *Corynebacterium striatum* suggests that this gene has recently entered the species and may be on a possible novel mobile element.

The TRACA technique was successfully employed in capturing plasmids resident in the metagenomic DNA from saliva samples, some of which were shown to contain either one or two antibiotic resistance genes that were demonstrated to be functional via MIC testing.

A method to quickly determine whether a bacterial isolate is carrying the common transposon Tn*916* was developed using a control isolate from the species *Bacillus subtilis* that is known to contain Tn*916*. This method was based upon amplifying two fragments of the Tn*916* transposon over two PCR reactions, performing a restriction digest on these fragments and analysing the restriction profile. This proved to be a successful method and was subsequently used to detect the presence of Tn*916* in bacterial isolates taken from wounds. This test could be further developed to generate known restriction profiles of other frequently occurring transposons. Such a technique could be a useful tool both in research that is aiming to characterise novel transposons by quickly screening out known common transposons as well as research tracking the movement and distribution of transposable elements.

Antimicrobial resistance genes were detected in bacterial isolates taken from

the oral cavity, as well as oral metagenomic DNA and isolates obtained from wound swabs.The resistance genes were found to be functional.

The genes within the resistome that confer resistance to the antimicrobial agents tetracycline and erythromycin were predominantly the same class, these findings suggest that the resistance gene profiles are similar between different individuals in the absence of disease.

6.1 Future Work

Based on the findings of this study, future work should involve research into inhibiting the mechanisms that are involved in the mobilisation of transposons, in particular those that belong to the Tn*916* family due to their frequent presence in individuals that have not been exposed to any antimicrobial therapy recently. The surveillance of the transposable elements should continue in order to determine whether the elements contributing to the maintenance of the resistome are changing and if so how they are changing. Research into antibiotic resistance genes has shifted away from culture based studies and towards a metagenomic approach. As technology advances, it will be possible to sequence entire metagenomes therefore allowing for the complete characterisation of the resistome of an environment. Without this knowledge the ability to understand and characterise the origin and evolution of resistance determinants is limited (Martinez *et al.*, 2015). Though this may require the development of new approaches as the current methodology for the identification of functional novel resistance genes is laborious and has relatively low throughput (Schmieder and Edwards, 2012). The increase in understanding of resistance determinants and mobile genetic elements will

lead to insights into what triggers resistance transmission allowing for methods to control or potentially stop the transfer of resistance to be developed (Roberts and Mullany, 2010).

The ongoing inappropriate prescription and misuse of antibiotics across medicine, dentistry and agriculture will continue to contribute to the maintenance of the antibiotic resistome resulting in difficulties in infection management and therapeutic failure(Cope *et al.*, 2014).

The rise in metagenomics is also likely to have an impact within the clinical setting that goes beyond expanding knowledge in antimicrobial resistance genes and their transfer (Sukumar *et al.*, 2016). As large volumes of metagenomic sequence data are collected and made available it will be possible to combine information regarding antimicrobial resistance genes, mobile genetic elements, the composition of the microbial community and information regarding metabolic pathways. Such analysis could be able to determine whether and how these different factors are linked which may provide information that is useful in reducing the selection for resistance (Sukumar *et al.*, 2016).

Until methods to inhibit the transfer of antibiotic resistance have been developed, the evolution towards antimicrobial resistance is unavoidable (Courvalin, 2016). It is also important to search for antivirulence drugs that inhibit the pathogenic aspect of a microorganism without interfering with growth, for as soon as growth is effected negatively then there is a selection pressure that favours resistance (Courvalin, 2016).

Further research will allow for a more detailed understanding of the diversity antimicrobial resistome and how it involved in the interactions between commensal and pathogenic bacteria creating potential targets to control this threat to public health.

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Appendix

.1 Table of strains

ID	Species	Properties	Reference	Obtained From
BS34A	*Bacillus subtilis*	Contains Tn*916*, *tetM* resistance gene	(Roberts *et al.*, 2003)	ARU culture collection
CU2189	*Bacillus subtilis*	Does not contain resistance genes	(Christie *et al.*, 1987)	ARU culture collection
QE01	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE02	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE03	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE04	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE05	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE06	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE07	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE08	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE09	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE10	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE11	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE12	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE13	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE14	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk

ID	Species	Properties	Reference	Obtained From
QE15	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE16	*Staphylococcus aureus*	Resistant to tetracycline and erythromycin	This study	Queen Elizabeth Hospital, Norfolk
QE17	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE18	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE19	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE20	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE21	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE22	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE23	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE24	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE25	*Staphylococcus aureus*	Resistant to erythromycin	This study	Queen Elizabeth Hospital, Norfolk
QE26	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE27	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE28	*Staphylococcus aureus*	Resistant to tetracycline and erythromycin	This study	Queen Elizabeth Hospital, Norfolk
QE29	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE30	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE31	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk

ID	Species	Properties	Reference	Obtained From
QE32	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE33	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE34	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE35	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE36	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE37	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE38	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE39	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE40	*Streptococcus agalactiae*	Resistant to tetracycline and erythromycin	This study	Queen Elizabeth Hospital, Norfolk
QE41	*Streptococcus dysgalactiae*	Resistant to tetracycline and erythromycin	This study	Queen Elizabeth Hospital, Norfolk
QE42	*Staphylococcus hominis*	Resistant to erythromycin	This study	Queen Elizabeth Hospital, Norfolk
QE43	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE44	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE45	*Streptococcus agalactiae*	Resistant to tetracycline	This study	Queen Elizabeth Hospital, Norfolk
QE46	*Streptococcus* group C	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE47	*Staphylococcus aureus*	Resistant to erythromycin	This study	Queen Elizabeth Hospital, Norfolk
QE48	*Streptococcus agalactiae*	Resistant to tetracycline	This study	Queen Elizabeth Hospital, Norfolk

ID	Species	Properties	Reference	Obtained From
QE49	*Streptococcus agalactiae*	Resistant to tetracycline and erythromycin	This study	Queen Elizabeth Hospital, Norfolk
QE50	*Staphylococcus aureus*	Resistant to erythromycin	This study	Queen Elizabeth Hospital, Norfolk
QE51	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE52	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE53	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE54	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE55	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE56	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE57	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE58	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE59	*Staphylococcus hominis*	Resistant to erythromycin	This study	Queen Elizabeth Hospital, Norfolk
QE60	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE61	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE62	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE63	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE64	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE65	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk

ID	Species	Properties	Reference	Obtained From
QE66	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE67	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE68	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE69	*Staphylococcus aureus*	Resistant to erythromycin	This study	Queen Elizabeth Hospital, Norfolk
QE70	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE71	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE72	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE73	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE74	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE75	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE76	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE77	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE78	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE79	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE80	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE81	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE82	*Staphylococcus aureus*	Resistant to erythromycin	This study	Queen Elizabeth Hospital, Norfolk

ID	Species	Properties	Reference	Obtained From
QE83	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE84	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE85	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE86	*Staphylococcus aureus*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE87	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE88	*Streptococcus* group A	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE89	*Streptococcus agalactiae*	Resistant to tetracycline	This study	Queen Elizabeth Hospital, Norfolk
QE90	*Streptococcus* group G	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE91	*Streptococcus pyogenes*	Resistant to tetracycline	This study	Queen Elizabeth Hospital, Norfolk
QE92	*Corynebacterium striatum*	Resistant to tetracycline and erythromycin	This study	Queen Elizabeth Hospital, Norfolk
QE93	*Corynebacterium striatum*	Resistant to tetracycline and erythromycin	This study	Queen Elizabeth Hospital, Norfolk
QE94	*Staphylococcus aureus*	Resistant to erythromycin	This study	Queen Elizabeth Hospital, Norfolk
QE95	*Streptococcus dysgalactiae*	Resistant to tetracycline	This study	Queen Elizabeth Hospital, Norfolk
QE96	*Streptococcus agalactiae*	Resistant to tetracycline	This study	Queen Elizabeth Hospital, Norfolk
QE97	*Streptococcus pyogenes*	Resistant to tetracycline	This study	Queen Elizabeth Hospital, Norfolk
QE98	*Streptococcus agalactiae*	Resistant to tetracycline	This study	Queen Elizabeth Hospital, Norfolk
QE99	*Streptococcus dysgalactiae*	Resistant tot tetracycline	This study	Queen Elizabeth Hospital, Norfolk

ID	Species	Properties	Reference	Obtained From
QE100	*Streptococcus mitis/oralis*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE101	*Streptococcus* group G	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE102	*Streptococcus agalactiae*	Resistant to tetracycline	This study	Queen Elizabeth Hospital, Norfolk
QE103	*Streptococcus* group B	Resistant to tetracycline	This study	Queen Elizabeth Hospital, Norfolk
QE104a	*Streptococcus pyogenes*	Resistant to tetracycline and erythromycin	This study	Queen Elizabeth Hospital, Norfolk
QE104b	*Stapylococcus simulans*	Resistant to erythromycin	This study	Queen Elizabeth Hospital, Norfolk
QE105	*Streptococcus* group G	Resistant to tetracycline	This study	Queen Elizabeth Hospital, Norfolk
QE106	*Corynebacterium striatum*	Resistant to tetracycline and erythromycin	This study	Queen Elizabeth Hospital, Norfolk
QE107	*Streptococcus dysgalactiae*	Resistant to tetracycline	This study	Queen Elizabeth Hospital, Norfolk
QE108	*Streptococcus milleri*	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE109	*Streptococcus* group G	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE110	*Streptococcus pyogenes*	Resistant to tetracycline	This study	Queen Elizabeth Hospital, Norfolk
QE111	*Streptococcus dysgalactiae*	Resistant to tetracycline	This study	Queen Elizabeth Hospital, Norfolk
QE112	*Streptococcus* group G	Tetracycline and erythromycin sensitive	This study	Queen Elizabeth Hospital, Norfolk
QE113	*Enterococcus faecalis*	Resistant to vancomycin	This study	Queen Elizabeth Hospital, Norfolk
QE114	*Enterococcus faecalis*	Resistant to vancomycin	This study	Queen Elizabeth Hospital, Norfolk
QE115	*Enterococcus faecalis*	Resistant to vancomycin	This study	Queen Elizabeth Hospital, Norfolk

ID	Species	Properties	Reference	Obtained From
QE116	*Enterococcus faecalis*	Resistant to vancomycin	This study	Queen Elizabeth Hospital, Norfolk
QE117	*Enterococcus faecalis*	Resistant to vancomycin	This study	Queen Elizabeth Hospital, Norfolk
QE118	*Enterococcus faecalis*	Resistant to vancomycin	This study	Queen Elizabeth Hospital, Norfolk
QE119	*Enterococcus faecalis*	Resistant to vancomycin	This study	Queen Elizabeth Hospital, Norfolk
QE120	*Enterococcus faecalis*	Resistant to vancomycin	This study	Queen Elizabeth Hospital, Norfolk
QE121	*Enterococcus faecalis*	Resistant to vancomycin	This study	Queen Elizabeth Hospital, Norfolk
QE122	*Enterococcus faecalis*	Resistant to vancomycin	This study	Queen Elizabeth Hospital, Norfolk
QE123	*Enterococcus faecalis*	Resistant to vancomycin	This study	Queen Elizabeth Hospital, Norfolk
QE124	*Enterococcus faecalis*	Resistant to vancomycin	This study	Queen Elizabeth Hospital, Norfolk
QE125	*Enterococcus faecalis*	Resistant to vancomycin	This study	Queen Elizabeth Hospital, Norfolk
QE126	*Enterococcus faecalis*	Resistant to vancomycin	This study	Queen Elizabeth Hospital, Norfolk
QE127	*Enterococcus faecalis*	Resistant to vancomycin	This study	Queen Elizabeth Hospital, Norfolk
QE128	*Enterococcus faecalis*	Resistant to vancomycin	This study	Queen Elizabeth Hospital, Norfolk
QE129	*Enterococcus faecalis*	Sensitive to vancomycin	This study	Queen Elizabeth Hospital, Norfolk
QE130	*Enterococcus faecalis*	Resistant to vancomycin	This study	Queen Elizabeth Hospital, Norfolk
QE131	*Enterococcus faecalis*	Resistant to vancomycin	This study	Queen Elizabeth Hospital, Norfolk
QE132	*Enterococcus faecalis*	Resistant to vancomycin	This study	Queen Elizabeth Hospital, Norfolk

ID	Species	Properties	Reference	Obtained From
QE133	*Enterococcus faecalis*	Resistant to vancomycin	This study	Queen Elizabeth Hospital, Norfolk
QE134	*Enterococcus faecalis*	Resistant to vancomycin	This study	Queen Elizabeth Hospital, Norfolk
QE135	*Enterococcus faecalis*	Resistant to vancomycin	This study	Queen Elizabeth Hospital, Norfolk
QE136	*Enterococcus faecalis*	Sensitive to vancomycin	This study	Queen Elizabeth Hospital, Norfolk
TRACA1	*Escherichia coli* K-12	Contains plasmid pSPW01	This study	Clone from salival DNA
TRACA2	*Escherichia coli* K-12	Contains plasmid pSPW11	This study	Clone from salival DNA
TRACA3	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA4	*Escherichia coli* K-12	Contains plasmid pSPW12	This study	Clone from salival DNA
TRACA5	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA6	*Escherichia coli* K-12	Contains plasmid pSPW02	This study	Clone from salival DNA
TRACA7	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA8	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA9	*Escherichia coli* K-12	Contains plasmid pSPW03	This study	Clone from salival DNA
TRACA10	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA11	*Escherichia coli* K-12	Contains plasmid pSPW20	This study	Clone from salival DNA
TRACA12	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA13	*Escherichia coli* K-12		This study	Clone from salival DNA

ID	Species	Properties	Reference	Obtained From
TRACA14	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA15	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA16	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA17	*Escherichia coli* K-12	Contains plasmid pSPW21	This study	Clone from salival DNA
TRACA18	*Escherichia coli* K-12	Contains plasmid pSPW14	This study	Clone from salival DNA
TRACA19	*Escherichia coli* K-12	Contains plasmid pSPW09	This study	Clone from salival DNA
TRACA20	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA21	*Escherichia coli* K-12	Contains plasmid pSPW15	This study	Clone from salival DNA
TRACA22	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA23	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA24	*Escherichia coli* K-12	Contains plasmid pSPW10	This study	Clone from salival DNA
TRACA25	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA26	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA27	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA28	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA29	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA30	*Escherichia coli* K-12		This study	Clone from salival DNA

ID	Species	Properties	Reference	Obtained From
TRACA31	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA32	*Escherichia coli* K-12	Contains plasmid pSPW16	This study	Clone from salival DNA
TRACA33	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA34	*Escherichia coli* K-12	Contains plasmid pSPW22	This study	Clone from salival DNA
TRACA35	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA36	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA37	*Escherichia coli* K-12	Contains plasmid pSPW04	This study	Clone from salival DNA
TRACA38	*Escherichia coli* K-12	Contains plasmid pSPW05	This study	Clone from salival DNA
TRACA39	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA40	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA41	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA42	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA43	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA44	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA45	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA46	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA47	*Escherichia coli* K-12		This study	Clone from salival DNA

ID	Species	Properties	Reference	Obtained From
TRACA48	*Escherichia coli* K-12	Contains plasmid pSPW17	This study	Clone from salival DNA
TRACA49	*Escherichia coli* K-12	Contains plasmid pSPW18	This study	Clone from salival DNA
TRACA50	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA51	*Escherichia coli* K-12	Contains plasmid pSPW19	This study	Clone from salival DNA
TRACA52	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA53	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA54	*Escherichia coli* K-12	Contains plasmid pSPW07	This study	Clone from salival DNA
TRACA55	*Escherichia coli* K-12	Contains plasmid pSPW08	This study	Clone from salival DNA
TRACA56	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA57	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA58	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA59	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA60	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA61	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA62	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA63	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA64	*Escherichia coli* K-12		This study	Clone from salival DNA

ID	Species	Properties	Reference	Obtained From
TRACA65	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA66	*Escherichia coli* K-12	Contains plasmid pSPW23	This study	Clone from salival DNA
TRACA67	*Escherichia coli* K-12		This study	Clone from salival DNA
TRACA68	*Escherichia coli* K-12	Contains plasmid pSPW24	This study	Clone from salival DNA
S2-1	*Streptococcus salivarius*	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S2
S2-2	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S2
S2-3	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S2
S2-4	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S2
S2-5	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S2
S2-7	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S2
S2-8	*Streptococcus salivarius*	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S2
S2-9	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S2
S2-10	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S2
S2-11	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S2
S2-12	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S2
S2-13	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S2
S2-14	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S2

ID	Species	Properties	Reference	Obtained From
S2-15	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S2
S2-16	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S2
S2-17	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S2
S28-1	*Rothia dentocariosa*	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-2	*Rothia dentocariosa*	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-3	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-4	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-5	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-6	*Streprococcus sanguinis*	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-7	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-8	ND	Resistant to tetracycline	This study	Isolate from saliva sample S28
S28-9	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-10	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-11	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-12	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-13	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-14	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28

ID	Species	Properties	Reference	Obtained From
S28-15	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-16	ND	Resistant to erythromycin	This study	Isolate from saliva sample S28
S28-17	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-18	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-19	ND	Tetracycline and erythromycin sensitive	This study	Isolate from saliva sample S28
S28-20	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-21	*Streptococcus haemolyticus*	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-22	ND	Resistant to tetracycline	This study	Isolate from saliva sample S28
S28-23	ND	Resistant to tetracycline	This study	Isolate from saliva sample S28
S28-24	ND	Resistant to tetracycline	This study	Isolate from saliva sample S28
S28-25	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-26	ND	Tetracycline and erythromycin sensitive	This study	Isolate from saliva sample S28
S28-27	ND	Resistant to tetracycline	This study	Isolate from saliva sample S28
S28-28	ND	Resistant to tetracycline	This study	Isolate from saliva sample S28
S28-29	ND	Tetracycline and erythromycin sensitive	This study	Isolate from saliva sample S28
S28-30	*Neisseria subflava*	Resistant to tetracycline	This study	Isolate from saliva sample S28
S28-31	ND	Resistant to tetracycline	This study	Isolate from saliva sample S28

ID	Species	Properties	Reference	Obtained From
S28-32	ND	Resistant to tetracycline	This study	Isolate from saliva sample S28
S28-33	ND	Resistant to tetracycline	This study	Isolate from saliva sample S28
S28-34	ND	Resistant to tetracycline	This study	Isolate from saliva sample S28
S28-35	ND	Resistant to tetracycline	This study	Isolate from saliva sample S28
S28-36	ND	Resistant to tetracycline	This study	Isolate from saliva sample S28
S28-37	ND	Resistant to tetracycline	This study	Isolate from saliva sample S28
S28-38	ND	Resistant to tetracycline	This study	Isolate from saliva sample S28
S28-39	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-40	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-41	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-42	*Streptococcus salivarius*	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-43	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-44	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-45	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-46	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-47	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-48	ND	Resistant to tetracycline	This study	Isolate from saliva sample S28

ID	Species	Properties	Reference	Obtained From
S28-49	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-50	ND	Resistant to tetracycline	This study	Isolate from saliva sample S28
S28-51	ND	Resistant to tetracycline	This study	Isolate from saliva sample S28
S28-52	ND	Resistant to tetracycline and erythromycin	This study	Isolate from saliva sample S28
S28-53	ND	Tetracycline and erythromycin sensitive	This study	Isolate from saliva sample S28