Transfer of antibiotic resistances between enterococci in biofilm

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DECLARATION

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ABSTRACT

Antibiotic resistance is an urgent and increasing problem in human and animal healthcare. While it is recognised that the environment must provide an opportunity for resistance to develop and spread, direct evidence for the mechanisms involved is still lacking. This research utilised enterococci from an agrarian environment to demonstrate horizontal gene transfer (HGT) of antibiotic resistance genes in specific reservoirs.

Screening a biobank of over 600 environmental isolates referencing previous partial characterisation data resulted in the selection of eleven *Enterococcus faecalis* and four *E. faecium* strains that exhibited potential for conjugation via a pheromone-dependent pathway. These isolates had prolific and diverse antimicrobial resistance profiles. Conjugal transfer of antibiotic resistance phenotypes was determined using a solid agar mating method followed by a standard antibiotic selection test resulting in different transfer patterns. Multiple gene transfer was observed in single reactions. An interspecies conjugal transfer of vancomycin resistance from *E. faecalis* to *E. faecium* was identified while the remaining reactions were within the same species. Transfer efficiencies ranging from 2×10^{-1} to 2.3×10^{-5} were determined. Interspecies transfer of vancomycin resistance among environmental isolates of enterococci had not previously been characterised, along with alternating transfer of different determinants from the same donor to different recipients. In certain cases, antimicrobial resistance to non-transferred resistance was elevated in transconjugants (T1, T2 and T4).

A novel biofilm apparatus model, based upon a Gene Frame, was developed to allow non-destructive analysis of *Enterococcus* biofilm. Fluorescently tagged Concanavalin A was used to label extracellular matrix material and bacteria were identified by fluorescent *in situ* hybridization (FISH) and DAPI staining. This unique model was more reproducible

than standard biofilm assays and it proved to be flexible in that it was adapted to identify antibiotic resistance genes. This novel system was used to demonstrate that interspecies transfer of vancomycin resistance takes place in bacterial biofilms, which are considered to be the natural state for environmental bacteria. Additionally, multiprobe FISH targeted to vanA on mobile elements demonstrated for the first-time vancomycin staining inside enterococcal biofilm.

Another potential reservoir of bacteria in an aquatic environment is the freshwater sponge. *Enterococcus* conjugation experiments were performed on *Ephydatia fluviatilis* and *Spongilla lacustris*, two sponge species that exist in similar geographical topography to where the enterococci were isolated. Enterococci were shown to bind to sponge material and HGT of vancomycin resistance was demonstrated in both sponge species by the modified FISH assay and by direct antibiotic selection methods.

Overall, this thesis highlights that enterococci of environmental origin are capable of transferring important resistance determinants in and out of biofilm, of their own construction. Their survival under harsh environmental conditions, such as low temperature and nutrient limitation, reduced but did not eliminate their ability to conjugate. *Enterococcus faecalis* and *faecium* have the potential to propagate antimicrobial resistance in the natural environment. An assessment of the impact of environmental conditions on HGT rates could help to preserve useful antibiotics from selection for resistance.

ABBREVIATIONS

µg/L	Micrograms per litre
30/50S	30/50 subunit of the ribosome
aac(6')-li aph(2'')-Ia	Aminoglycoside acetyltransferase
aada	Aminoglycoside 3"-adenylyltransferase gene
ace	Collagen binding molecule gene
ALS1,2,3	Alkaline lysis solution 1,2,3
AMR	Antimicrobial resistance
ant(6)	Streptomycin resistance gene
APH(2'')-I	Aminoglycoside phosphotransferase
asa1	Aggregation substance gene
Asc10	Alternative name for aggregation substance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BHI	Brian heart infusion
BLAST	Basic local alignment search tool
BopD	Putative maltose binding protein
CBD	Calgary biofilm device
CFU	Colony forming units
cls	Cardiolipin synthetase
CLSI	Clinical and Laboratory Standards Institute
ComEA	DNA binding membrane protein
conA	Concanavalin A
CTnDOT	Conjugative transposon

CV	Crystal violet
DBR	Drip-flow biofilm reactor
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide mix
DprA	mediator for DNA internalisation
dsDNA	Double stranded-deoxyribonucleic acid
ECDC	European centre for disease prevention and control
ECOFF	Environmental cut-off value
eDNA	Extracellular- deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
Eep	Enhanced expression of pheromone gene
EfmM	Intrinsic rRNA methyltransferase
EmeA, EfrAB	ABC multidrug pump
EndA	DNA nuclease
ENF	*Enterococcus faecalis* probe
EPS	Extracellular polymeric substance
ermB	Erythromycin resistance gene
esp	Enterococcal surface protein gene
EU/EEA	European union/European economic area
EUB	General bacteria probe
EUCAST	European committee on antimicrobial susceptibility testing
FASTA	FAST-All nucleotide sequence format

FISH	Fluorescent *in situ* hybridisation
Fsr	Quorum sensation system
G2-A	Green filter - absorbance
GBA	Gene-frame biofilm apparatus
gdpD	Glycerophosphoryl diester phosphodiesterase gene
gelE	Gelatinase gene
GFP	Green Fluorescent Protein
GI	Gastro-intestinal
GyrA/B	DNA gyrase subunit A/B
H_2O	Dihydrogen oxide
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HGT	Horizontal gene transfer
iCF10	Inhibitor peptide for pCF10
Inc	Incompatibility plasmids
INT	Iodonitrotetrazolium chloride
kb	Kilobase
Ldt_{fm}	Transpeptidase
LexA	Damage response suppressor gene
liaF	Cell envelope remodelling gene
Lsa	ABC transporter gene
MDR	Multidrug resistant
mecA	Methicillin resistance gene
mefA	Macrolide resistance determinant

$Mg2^{+}$	Magnesium
$MgCl_2$	Magnesium chloride
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
MIC90	Minimum inhibitory concentration for 90% inhibition
ml	Millilitre
MLS_B	Macrolide-lincosamide-streptogramin B
MLST	Multilocus sequence typing
mm	Millimetre
mM	Millimolar
Mob	DNA mobilisation genes
Mpf	Mating pair formation genes
MprF	Lysylphosphatidylglycerol synthetase gene
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMM	Microbial surface components recognising adhesive matrix molecule's
N	Normality
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National centre for biotechnology information
ng/L	Nanograms per litre
NIH	National institutes of health
NIS	Nikon instruments software
NPET	Nascent peptide exit tunnel
OD	Optical density

oriT	Origin of replication gene
p	Probability
ParC/E	Topoisomerase IV genes
PBP	Penicillin binding proteins
PBS	Phosphate buffered saline
pCF10	Conjugal plasmid
PCR	Polymerase chain reaction
PilQ	Type 4 pilin gene
PrgB	Alternative name for aggregation substance gene
PrgY	Inhibitor for plasmid pCF10 gene
PrgZ/X	Membrane bound receptor for aggregation signalling gene
PTC	peptidyl transferase centre
PVC	Polyvinyl chloride
qepA	Quinolone efflux pump gene
qnrA/B/C/D/S	Pentapeptide repeat protein genes
QRD	Quinolone resistance determinants
RCF	Relative centrifugal force
RecA	Recombinase A
RI	Resistance integrons
Rif	Rifampicin
RING	Recognition of individual genes
RNA	Ribonucleic acid
rpm	Revolutions per minute
rpoB	Beta subunit of RNA polymerase

rRNA	Ribosomal-ribonucleic acid
scm	Collagen binding molecule gene
SDS	Sodium dodecyl sulphate
SM*qnr*	Quinolone resistance gene
SNP	Single nucleotide polymorphisms
SodA	Enterococcal superoxide dismutase gene
sp	Species
SprE	Fratricide gene
SYTO9	Green fluorescent nucleic acid stain
T1,2,3,4	Transconjugants
T4P	Type 4 pilin
TAMI	The antimicrobial index
Taq	Taq polymerase
TBR	Tubular biofilm reactor
TDR	Totally drug resistant
TE	Tris-EDTA buffer
tetK	Tetracycline resistance gene
tetL	Tetracycline resistance gene
tetM	Tetracycline resistance gene
TetO	Tetracycline resistance gene
tetS	Tetracycline resistance gene
tetT	Tetracycline resistance gene
tetW	Tetracycline resistance gene
tfp	Transformation pilus

Tn	Transposon
Tra	Transfer genes encoding pili
Tris-Cl	Tris hydrochloride
tRNA	Transfer-ribonucleic acid
TSA	Tryptone soy agar
TSB	Tryptone soy broth
UK	United Kingdom
USA	United states of America
UTI	Urinary tract infection
UV	Ultra violet
vanA	Vancomycin resistance type A gene
vanB	Vancomycin resistance type B gene
VBNC	Viable but not culturable
vol/vol	Volume per volume percentage
VRE	Vancomycin-resistant enterococci
w/v	weight per volume percentage
XDR	Extremely drug resistant
YycG	Histidine kinase gene

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Chapter 1

General Introduction

1.1. Introduction

The continual escalation of antibiotic resistance in human and animal pathogenic bacteria is a serious threat to public health. Multi-resistant organisms such as methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant enterococci (VRE) are major threats to human health prognosis in hospitals across the world (Warnke *et al.,* 2013). These multi-resistant bacteria are also being identified in the natural and built environment (Huijbers *et al.,* 2015). Understanding the dynamics of resistance transfer will be essential in determining how best to minimise this problem. In the last decade, a fundamental reappraisal of how bacteria grow under natural conditions has taken place (Haruta and Kanno, 2015). It is now clear that many bacteria exist as part of complex communities attached to surfaces, embedded in polymeric matrices of their own devising (Flemming *et al.,* 2016). This is known as a biofilm. Biofilms were first identified in aquatic environments, such as rock surfaces in streams, but have now been recognised as major contributors to infection (Høiby *et al.,* 2014). They allow colonisation of catheters and other indwelling devices, as well as forming directly on body surfaces e.g. chronic wounds and the lung epithelia of individuals suffering from Cystic Fibrosis (CF) (da Silva *et al.,* 2015). In spite of the recognition of the ubiquitous nature of biofilms, it is not known how the physiological processes within them are regulated (Santos-Beneit, 2015; O'Toole and Wong, 2016). Antibiotic resistance transfer mechanisms are well described for bacteria growing in planktonic culture, but understanding on how efficiently they function in biofilm are limited (Van Acker *et al.,* 2014). This review recounts the mode of action, biochemical mechanisms and origins of antimicrobial resistance. It considers the current environmental sources of antibiotic resistance. Finally, it discusses the dissemination of antibiotic resistance through extensive gene transfer events, using

intermediaries such as biofilm, all with relevance to enterococci as a model organism for studying such processes.

Members of the genus *Enterococcus*, have developed resistance to a significant number of antibiotics, as discussed further in sections 1.3 and 1.4. They have increasingly become more infectious to humans and they are now widely implicated in bacterial disease progression. Their natural environment, in the gut of warm-blooded animals, is conducive for antibiotic resistance development. Many enterococcal antibiotic resistance genes are capable of being transferred to other bacteria. The unique characteristics of enterococci are discussed within this literature review and, collectively, they present enterococci as useful model organisms for studying processes of bacterial conjugation on these novel substrates in an effort to understand the escalating global emergence of antibiotic resistance.

1.2. *Enterococcus* – genus, species and infection

Enterococcus is a genus of lactic acid gut bacteria that are facultative anaerobes (Domig *et al.,* 2003). Different species have been found in human (*E. faecalis, E. faecium, E. caccae*), animal (*E. faecium, E. canintestini*) and insect guts (*E. casseliflavus, E. faecalis, E. faecium*). They have been recovered from fermented foodstuffs (*E. hermanniensis, E. thailandicus*) and dairy produce (*E. italicus, E. lactis*), as well as from soil (*E. durans, E. hirae*) plant (*E. plantarum*) and aquatic (*E. aquimarinus, E. quebecensis*) microcosms (Mundt, 1961; Mundt, 1963; Ator and Starzyk, 1976; Mundt, 1986; Lebreton *et al.*, 2014). Their characteristic ubiquitous nature reflects their hardiness, with the ability to grow under elevated temperature (45^{o}C), pH (pH 9.6) and salinity (6.5% NaCl) (Sherman, 1938). There are 36 identified species within the genus and, to date, 26 have been

associated with human infection (Ruoff *et al.,* 1990; Teixeira and Merquior, 2013; Lebreton *et al.,* 2014).

Enterococci have become some of the most prevalent healthcare associated pathogens (Arias and Murray, 2012). Modernisation of bacterial disease interventions as well as prophylactic treatments prior to invasive medical procedures has contributed to increased prevalence of enterococci (DiazGranados *et al.,* 2005). Enterococcal infections can be extensively resistant to antibiotics (Muray, 1998). Vancomycin resistance in enterococci historically has been the most concerning in the nosocomial environment due to the importance of vancomycin as a drug of last resort (Taneja *et al.,* 2004). Nosocomial enterococci have been identified as resistant to penicillin, aminoglycosides, linezolid, quinupristin/dalfopristin, daptomycin, tetracycline and erythromycin (Gilmore *et al.,* 2014).

The most infectious multidrug resistant enterococci are *E. faecium* and then *E. faecalis* (Moellering, 1992). Over 50% of all identified pathogenic *E. faecium* are multidrug resistant (Hidron *et al.,* 2008). In the same study Hidron *et al.* (2008) identified that 40% of medical device associated infections were due to vancomycin and ampicillin resistant *E. faecium* only. *E. faecalis* is less commonly resistant to vancomycin and is the primary causative agent for human endocarditis (Murdoch *et al.,* 2009). Other infective enterococci include *E. durans, E. avium, E. gallinarum* and *E. casseliflavus* (Gordon *et al.,* 1992). Enterococcal pathogenesis typically results in urinary tract infections, abdominal, pelvic and soft tissue infections and they can translocate to cause bacteraemia, endocarditis and even septicaemia (Fisher and Phillips, 2009).

Antibiotics have been effective antimicrobial agents used to combat bacterial infection (Lorian, 2005). They are split into classes based on their chemical structure and mode of

action (Walsh, 2003). They act through several pathways to either kill or inhibit the growth of bacteria, as discussed below.

1.3. Antibiotics: mode of action

1.3.1. Macrolides

Macrolides are an effective group of antibiotics against bacteria and have historically been used to treat serious infection. Their general mode of action works through inhibiting bacterial protein synthesis, thus arresting bacterial growth (Dunkle *et al.,* 2010). Macrolides interact with the ribosomes in the nascent peptide exit tunnel (NPET) (Schlünzen *et al.,* 2001). The NPET is a channel that allows the passage of newly assembled polypeptides from the peptidyl transferase centre (PTC) (Tu *et al.,* 2005). The macrolide-ribosome binding interaction in these narrow conduits acts as a barrier for the transfer of polypeptides from the PTC through the NPET, effectively reducing protein synthesis through a bottleneck (Menninger, 1985). As the macrolide-ribosome interaction blocks the NPET it has been suggested that proteins can still pass through the blockade and that polypeptide assembly does not fully shut down (Tu *et al.,* 2005; Kannan *et al.,* 2012). Recent work has determined that the macrolide mode of action has additional functions (Ramu *et al.,* 2011). Not only do macrolides create a blockade in the NPET, they can stop synthesis of peptides at the amino acid level when bound to the ribosome (Arenz *et al.,* 2014; Sothiselvam *et al.,* 2014). The ribosome-macrolide interaction can act specifically on recognisable C-terminal amino acids during assembly from the peptidyl donors and the aminoacyl-tRNA acceptor, arresting translation (Kannan *et al.,* 2014).

1.3.2. Streptogramins and lincosamides

Streptogramins and lincosamides are structurally different classes of antibiotics that utilise a general mode of action for antibacterial action (Wilson, 2009). They all bind to the ribosomal peptidyl transferase centre on the 50S ribosome. Specific binding occurs at the bottom of the cleft where peptide transfer occurs within overlapping binding sites of the oxazolidinones (Long and Vester, 2016). Streptogramins bind adjacent to the peptidyl transferase centre at the entrance to the NPET, similar to the macrolides (Poehlsgaard and Douthwaite, 2005). They work by interfering with peptide transfer of peptidyl/amino acyl-tRNA or largely blocking transfer. Lincosamides bind adjacent to the A site of the peptidyl transferase centre (Gaillard *et al.*, 2016).

1.3.3. Beta-lactams

Beta-lactam antibiotics are some of the oldest and most utilised used drugs against bacteria. The general mode of action of beta-lactams functions through disruption of the peptidoglycan cell wall of bacteria (Page, 2012). The drugs target a variety of high and low molecular weight penicillin binding proteins (PBP) (Tipper and Strominger, 1965). These PBPs, of which there are two classes (A and B), carry out the functions of polymerising glycan strands (glycotransferase) and crosslinking them (transpeptidase) during peptidoglycan synthesis (Sauvage *et al.*, 2008). Class A PBPs contain both glycotransferase and transpeptidase functions, whereas class B contain only transpeptidase activity (Cho *et al.*, 2014). Beta-lactams directly target the transpeptidase sites located on PBPs covalently modifying them (Uehara and Bernhardt, 2011). Additionally, inhibition of transpeptidase acts to deplete glycan and other precursors, which has a compounding effect on all aspects of peptidoglycan complexes, even those unrelated to the targets of beta-lactams (Cho *et al.*, 2014). The targeting of several PBP's

and the secondary depletion of peptidoglycan precursors acts to effectively stave off peptidoglycan modelling, initiating breakdown and compromising the bacterial cell wall leading to cell death and, ultimately, lysis (Uehara *et al.*, 2009).

1.3.4. Aminoglycosides

Aminoglycosides are a class of broad-spectrum of antibiotics with variations in their structural composition, containing amino-modified glycosides (Wang and Bertozzi, 2001). Aminoglycosides mode of action works through binding to the 30S subunit of ribosomes (Bryan and Kwan, 1983). Aminoglycosides act on the rRNA and binding disrupts protein synthesis, primarily by disabling ribosome proofing during protein translation, a mechanism vital for cell survival (Davis, 1987). Aminoglycoside binding to rRNA varies between drugs. Neomycin and gentamycin can bind to the A site targeting the 16S rRNA via interaction with bases A1409 and G1494, whereas paromomycin binds to four bases in the same site (A1408, A1493, C1407-G1494, and U1495) (Noller, 1991; Recht *et al.,* 1996). They also destabilise the cell membrane through altering the Mg^{2+} bridges (Shakil *et al.,* 2008).

1.3.5. Fluoroquinolones

Fluoroquinolones are a group of topoisomerase inhibitors that can form a complex with DNA gyrase and topoisomerase IV, disabling DNA replication and instigating dsDNA breaks (Hooper, 2001). Fluoroquinolones directly act upon topoisomerase IV, which contains subunits ParC and ParE, which function to bind DNA and hydrolyse ATP respectively (Champoux, 2001; Wang, 2002). They also act upon the subunits GyrA and GyrB of DNA gyrase which function in the same way as ParC and ParE respectively

(Bates *et al.*, 2011; Vos *et al.*, 2011). Fluoroquinolones form a ternary complex with the topoisomerase-DNA complex inhibiting DNA replication through arrest of topoisomerase functionality (Heddle *et al.*, 2000; Anderson and Osheroff, 2001; Drlica and Malik, 2003). They can also overstimulate topoisomerase dsDNA strand breakage consequentially inducing cell death (Drlica, and Malik, 2003; Nitiss, 2009).

Anderson *et al.* (1998) demonstrated that fluoroquinolones stimulate topoisomerase IV to carry out simultaneous dsDNA breakage and arrest re-ligation. Oppegard *et al*. (2016) determined that when fluoroquinolones form a ternary complex with the topoisomerase-DNA complex, they either arrest re-ligation through low levels of Mg^{2+}, or they catalyse dsDNA breakage through high levels of Mg^{2+}, leading to unstable DNA interactions. The fluoroquinolones alter the DNA structure affecting Mg^{2+} metal binding affinity explaining the Mg^{2+} induced variation in the drugs modes of action.

1.3.6. Glycopeptides

Glycopeptide antibiotics general mode of action works through inhibition of peptidoglycan biosynthesis which results in cell death (Cegelski *et al.*, 2002). Glycopeptides bind to the peptidoglycan precursor lipid II, preventing the transportation of lipids essential for peptidoglycan assembly, through the inhibition of the bactoprenol-phosphate (C_{55}-P) recycling process (Schneider *et al.*, 2009). This also negatively affects the assembly of wall teichoic acid, thereby affecting two of the major components of the Gram positive cell wall compromising cellular integrity and killing the cell (Swoboda *et al.*, 2010).

Vancomycin is a well-known glycopeptide (Kirst *et al.*, 1998). Its mechanism of peptidoglycan inhibition functions through attachment to the D-Ala-D-Ala terminus on

lipid II (Barna and Williams, 1984). Vancomycin-lipid II complex becomes sequestered, unable to undergo transglycosylation terminating C_{55}-P regeneration (Arthur *et al.,* 1992; Billot-Klein *et al.,* 1992). The sequestered complex (Park's nucleotide) builds in the cytoplasm, unable to be recruited for peptidoglycan synthesis (Cegelski *et al.,* 2002). The lipoglycopeptide oritavancin was developed to target VRE and MRSA and it has been discovered that the mode of action of this drug acts primarily through inhibition of the wall teichoic acid which yields the same biocidal effects as other glycopeptides (Singh *et al.,* 2017).

1.3.7. Lipopeptides

Lipopeptides are a diverse group of antibiotics with a good degree of variation in their mode of action (Baltz *et al.,* 2005). Daptomycin is one of the most recent lipopeptides to be used as an effective antibiotic for Gram positive infections (Straus and Hancock, 2006). The mechanism is therefore not fully understood (Miller *et al.,* 2016). The molecule shares similarity to the cationic antimicrobial peptides found as part of the mammalian immune response. Their molecular mechanisms have been postulated based on shared structure and therefore antibiotic activity (Straus and Hancock, 2006).

The two major postulations on daptomycin mode of action share similarity and are as follows. Calcium-bound daptomycin eases its way inside the bacterial membrane, where it forms a pore-like structure negating membrane potential through ion leakage (Silverman *et al.,* 2003). Zhang *et al.* (2014a) demonstrated that daptomycin can associate itself into a pore-like structure on the cell membrane in the presence of the phospholipid phosphatidylglycerol, with two oligomers arranged individually two-by-two. Another article from members of the same group, Zhang *et al*. (2014b) determined that daptomycin

action on liposomes permeabilised membranes to anions but not cations. This agrees with the postulated one way de-polarisation of cellular membrane potential mode of action.

The second postulation comes from work carried out by Chen *et al.* (2014). This research group identified that daptomycin membrane incorporation yields a calcium and phospholipid phosphatidylglycerol expansion of the outer membrane vesicle. Continuous incorporation of daptomycin causes lipids to accumulate at the surface of the membrane, whilst the vesicle reduces in size. This increase and then decrease of the vesicle suggests recruitment of lipids from the vesicle membrane. As this process reaches maturation, water-pores temporarily form, potentially allowing ion leakage and membrane destabilisation. This would agree with work conducted by Pogliano *et al.* (2012) Whereby they visualise daptomycin-mediated distortion of *B. subtilis* cellular membranes by using fluorescent microscopy.

1.3.8. Rifampicin

Rifampicin is a semisynthetic antibiotic that was primarily designed to target *Mycobacterium tuberculosis* (Heifets, 1994). The exact details of its mechanism of action have not yet been fully elucidated (Unissa and Hanna, 2017). What is understood is that rifampicin binds to RNA polymerase, an enzyme which catalyses RNA polymerisation during DNA transcription. Rifampicin attaches directly to the beta-subunit of the polymerase, genetically encoded by *rpoB* (Campbell *et al.*, 2001). The binding complex inhibits the bound beta-subunit, terminating RNA polymerisation after it starts to function, leading to cell death (Somoskovi *et al.*, 2001; Floss and Yu, 2005).

1.3.9. Oxazolidinones

Oxazolidinones such as linezolid and tedizolid are fully synthetic antibiotics with strong bacteriostatic effects on Gram positive bacteria. Their general mode of action acts through inhibition of bacterial protein synthesis. Like the macrolides, oxazolidinones bind to the A site of the peptidyl transferase centre on the 50S ribosomal subunit only (Leach *et al.*, 2007). They therefore block the attachment of the growing amino acyl chain to the amino acyl tRNA during protein translation (Shinabarger, 1999). The binding sites for oxazolidinones overlaps with those of clindamycin and chloramphenicol (Douros *et al.*, 2015). Shaw *et al.* (2008) determined that tedizolid also had affinity with 23S rRNA peptidyl transferase, suggesting secondary antibacterial functionality. However, as tedizolid is a new drug, further research is required to identify the mechanism (Zhanel *et al.*, 2015).

The antibiotic classes mentioned here have been historically used to treat Gram positive infections amongst others. The caveat with their mode of action is that bacteria such as *E. faecalis*, *E. faecium*, *S. aureus* and *M. tuberculosis* display resistance mechanisms to all these antibiotics.

1.4. Antibiotic resistance: biochemical mechanisms

The specific mechanism of antibiotic resistance to the antibiotics mentioned above are discussed here. All these resistance mechanisms can be found with a degree of smiliarity in *E. faecalis* and *E. faecium*.

1.4.1. Macrolides, streptogramins, and lincosamides

The general mechanism of bacterial macrolide, streptogramin and lincosamide resistance involves the use of an enzyme (MLS_B) encoded in *ermB* (Pérez-Trallero *et al.,* 2007). This enzyme can methylate an adenine subunit on the 23s rRNA (50S ribosome) reducing the ribosome binding affinity for these drugs (Johnston *et al.,* 1998). Additional biochemical mechanisms include the macrolide efflux pump *mefA* in *Streptococcus pyogenes* (Clancy *et al.,* 1996). The presumptive ABC transporter *Lsa* in *E. faecalis V583* is able to increase the MIC of members of the streptogramin family (quinupristin-dalfopristin) as compared to *E. faecium* without *Lsa* (Singh and Murray, 2005).

1.4.2. Beta-lactams

Beta-lactam resistance varies slightly depending on the specific drug member of the family. Penicillins, carbapenems and cephalosporins have decreasing effectiveness in enterococci. Penicillins are effective on susceptible isolates, while use of cephalosporins can actually promote enterococcal infection due to the prevalence of high levels of resistance (Shepard and Gilmore, 2002). Resistance to beta-lactams involves Pbp5 in enterococci and Pbp2a in *S. aureus* (Gonzáles *et al.,* 2001). These are low affinity class B penicillin binding proteins, which can continue to synthesise peptidoglycan in the presence of beta-lactams (Canepari *et al.,* 1986). Pbp5 in enterococci has been shown to confer resistance to ampicillin and cephalosporins (Rice *et al.,* 1991; Arbeloa *et al.,* 2004).

1.4.3. Aminoglycosides

Aminoglycoside resistance mechanisms can vary depending on the species of bacteria. Enterococci can have a moderate tolerance to aminoglycosides due to poor drug uptake. This often can result in species of enterococci with significant minimal inhibitory concentration (MIC) values for aminoglycosides (Chow, 2000). Aslangul *et al*. (2006) demonstrated poor uptake of gentamycin in gentamycin resistant enterococci, without identification of specific resistance genes. An intrinsic rRNA methyltransferase (EfmM) was identified as contributing to kanamycin and tobramycin resistance in *E. faecium* through methylation of the C1404 residue on 16S rRNA (30S ribosome) (Galloway-Peña *et al.,* 2012). This significantly reduced aminoglycoside binding affinity. Secondarily, aminoglycoside acetyltransferases such as encoded by *aac(6')-li* contribute to intermediate levels of aminoglycoside resistance in enterococci (Costa *et al.,* 1993)

Enterococci also possess significantly higher levels of aminoglycoside resistance located on their 'mobilome' and the biochemical mechanism operates through enzymatic drug modification. Examples include other aminoglycoside acetyltransferases (*AAC(6')-Ie-APH(2'')-Ia)* and phosphotransferases (*APH(2'')-I*) (Kristich *et al.,* 2014). These enzymes typically phosphorylate (ATP) a hydroxyl group or acetylate (acetyl-CoA) amino groups. Nucleotidyltransferases also function to adenylate (ATP) hydroxyl groups on aminoglycosides.

1.4.4. Fluoroquinolones

Quinolone (fluoroquinolone) resistance is expressed through several biochemical resistance mechanisms. The quinolone resistance determinants (QRD) are a range of genes across several species of bacteria which code for mutated topoisomerase IV and

DNA gyrase. Examples include SM*qnr, qnrA/B/C/D/S*, (pentapeptide repeat proteins). These resistance determinants have been identified in *Stenotrophomonas maltophilia, Escherichia coli, Enterobacteriaceae,* and some homologues in *E. faecalis* (Arsene & Leclercq, 2007; Aedo *et al.,* 2014; Kanamori *et al.,* 2015; Yanat *et al.,* 2017). These QRDs cannot complex with quinolones and as such abolishes the bactericidal effects of the drug.

The second mechanism of quinolone resistance involves the use of multidrug efflux pumps such as the *qepA* quinolone efflux pump in *S. maltophilia* and *Enterobacteriaceae*. Enterococci, such as *E. faecalis V583* have been predicted to have multidrug efflux functionality, with 34 presumptive pumps (Pazoles *et al.,* 2001). Two *E. faecalis* efflux pumps *EmeA* and *EfrAB (ABC multidrug pump)* have presumed function as quinolone efflux pumps. *EmeA* has had quinolone efflux confirmed and *EfrAB* has been suggested to function in the same way due to similarity to the *E. coli* pump (Lefort *et al.,* 2000; Moon *et al.,* 2010).

1.4.5. Glycopeptides

The biochemical mechanism of glycopeptide resistance involves modification of the antibiotic target. Enterococci that are glycopeptide resistant encode the production of mutated peptidoglycan precursors which significantly reduce the binding affinity of glycopeptide antibiotics. The usual D-Ala-D-Ala end termini are either modified to a D-lactate (x1000 reduction in binding affinity) or D-ser (x7 reduction in binding affinity). These mutated precursors retain their original functionality and can be used to produce peptidoglycan.

Cremniter *et al.* (2006) described a secondary glycopeptide resistance mechanism in *E. faecium* involving activation of a secondary transpeptidase (Ldt_{fm}). This transpeptidase

could crosslink peptidoglycan chains at the third position (L-Lys) rather than the fourth position (D-Ala) that typically is used by the penicillin binding proteins. Additionally, the peptide precursors were synthesised as tetrapeptides (using D, D-carboxypeptidase) over the traditional pentapeptides, producing a zero-binding affinity for glycopeptides.

1.4.6. Lipopeptides

The mode of action of daptomycin has not been unequivocally identified. Resistance to the drug has mostly been identified in bacteria (*S. aureus*) isolated from patients, post-therapy (Hayden *et al.*, 2005). Biochemical resistance mechanisms remain elusive, but have been associated with staphylococcal intrinsic gene mutations in *rpoB/C* (RNA polymerase subunits), *MprF* (lysylphosphatidylglycerol synthetase) and *YycG* (histidine kinase) (Galimand *et al.*, 2011). However, in daptomycin resistant *E. faecium* these genes were not mutated in the tested strains (Muller *et al.*, 2006). Arias *et al.* (2011) created daptomycin resistant enterococcal strains in a similar fashion to the daptomycin resistant *S. aureus*, and did not identify mutations in the four associated staphylococcal resistance genes within the enterococci. They did identify polymorphisms in the phospholipid associated *cls* and *gdpD* genes in enterococci, as well as *liaF* (part of the cell envelope remodelling in response to antibiotics), however this did not translate to gene mutations in *S. aureus*. This has suggested separate mechanisms of antibiotic resistance for different Gram positive species of bacteria.

Daptomycin resistant enterococci had distinct alterations to the cell envelope requiring further investigations for significance. These mutations along with the structural alterations have been suggested to modify the cell envelope in such a way as to reduce daptomycin membrane interactions, including cellular uptake.

1.4.7. Rifampicin

The majority of rifampicin resistance originates in mutations to the *rpoB* subunit of RNA polymerase as it is the binding site of the drug. These mutations lower the binding affinity of the drug to the subunit. A secondary mechanism of enzymatic drug inactivation has also been documented. *RpoB* gene polymorphisms have been identified in a large number of different bacterial species. Many of the specific mutations have also been identified across species. Point mutations are enough to significantly reduce rifampicin binding affinity and increase drug MIC. Multiple antibiotic resistance has been documented with *rpoB* mutations. The *rpoB* H486Y mutation significantly increased the MIC of cephalosporin resistance in both *E. faecalis* and *E. faecium* (Kristich and Little, 2012).

The mechanism is not fully understood, but multidrug efflux pumps have been implicated in *M. tuberculosis* as being overexpressed in rifampicin resistance mutations (Louw *et al.*, 2011). Additionally, Cui *et al.* (2010), determined that a *rpoB* mutation (Position 1862 alanine to glutamic acid substitution) in *S. aureus* caused heteroresistance to vancomycin and daptomycin. Conversely, and for reasons yet to be fully identified, daptomycin therapy against a rifampicin resistant enterococci abolished antibiotic resistance (Reynolds and Courvalin, 2005).

1.4.8. Oxazolidinones

Oxazolidinone (Linezolid) resistance originates from mutations to the V domain of 23S rRNA. The G2576U mutation in the central loop of the V domain has been identified as a linezolid resistance mutation (Bozdogan and Applebaum, 2004). It is believed that this mutation reduces the binding affinity of linezolid (Marshall *et al.*, 2002). Martínez-Martínez *et al.* (1998) confirmed observations that selected linezolid resistant *E. faecalis* contain a higher concentration of ribosomes with the G2576U mutation. This has been

suggested as the principle mechanism of amplification of linezolid resistance based on selective pressure from antibiotic therapy (Kristich *et al.,* 2014).

1.5. Development of antibiotic resistance

The number and variation of biochemical and physiological mechanisms responsible for the development of tolerance/resistance to biologically active compounds are vast (Davies, 2010). Bacterial evolution is based on "out surviving" other organisms and comes from significant genetic plasticity allowing varied mutational augmentations, acquisition and exchange of genetic material affecting transcriptional activity. These adaptations of bacterial genetics have allowed the development of resistance to the majority of antibiotics used therapeutically. Several of these resistance mechanisms are not fully understood, such as the specific interactions of the multidrug efflux systems in enterococci or the "spontaneous" generation of total resistance in *Mycobacterium tuberculosis* (Davies and Davies, 2010; Hürlimann *et al.,* 2016; Unissa and Hanna, 2017).

The lack of complete understanding of the dynamics of these evolved antibiotic resistant pathogens and their dissemination has significantly contributed to the rapidly proliferating antibiotic resistance crisis (Losos and Lenski, 2016). Louis Pasteur and Robert Koch identified that bacteria can cause infectious disease. The identification and deployment of benzylpenicillin by Alexander Fleming in the early 20th Century proved that antibiotics were effective therapeutic agents against bacterial infection (Davies and Davies, 2010). Shortly after the deployment of benzylpenicillin, antibiotic development and usage flourished, and so too did the appearance of antibiotic resistance (Hotchkiss, 1951). Antibiotics were exclusively natural compounds for a long period until technology caught up to their complexity. Their biosynthetic pathway and dynamic biological effects

were challenging to identify and understand (Strohl, 1997; Brötze-Oesterhelt and Brunner, 2008). It took 31 years to synthesise benzylpenicillin in the laboratory, owing to its complex chirality and functionality (Nikolaou and Montagnon, 2008). The field of genetics has origins in the physiological modes of action of benzylpenicillin (Vazquez *et al.,* 1969). To this day, knowledge of the fundamental understandings of the natural biological interactions of antibiotics is still limited (Yeh, 2009; Chevereau, 2015).

Bacterial natural resistance to antimicrobials existed before human influence and their mechanisms of resistance largely remain unchanged (Giedraitienė, 2011; Miller, 2014). Before benzylpenicillin could be manufactured for therapeutic use, bacterial resistance (penicillinase) was discovered by members of the Nobel Prize winning penicillin research team (Abraham and Chain, 1940). Sulphonamides were deployed in 1937 targeting bacterial C1 metabolism; and efflux/target alteration resistance emerged in less than three years (Kashmiri and Hotchkiss, 1975; Davies and Davies, 2010).

The conserved nature of bacterial resistance mechanisms and the rapid identification of resistance shortly after, or even before the discovery of the therapeutic use of an antibiotic, indicated that a significant quantity of bacterial resistance genes evolved before human influence (D'Costa, 2011; Gaze, 2013). This occurred most likely in microcosms of natural selection between microbiota (D'Costa *et al.,* 2006). Usage of therapeutic concentrations of antibiotics selects for heterogeneously resistant clones of an individual species of bacteria. Therefore, perpetuating pre-existent resistant genes initially generated from instances of natural selection (Baquero *et al.,* 2002). This heterogeneous makeup of antimicrobial resistance is a possible explanation for the emergence of streptomycin resistant *Mycobacterium tuberculosis* during the first ever patient treatment of tuberculosis with streptomycin in 1944 within the same patients (Davies and Davies, 2010).

Bacterial resistance in many cases may have evolved long before human intervention. However, the antibiotic resistance crisis is proliferating at a rate much faster than only selecting for heterogeneously resistant clones of a susceptible bacterial isolate. The rapid proliferation can be explained by horizontall gene transfer.

1.6. Fitness cost of antimicrobial resistance

Contrary to the generalised belief that AMR is the natural mode of microbial evolution, it is in fact a pathway of fitness cost for many microorganisms (Andersson and Hughes, 2010). These fitness advantages are survival of each resistant clone, however at a cost of growth rate and metabolic adaptability (Vogwill and MacLean, 2015). Fitness cost is not always observed in tested microorganisms, but when it is, it usually arrives through counter selection against antibiotic resistance in an antibiotic-free environment (zur Wiesch *et al*., 2011). Chromosomal mutations or insertions have the greatest cost to microbial fitness as they can alter the nature of conserved genes with undesired microbial metabolic changes (MacLean *et al*., 2010). MGE introduction to a new host, which can contain a great degree of varied genes encoding metabolically inclusive functions are significantly less of a burden on the new host compared to genomic mutations/inclusions (Vogwill and MacLean, 2015). The apparent lack of serious fitness cost of the acquisition of AMR, which is most commonly by means of MGE highlights the inherent danger of plasmid mediated AMR propagation.

1.7. Horizontal gene transfer: the crux of bacterial evolution

The mobile nature of antibiotic resistance was first discovered in the late 1950's highlighting how localised sources of ancient antibiotic resistance genes could disseminate to the members of a pathogenic microcosm of bacteria (Davies, 2008). Horizontal gene transfer of antibiotic resistance is as ancient as the naturally evolved antibiotic resistance genes themselves (Brown, 2003).

Whilst it has been determined that a great variety of antimicrobial resistance genes existed before human influence, there is no doubt that dissemination of resistance has occurred due to the use of antibiotics (Berglund, 2015). Tuberculosis has remained a serious human pathogen for as long as recorded human history, despite effective antimicrobial therapies (Rothschild *et al.,* 2001; Lawn and Zumla, 2012). Streptomycin and isoniazid were effective antibiotics for patients with tuberculosis and dissemination of resistance was prompt (Finken *et al.,* 1993; Mitchison, 1998). Combination therapies comprising as many as five second line drugs such as, cycloserine, amoxicillin, clarithromycin, moxifloxacin, and kanamycin in combination with neurotoxic concentrations (1200mg daily dose) of linezolid are currently the only way to defeat tuberculosis (Koh *et al.,* 2009). Even with inclusive combinations of anti-TB drugs, in many places extremely drug resistant tuberculosis (XDR) is rife (Shah *et al.,* 2007; Sotgiu *et al.,* 2009). Similarly, to the totally drug resistant *K. pneumoniae* (which can partake in horizontal gene transfer of antimicrobial resistance genes), totally resistant (TDR) tuberculosis has indications of, but non-confirmatory evidence for horizontal gene transfer events (Davies and Davies, 2010). For now, it appears that TDR *M. tuberculosis* has evolved due to mutations as a result of selective pressure alone. Perhaps the only positive outcome from the rise of bacterial antimicrobial resistance is that through studying their mechanisms, improved understanding of bacterial cell structure and function has been achieved.

The horizontal gene exchange process generates extremely varied recombinant bacterial genomes which can involve introduction or removal of large quantities of DNA into the transcriptionally active genome (Ochman *et al.,* 2000). This process can occur in three primary forms as described and illustrated below (Figure 1.1):

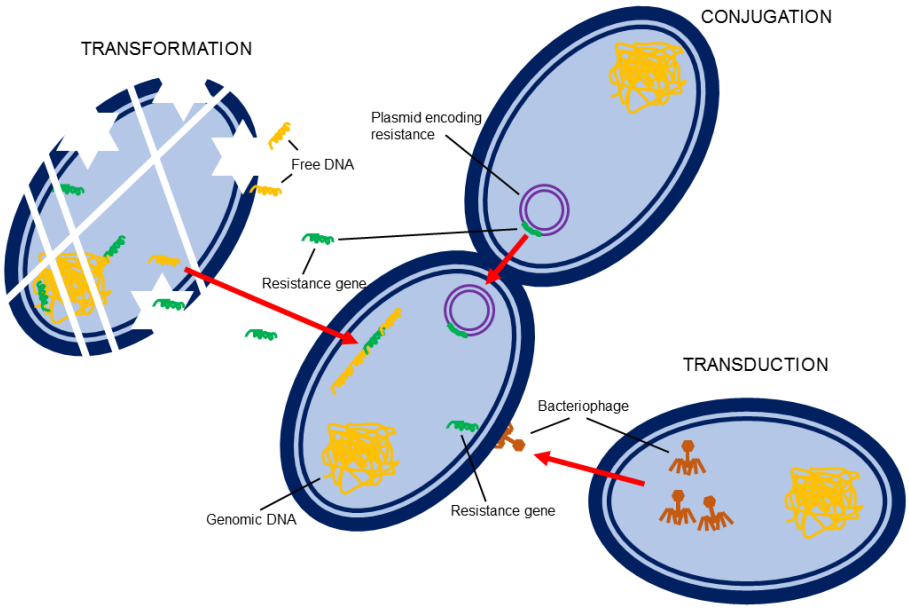


Figure 1.1. Modes of bacterial horizontal gene transfer. Transformation involves the uptake of naked DNA which may contain resistance genes (green). Conjugation involves the transfer of mobile genetic elements, most commonly plasmids (purple) which likely contain resistance genes. Transduction occurs through the unpacking of DNA from a previous host by adsorbed bacteriophages. Adapted from: Kim, G., 2007. Attack of the superbugs: antibiotic resistance. *Science Creative Quarterly*. 01.07: *Cellular Resistance.*

1.7.1. Transduction

Transduction is the process of infection of a bacterial host with a bacteriophage (phage) that has completed a lytic cycle in a prior host and incorporated some host DNA. This can involve generalised transduction, repacking random DNA fragments from the host, or specialised transduction which involves taking DNA from the initial integration site as illustrated in figure 1.1 (Schicklmaier and Schmieger, 1995). Bacteriophages are viruses that infect bacteria and require the metabolic activity of a bacterial host to replicate and package their viral genetic material. Phages can be categorised based on their replication strategy as temperate or lytic. Temperate phage replication cycle involves lysis, or recombination using a phage associated integrase. Lytic phages have a host lysis replication cycle only. Temperate phages are known for their ability to carry out horizontal gene transfer. The temperate phages ΦFL1C, ΦFL2A, and ΦFL3B were shown to be capable of generalised transduction of antibiotic resistance into *E. faecalis* OG1RF and JH2-2 recipients (Yasmin *et al.*, 2010). The lytic phage EFRM31, isolated from a pig farm, amplified in *E. faecalis* was capable of transferring gentamicin resistance to *E. faecium* and was the first interspecies transduction event reported in enterococci (Mazaheri Nezhad Fard *et al.*, 2011). Viral transduction is limited by the size of the phage capsid to a maximum of 100 Kilobases (Jiang and Paul, 1998). Phage global population is postulated to be more than 10^{31} with an estimated 10^{25} infections per second (Hendrix, 2003; Lima-Mendez *et al.*, 2007). With an associated phage for every bacterial species and a large infection frequency there is little doubt that phages are a large component of bacterial evolution, including antibiotic resistance development.

1.7.2. Transformation

Transformation is the process of a bacterium internalising and expressing extracellular (naked) DNA, taken from the surrounding milieu, through homologous recombination as illustrated in figure 1.1 (Chen and Dubnau, 2004). This uptake of naked DNA allows direct integration of adaptive genetic traits facilitating bacterial survival against biocidal attack (Croucher *et al.,* 2011). There are now over 80 species of bacteria that have demonstrated transformative uptake of naked DNA, such as *Bacillus subtilis* 168, *Streptococcus pneumoniae* R6, *Neisseria meningitidis* MC58 and *Vibrio cholera* N16961 (Lorenz & Wackernagel, 1994; Johnsborg *et al.*, 2007; Johnston *et al.*, 2014). The unique characteristic of bacterial transformation is that the mechanism is contained within the bacterium, which is typically inactive until competency has been achieved (Lorenz & Wackernagel, 1994). Therefore, transformation can only be triggered as an opportunistic process. The mechanism of DNA internalisation is conserved across the identified 80+ species of bacteria, accounting for the transport processing differences between Gram positive and negative bacteria (PilQ secretin channel) (Chen & Dubnau, 2004; Johnston *et al.,* 2014).

Many aspects of the transformation mechanism require species specific validation. The general process will be explained here with the Gram positive *B. subtillis* and Gram positive *S. pneumoniae* systems discovered so far. Competence is regulated through bacterial growth phases, which vary from species to species, *B. subtillis* (stationary phase) *S. pneumoniae* (early log phase). Other species can receive DNA at any physiological state e.g. *N. gonorrhoeae* (Dubnau, 1999). Competence is variable between species during transformation and is regulated by alternative sigma factors, transcription activators and regulators (Johnston *et al.,* 2014). *LexA*, the suppressor to the "SOS" bacterial DNA damage response mechanism also functions as a competency suppressor

(Butala *et al.,* 2009). Competency can be induced as a response to stress that threatens the viability of bacterial cells, such as antibiotics (streptomycin in *S. pneumoniae*) and nutrient deprivation (*H. influenza*) and DNA stress (UV exposure in *Legionella pneumophila*) (Redfield, 1991; Prudhomme *et al.,* 2006; Charpentier *et al.,* 2011).

Once competency has been reached, Gram negative T4P proteins (similar to type II secretion and type IV pilli systems), and Gram positive T4P (ComGC pilin) comprise a transformation pilus (Tfp). This pilus protrudes from the outer cell surface (observed up to 3µm) and binds to extracellular dsDNA (Johnsborg *et al.,* 2007; Laurenceau *et al.,* 2013; Mann *et al.,* 2013). The Tfp brings the bound DNA to a dsDNA cell surface receptor (ComEA), which delivers the DNA to a nuclease (EndA) that degrades one strand leaving ssDNA for internalisation through ComEC (Puyet *et al.,* 1990; Provvedi & Dubnau, 1999; Bergé *et al.,* 2002). The ssDNA can now be integrated using homologous recombinases (RecA) with the assistance of a single strand protecting mediator (DNA processing protein A (DprA) in *S. pneumoniae*) (Beernink & Morrical, 1999; Mortier-Barrière *et al.,* 2007; Claverys *et al.,* 2009).

Transformation heredity can be far reaching. Kay *et al*. (2002) demonstrated transformative uptake of *aadA* antibiotic resistance gene from transplastomic tobacco plants into *Acinetobacter* sp. BD413 bacteria. Conferring resistance to spectinomycin and streptomycin. Additionally, studies discussed by Woo *et al*. (2003) demonstrated the uptake of antibiotic resistance genes by *B. subtillis* from *S. aureus* when treated with antibiotics that degraded *S. aureus* bacterial cell wall. Although the number of confirmed species of bacteria capable of taking up DNA by transformation is less than 100, the reality of undocumented uptake of naked DNA and genetic fragments from other

organisms is that this is likely to occur on a large scale with DNA fragments as large as 150 kilobases (Song *et al.,* 2003).

1.7.3. Conjugation

Conjugation is a common process of transferring large quantities of DNA between bacteria, and is instigated through direct contact as illustrated in figure 1.1 (Buchanan-Wollaston *et al.,* 1987). Plasmids are some of the most common elements of DNA transferred through conjugation, such as the Inc18 incompatibility, and sex pheromone plasmids in enterococci (discussed in subsequent sections) (Ochman *et al.,* 2000; Gilmore *et al.,* 2014).

The general mechanism of bacterial conjugation, as originally described for members of the *Enterobacteriaceae*, is as follows When two bacteria come into close contact, the plasmid containing donor bacterium utilises an F pilus (sex pilus) to make physical contact with the plasmid free recipient bacterium (Zechner *et al.,* 2012). Depolymerisation shrinks the F pilus to such an extent that the two bacterial cell envelopes are touching (Sana *et al.,* 2014). Membrane proteins synthesised by the plasmid form a channel between the two connected bacteria (Grohmann *et al.,* 2003). Nucleases (assisted with auxiliary proteins) break the plasmid DNA at the origin of replication (*oriT*) separating a single strand of plasmid DNA into a relaxosome intermediate (Howard *et al.,* 1995). This relaxosome can pass through the channel into the recipient whilst the other strand remains in the donor. Both strands will be re-synthesised creating two identical plasmids. This process is known as rolling circle replication (Khan, 2005).

Conjugative plasmids contain *Mob* (DNA mobilisation genes), *Mpf* (mating pair formation genes) and *Tra* (transfer genes encoding pili) which allow them to transfer from the donor to the recipient, hence they are known as autonomically self-replicating

(Garcillán-Barcia *et al.,* 2009; De la Cruz *et al.,* 2010). Mobilisable plasmids such as RSF1010 can be horizontally transferred between bacteria, but do not contain the *Tra* machinery necessary to accomplish this autonomously (Davison, 1999). They require assistance from *Tra* containing helper plasmids, such as RP4 in the case of RSF1010 or from the recipient (Mergeay *et al.,* 1985).

Genome integrating elements can transfer genes into the host genome, specifically antibiotic resistance genes (Ochman *et al.,* 2000; Wang *et al.,* 2004). These conjugative elements, such as Tn*916* (*E. faecalis* – tetracycline resistance) and CTnDOT (*Bacteroides thetaiotaomicron* – tetracycline and erythromycin resistance) can self-excise from the host and intermediate across to a recipient cell and integrate into the genome (Scott and Churchwood, 1995; Whittle *et al.,* 2002; Burrus and Waldor, 2004).

Conjugative plasmids and elements have the ability of transferring cross a wide range of hosts. The IncQ mobilizable plasmids have been postulated to be compatible with all Gram negative bacteria, such that they were frequently utilised as a cloning vector (Davison *et al.,* 1990). The enterococcal Tn916 element has been identified in over 36 genera of bacteria within the *Deinococcus-Thermus, Actinobacteria, Firmicutes, Fusobacteria* and *Proteobacteria* phyla (Roberts and Mullany, 2009; Ciric *et al.,* 2013).

As with all other methods of horizontal gene transfer, conjugation has played an important role in the evolution of bacteria, particularly the evolution of antibiotic and ultraviolet resistance within localised sources of diverse species of bacteria under external stress (Dahlberg *et al.,* 1998; Davies and Davies, 2010; Clewell *et al.,* 2014). Antibiotic resistance horizontal gene transfer is postulated to occur in the natural environment. Many species of bacteria, harbouring antibiotic resistance genes have been identified in the natural environment as discussed below.

1.8. Sources of antibiotic resistant bacteria in the environment

Antibiotics are most commonly ascribed as biologically active compounds that elicit bactericidal effects. However, from an early stage these compounds demonstrated secondary, beneficial biological processes when administered to complex eukaryotic organisms (Hao *et al.*, 2014). These beneficial processes are still not fully understood, yet antibiotic functions are mostly exploited in this way (Prescott and Dowling, 2013). When industrial antibiotic production improvements yielded economically viable products, a massive influx of non-prescription based purchasing perpetuated their usage in numerous applications, mainly in the food production and pharmacological sectors (Boyd, 2001; Adorka *et al.,* 2015). Over the years this has resulted in millions of tons of antibiotics saturating the natural environment (Li *et al.,* 2015). The decades-long overuse of antibiotics by humans has placed significant stress on bacteria, producing antibiotic resistance phenotypes at rates much greater than "survival of the fittest" selection from microbial soil microcosms (Amábile-Cuevas, 2015).

The natural environment is the communal bacterial macrocosm of the planet, providing a link between all living organisms and bacteria (Finlay, 2002). Saturating the natural environment with antibiotic stress has created heterogeneous sources of antibiotic resistant microbes with capabilities of global dissemination. As such, antibiotic resistant bacteria have been identified from sources both directly, and linked to the natural environment.

1.8.1. Livestock practices facilitating antibiotic resistance

Industrial livestock production consumes up to two thirds of all produced antibiotics globally (Van Boeckel *et al.*, 2015). Huge pressure applied to this sector has given way

to a drive for maximum yields in meat and dairy production (Delgado, 2003; Walker *et al.,* 2005). Outside the EU, Animals are packed in confined spaces and fed significant quantities of animal feed (Fraser, 2001; Grave *et al.*, 2006). Prophylactic antibiotic supplementation in these feeds acts as a growth promoter through modifications of animal GI tract microbiota and reduces inter-animal infection in the short term (Gaskins *et al.,* 2002; Sarmah *et al.,* 2006). The result of this intensive approach is an environment which is conducive for the creation of resistant bacteria with capabilities of zoonotic infection. Examples include the multidrug resistant *E. faecalis*, *E. faecium* and *E. coli* which have been isolated from animal meats for human consumption (Garcia-Migura *et al.*, 2014).

A lesser known example of antibiotic selection pressure exerted on livestock rests within the fish farming sector. Fish husbandry aquaculture utilises similar confinement strategies as seen in the battery farming practices used for mammalian and avian livestock (Barton and Iwama, 1991). However, for fish such as trout these strategies are detrimental to their immune system (Leonardi and Klempau, 2003). In an attempt to combat immunodeficiency and lack of good sanitary practice, there has been increase in the prophylactic use of antibiotics relevant to human disease in the aquaculture of fish (Barton and Iwama, 1991; Naylor and Burke, 2005). Common examples include overuse of chloramphenicol, florphenicol and oxytetracycline in trout and salmon aquaculture which leach into sediments and the surrounding environment (Miranda and Zemelman, 2002; Lalumera *et al.,* 2004; Seoane *et al.,* 2014). Since antibiotics are used so generously, increased background concentrations of antibiotics deposited by fish excrement, and water contamination from antibiotic fish baths escape the limits of the farming zones into the natural environment, such as the communal waterways of rivers and lakes. Therefore, perpetuating selective pressures on indigenous bacteria as well as contaminants capable of human infection (Kim *et al.*, 2004; Sørum, 2006; Cabello, 2006).

One of the most pertinent examples of antibiotic supplementation in livestock farming practices gone awry, lies within the meat industry. Glycopeptide resistant enterococci were first discovered in poultry and pig farms used in meat production in Denmark in 1997 (Bager *et al.*, 1997). Overuse of the glycopeptide avoparcin as a prophylactic growth stimulator in pig and poultry to prevent necrotic enteritis was widespread throughout Europe in the mid-1990's (Kaldhusdal and Hofshagen, 1992). Avoparcin shares a similar chemical structure to vancomycin, which was used for serious human drug resistant infection (Barna and Williams, 1984). Vancomycin (*vanA*) resistant enterococci were identified in humans that consumed pork from origins that had avoparcin dosing regimens Garofalo *et al.*, 2007). Enterococci are not known to be pathogenic in swine, however the discovery of drug resistant enterococci led to the implementation of antibiotic resistance surveillance in livestock (Mathew *et al.*, 2007). The *vanA* gene was the only vancomycin determinant linked to avoparcin usage (Simonsen *et al.*, 1998). The results of a 23 year surveillance study on swine VRE in Europe and the USA identified several clones that existed both in humans and pigs (Freitas *et al.*, 2011). *E. faecalis* CC2 and *E. faecium* CC17 and CC5 all possessed the same mobile genetic element (Tn1546) encoding *vanA* on unrelated plasmids.

The effects of overuse of antibiotics can be clearly observed in the effluents of livestock farming sectors. Slurry and organic waste from agrarian practices including meat and dairy production likely contain antibiotic residues and bacteria, many of which display limited biodegradability (Balcioğlu and Ötker, 2004). Sulfamethoxazole and ciprofloxacin were shown to have biocidal effects on wastewater bacteria (*Pseudomonas putida*) when first tested after a wastewater biodegradation assay by Al-Ahmad *et al.* (1999). The fluoroquinolone class of antibiotics were not fully removed (15% remained) during wastewater treatment and can be introduced into the natural environment, and

concentrated in sludge used for agricultural purposes (Duong *et al.,* 2008). Fluoroquinolone non-biodegradability is the cause of persistant contamination and advanced oxidation techniques may be the only way to treat contamination (Frade *et al.,* 2014). These non-biodegradable antibiotics will produce selective pressure at very low concentrations and sit in containment facilities, exposing indigenous and faecal contaminant microbes to selective pressure for antimicrobials. Avoparcin was banned at the turn of the 20th century and more recent European surveillance reports have seen a reduction in vancomycin resistance, further linking prophylactic overuse in the livestock industry to the development antibiotic resistance (de Jong *et al.,* 2012).

Examination of environmental propagation of zoonotic bacteria to humans is limited, and there has been a call for a more detailed understanding of environmental reservoirs of antibiotic resistance (Allen *et al.,* 2010). A significant pressure for the development of AMR strains of zoonotic pathogens comes from the metaphylactic processes in place from the livestock industry. Whereby a sick animal induces the mass treatment of all neighbouring animals in an attempt to prevent spread of any disease (Woolhouse *et al*., 2015). The geographical terrain of many of livestock farms and related industries exists in, or adjacent to hydrological processes with significant human interaction (wastewater) (Woli *et al.,* 2004; Ulén *et al.,* 2007; Daniels, 2011). Herein lies the potential for zoonotic infections from tainted environmental water sources linked back to livestock and meat production industries.

1.8.2. Antibiotic resistant bacteria in wastewater

Wastewater treatment plants deal primarily with human sewage (Adriano, 2001). They link human effluents to the natural environment through treatment processes that utilise the hydrological properties of waterways. Antibiotics commonly contaminate sewage from human origins, especially clinical sewage (Östman *et al.,* 2017). Therefore, antibiotics can be directly leached into the wider environment through wastewater treatment facilities using natural waterways (Batt *et al.,* 2006). Due to the constant pressures from hospital sources of effluent, high concentrations of MDR (multidrug resistant) human pathogenic bacteria are likely to concentrate in the receiving waters and sludge of wastewater treatment plants (Kemper, 2008). The mechanisms of wastewater treatment have the potential to bring diverse species of bacteria and the non-biodegradable antibiotics discussed above, into prolonged contact with each other (da Silva *et al.,* 2006). This coming together of waste contaminants will only contribute to the potential leaching of MDR pathogens and mobile AMR (antimicrobial resistance) genes into the natural environment, and even back to humans. These antibiotic resistant human pathogens have been previously shown to move into the environment through breaches of containment and elimination processes that occur during wastewater treatment (Lupo *et al.,* 2012).

The *Enterococcus* genus of bacteria is one of the most studied faecal indicator bacteria as well as an indicator of localised antibiotic resistance presence, such as vancomycin, erythromycin and tetracycline (da Silva *et al.,* 2010) Resilient pathogens which can survive wastewater treatment processes have been shown to enter receiving waters and therefore increase the potential for propagation back to humans (Rizzo *et al.,* 2013). Reinthaler *et al.* (2003) identified that the highest resistance rates of *E. coli* were identified throughout wastewater treatment facilities and receiving waters that functioned

to serve municipal and hospital sources. Lanthier *et al.* (2010) determined that enterococci with antibiotic resistance determinants, present in surface waters contained within a watershed in eastern Ontario, Canada, were more likely to originate from wastewater or agricultural sources. This identified the natural environment as a source of potential infective enterococci which originated from medical and farming practices, which themselves are postulated as entrenchment sites for antibiotic resistance.

Fick *et al.* (2009) identified concentrations of ciprofloxacin of 14µg/ml in the effluent of a wastewater plant in Hyderabad, India. This single effluent plant received wastewater from 90 drug manufacturers from the Patancheru industrial area. Water in adjoining lakes were determined to have contamination levels of ciprofloxacin up to 6.5µg/ml as a result of the complete failure of wastewater treatment in an area with a large consortium of global pharmaceutical industries (Tong *et al.,* 2008). This is an example of significantly increasing the base level of antibiotic selection on indigenous and foreign microbes through mishandling of water treatment, and many examples of this occurrence may go unreported (Mompelat *et al.,* 2009).

1.8.3. Sources of antibiotic resistance from agricultural practices

The use of animal slurry in agricultural practices has been shown to harbour bacteria with clinically relevant antibiotic resistances and the potential to infect humans. Examples include *Pseudomonas* spp and *Bacillus cereus* (tetracyclines and macrolides) (Jensen *et al.,* 2001; Sengeløv *et al.,* 2003, Byrne-Bailey *et al.,* 2008). Additionally, many farming practices utilise the fertilising properties of treated wastewater to cut down on fresh water usage in agrarian practices (Pedrero, 2010). Examples of breaches of wastewater treatment with antibiotic resistant bacteria are well documented. Gao *et al*. (2012)

identified tetracycline (*TetO*) and sulphonamide resistance in a large population of bacteria in the receiving waters of a wastewater treatment plant in Michigan USA. Huang *et al.* (2012) demonstrated that effluent water bacteria tested in wastewater treatment plants in Beijing China, 59% were resistant to ampicillin and 44% were resistant to chloramphenicol. They also demonstrated that the MIC's to penicillin, ampicillin, cephalexin, chloramphenicol, tetracycline and rifampicin were above the base level of antibiotic contamination. Taken together these suggest that prolonged exposure to antibiotic contamination will only further propagate these heterogenic isolates.

The base levels of antibiotic and heavy metal contamination in wastewater effluent can reach concentrations as high as µg/L (Segura *et al.,* 2009). Heavy metal contamination (copper, zinc and arsenic) can contribute to co-selection against antimicrobial resistance (Seiler and Berendonk, 2012). Berglund *et al.* (2015) detected four out of ten tested antibiotics downstream of wastewater effluent, with trimethoprim concentrations as high as 47ng/L and ciprofloxacin, clarithromycin and clindamycin at concentrations below 20µg/L. These concentrations of antibiotics detected downstream of wastewater treatment plants serve to increase the MIC's of indigenous bacteria residing in soils when irrigated with similarly sourced water (Gatica and Cytryn, 2013). Compounding these facts, horticultural practices employed during the growing seasons coupled with a dry climate create a high risk for the introduction of floods of antibiotic resistant bacteria (Gelsomino *et al*., 2006). This would occur during runoff from large hydrological process that could make their way back into recreational waters and drinking water treatment systems, exasperating incidences of the kinds of breaches currently observed across the world.

1.8.4. Animal sources of antibiotic resistant bacteria

Evidence suggests that wild animals that live in co-existence with the human built environment are more likely to harbour antibiotic resistant bacteria, as compared to wild animals independent of human influence (Rolland *et al.*, 1985; Allen *et al.*, 2010). They have also been suggested to help spread antibiotic resistance through indirect contact with human processes (Allen *et al.*, 2010). The majority of wild mice tested in England contained bacteria resistant to beta lactam antibiotics, something that would not be expected without human influence (Gilliver *et al.*, 1999). A study on the antibiotic resistance profiles of *E. coli* from mammals from densely populated human residencies of Mexico including: primates, rodents and marsupials determined that they harbour antibiotic resistances similar to those detected in human isolates (Souza *et al.*, 1999).

Migratory birds carry the greatest risk for sourcing and mechanically propagating antibiotic resistance, due to their dual compatibility with the built and natural environment (Middleton and Ambrose 2005; Sjölund *et al.*, 2008). Studies on bird species determined that birds localised close to the human environment (Great cormorants and mallards from ponds and lakes from Lower Silesia region, and swans from Wroclaw, Poland) yielded *E. coli* with a wide range of antimicrobial resistance profiles (Kuczkowski *et al.*, 2016). This could be compared to birds that spend significant amounts of time outside the human environment, and therefore have limited contact with humans. Such as greylag geese and Canada geese from park and grassland in the outskirts of Zoetermeer and Molengreend nature reserve, Netherlands, where lower rates of resistance were observed (Cole *et al.*, 2005; Dolejska *et al.*, 2007; Kuczkowski *et al.*, 2016). Feral pigeons can carry zoonotic pathogens and often come into close contact with humans (Simpson, 2002). Silva *et al.* (2009) determined that 38% of *E. coli* recovered from Brazilian feral pigeons were resistant to antibiotics. Radimersky *et al.* (2010)

isolated *E. faecalis* and *E. faecium* from urban feral pigeons in the Czech Republic. They discovered that these isolates harboured significant antibiotic resistance, including tetracycline (*tetM*) and vancomycin (*vanA*) resistance determinants. Butaye *et al.* (2002) recovered enterococci from racing pigeons with a higher antibiotic resistance frequency as compared to the feral pigeons described by Radimersky *et al.*, citing human influence over the selection for antibiotic resistance determinants of animals in closed environments.

Insects have been shown be carriers of human pathogens and have been implicated as a mobile reservoir for several human pathogens such as, *Salmonella spp, Pseudomonas aeruginosa*, and *Yersinia pseudotuberculosis* (Graczyk *et al.*, 2001; Zurek and Gorham 2008). Multi-drug resistant enterococci have been identified in house flies (*Musca domestica*) isolated from human food preparation sites (Macovei and Zurek, 2006). Macovei *et al.* (2008) also determined that these *M. domestica* had the capability to transfer MDR enterococci to human food. *M. domestica* possess many features that make them the perfect vehicle for the transmission of zoonotic pathogens from their origin source (often located in areas of low human population density) directly into human environments. They have a direct relationship with bacterial reservoirs during their life cycle (Zurek and Nayduch, 2016). The nature of faecal storage in the production of slurries, as well as containment facilities for organic waste by-products of livestock farming provides a niche environment for *M. domestica* and other mobile insects such as cockroaches (Nwosu, 2001; Beuchat, 2006; Zurek and Ghosh, 2014). *M. domestica* are attracted to food preparation processes, and have specific feeding/excretion methods that encourage the deposition of pathogenic enterococci onto foodstuffs for human consumption (Graczyk *et al.*, 2001; Zurek and Gorham, 2008).

The presence of antibiotic resistant bacteria in the natural environment is unquestionable. The majority of the identified species of bacteria are understood in terms of their human interaction (zoonotic, nosocomial, commensal, indigenous etc.). What is not understood, is the exact nature of horizontal gene transfer of AMR, facilitating the evolution of individual species of bacteria, in hotspot areas created by human practices. Knowledge of horizontal gene transfer in the environment is essential to combat the progression of the antibiotic crisis.

1.9. Identifying horizontal gene transfer Hotspots in the environment

The main issue with identifying and analysing the propagation of specific antibiotic resistance genes in potential environmental reservoirs (wastewater treatment plants, aquatic biofilms etc.) and animal reservoirs (GI tracts and excrement) is that culture plate and molecular detection assays cannot accurately identify horizontal gene transfer between two specific bacteria from an environment with thousands of species (Ochman *et al.,* 2000; Soucy *et al.,* 2015). This excludes the number of potential instances of transfer that are unknown amongst non-culturable bacteria, local uncharacterised species and intermediaries that may occur during propagation (Nielsen *et al.,* 2013).

Many studies on horizontal gene transfer describe a single known AMR transfer target; however, there may be several AMR genes transferred, in a process where detection assays may only scan for a single target (Crisp *et al.,* 2015; Ravenhall *et al.,* 2015). Christie *et al.* (1987) were one of the first groups to describe two different conjugation reactions with the *E. faecalis* plasmid pCF10. When an interspecies reaction was carried out, tetracycline resistance transferred along with a 16-kb region of the plasmid from *E. faecalis* into *Bacillus subtilis.* Compared to the initial intra-species reaction between two

E. faecalis transferring a 25-kb region containing tetracycline resistance. In this case the resistance phenotype was the same, however the genetic transfer involved a smaller region from the plasmid.

Moubareck *et al.* (2003) determined multiple gene transfers of *vanA* and *ermB* from four *E. faecium* isolates into another susceptible *E. faecium*. In every instance, both *vanA* and *ermB* were transferred *in vitro* conferring vancomycin and erythromycin resistance. Two of the donors had additional resistance genes (*tetL*-tetracycline and *ant(6)*-streptomycin) which were only identified in the transconjugants due to prior knowledge of the gene presence in the donors. Studies on conjugation of single gene phenotype/genotype may not account for these additional genetic determinants which may translate as additional AMR phenotypes. This study also utilised *in vivo* conjugation using the same isolates. Transconjugants isolated from *in vivo* conjugation were not uniformly resistant to vancomycin and erythromycin like the *in vitro* experimentation, with a small percentage (14%) of alternating vancomycin or erythromycin phenotypes. This suggests that like the pCF10 conjugation investigations, these two AMR genes are located on the same transferrable element but can be transferred in a two-component system. These research investigations highlight the potential for varied transfer of genetic determinants based on inter/intra-species conjugation reactions, which may include AMR genes.

Therefore, a more accurate disseminator of potential horizontal gene transfer (HGT) hotspots would be scanning for common structures associated with AMR, such as those on the mobile genetic elements that carry AMR genes (Lupo *et al.*, 2012). Rizzo *et al.* (2013) suggested integrons, such as the resistance integrons (RI) of MGE's as an alternative indicator for potential HGT hotspots. They have a common structure and are associated with many MGE's and AMR genes. Resistance integron gene cassettes have been determined to carry the majority of all antibiotic resistance groups

(aminoglycosides, beta-lactams, rifampicin, macrolides etc.) making them a stable general solution for identifying potential HGT hotspots (Partridge *et al.,* 2009). However, it is still important to understand the specifics of antibiotic resistance transfer as the presence of antibiotic resistance genes may facilitate persistence due to therapeutic failure.

Identifying potential hotspots for HGT is an essential step in the combat of AMR interbacterial propagation. Understanding the dynamics of HGT under totally natural conditions is another essential step towards understanding AMR propagation.

1.10. Horizontal gene transfer dynamics in the environment

The increasing incidences of AMR bacteria in correlation with the overuse and inadequate disposal of antibiotics have clearly been demonstrated (Davies and Davies, 2010). However, an in depth understanding of AMR in the complex natural and built environment remains elusive (Holmes *et al.,* 2016). There are two known methods with which the proliferation of AMR has occurred. Selective stress placed upon populations of bacteria and HGT (Courvalin, 2008). Studies on prevalence of drug resistant bacteria may not always point to a HGT origin, as is the case with rifampicin resistance generation and *M. tuberculosis* multidrug resistance (Telenti *et al.*, 1993).

There are an abundance of investigations detecting a large proportion of the known antimicrobial resistance genes in wastewater treatment systems (Dröge *et al.*, 2000; Reinthaler *et al.,* 2003; Szczepanowski *et al.,* 2008). Investigations such as these, give convincing conclusions that HGT occurs in these environments, as there are diverse bacterial species as well as mobile AMR genes. The issue with direct confirmation of

these statements is in confirming HGT itself with native bacteria, as the number of potential recipient isolates in the environment are high (Sørensen *et al.,* 2005).

A focused approach to understanding HGT in the environment is necessary to reduce the large number of variables at any given time. Whilst bacteria have been shown to conjugate under planktonic conditions, substrate conjugation may be a more likely mode of natural HGT due to the concentration of bacteria on solid surfaces. Bacterial biofilm has been postulated to be the substrate of choice for such processes.

1.11. Biofilm: an environment conducive for the dissemination of antimicrobial resistance

Bacterial evolution can be driven by horizontal gene transfer of AMR (Juhas *et al*., 2009). Bacterial biofilm is an environment that would facilitate this process. In the natural environment bacteria form communities encased in a protective sheath called a biofilm (Mohamed and Huang, 2007, Deligianni *et al*., 2010). Biofilms have definitively been shown as augmenters to nosocomial infection, as they allow anchorage of a community of bacteria to host tissue as well as medical devices providing a site of continual reproduction and shelter from biocides (O'Toole *et al.,* 2000).

Biofilms allow pathogenic bacteria to establish successful colonies, providing a continuous source of life threatening infection, protected from therapeutic interventions. An example of such infections includes enterococcal endocarditis, especially within the hospitalised communities of immunocompromised patients (Deligianni *et al.,* 2010). These biofilms form (figure 1.2) when an initial colonising bacterium loses its motility and permanently binds to a surface to avail of favourable growth conditions (O'Toole *et al.,* 2000). The initial coloniser will modulate the environment in a way that allows other

bacteria to adhere to the matrix (Mohamed and Huang, 2007, O'Toole *et al.,* 2000). Otherwise these bacteria could not grow at the site of initial attachment due to factors such as unfavourable oxygen saturation or iron availability (Whitchurch *et al.,* 2002).

Their adherence to the multi-polymer matrix allows them to contribute to the community with specific virulence factors (enterococcal *asa1, esp* and *gelE*) or to avail of the initial environment created by an initial colonising member (O'Toole *et al.,* 2000; Vuong *et al.,* 2004; Gill *et al.,* 2005; Farahani, 2016). The type IV pili present on *P. aeruginosa* aid in the formation of biofilm in the presence of *S. aureus* through pilin binding to the eDNA components of the extracellular polymeric substance (EPS) (Yang *et al.,* 2011). Multispecies biofilms avail of co-operative aggregation to further strengthen the EPS matrix structure making them harder to eradicate than single species biofilms (Rickard *et al.,* 2003).

Mature biofilms can have several different species of bacteria increasing the difficulty of total eradication of the biofilm community (Boles *et al.,* 2004, Rochex *et al.,* 2008). A multispecies biofilm will likely have members that have unique resistances to pH, salinity, temperature as wells as their own AMR profiles. Members that have varied AMR profiles increase the likelihood of displaying resistance to any given antimicrobial agents (Burmølle *et al.,* 2010).

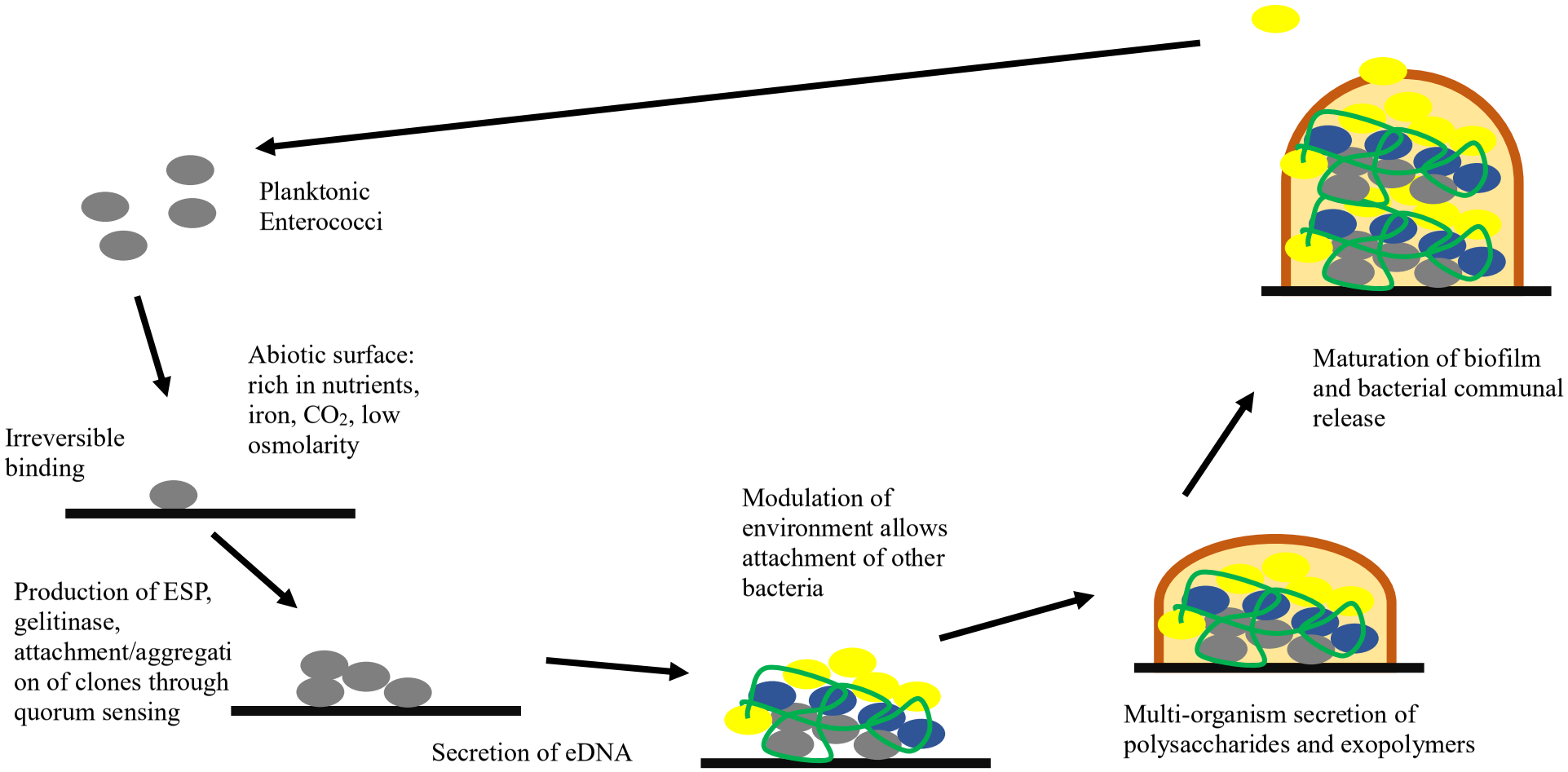


Figure 1.2 The biofilm cycle. A generalised Illustration highlighting the key stages of biofilm establishment and maturation. Created using information from Dunny *et al*. (2014).

Examination of natural HGT processes occurring in host bacterial biofilm is inherently a difficult task due to the number of unknown variables at play. Variables such as MGE compatibility, compatibility between two isolates of bacteria, and finally getting them inside biofilm and capturing successful HGT. Assessment of conjugation under these conditions has never truly been accomplished without some molecular modification (Green Fluorescent Protein (GFP) reporter incorporation) to donor bacteria plasmids (Hausner and Wuertz, 1999; Cook *et al.*, 2011). Identifying conjugation partners that can carry out HGT naturally, and form biofilm of their own creation would make them suitable model organisms for studying this important phenomenon.

1.12. *Enterococcus* – a genus of bacteria that can model antibiotic resistance, biofilm formation and subsequent horizontal gene transfer

The difficulty associated with acquiring knowledge of environmental conjugation can be reduced to a straightforward investigation by using members of the *Enterococcus* genus to model natural conjugal mating pair HGT. *Enterococcus faecalis* and *E. faecium* are well known for their proficiency in this process, they possess significant AMR genes as discussed in sections 1.3 and 1.4 and they can form biofilm (discussed further here).

Whilst front line care deals with the consequences of the antibiotic resistance crisis and research investigations aim to prevent further generation and propagation of antibiotic resistance (Edwards *et al.*, 2013), there is a gap in knowledge linking these unique traits of enterococci to antibiotic resistance propagation. As mentioned previously, high levels of antibiotic resistance can be transferred through horizontal gene transfer, and bacterial biofilm matrices can make this possible under a great variety of environmental conditions.

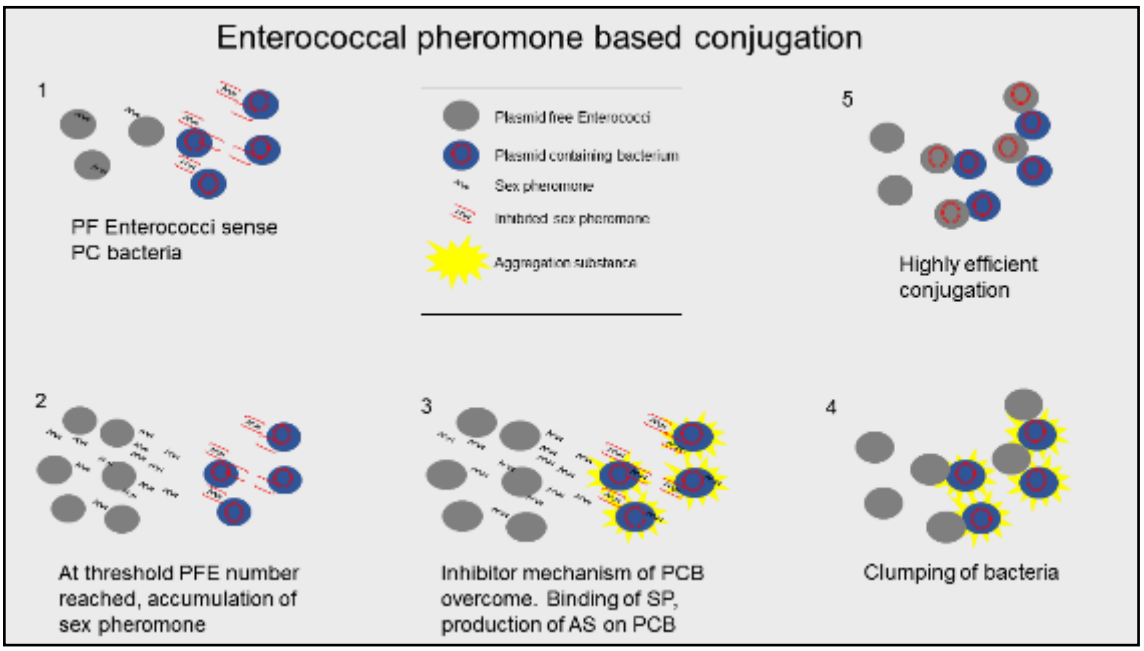


Figure 1.3. Overview of enterococcal pheromone based conjugation. Plasmid free enterococci secrete sex pheromones into the extracellular environment. When the plasmid free enterococci sense a compatible plasmid containing bacteria, pheromone production is directed towards out-competing the inhibitor production in the plasmid containing bacteria. Once a threshold has been reached and the inhibition mechanism has been overcome, binding of the pheromone occurs to the cell surface binding sites on the plasmid containing bacteria. Induction of aggregation and the production of aggregation substance occurs. The plasmid containing bacteria clump together along with the plasmid free enterococci increasing surface area and allowing conjugation to occur. Created with information from Dunny and Berntsson, (2013).

1.12.1. Enterococcal HGT practices

The most prolific enterococcal characteristic is their ability to conjugate efficiently with different genera of pathogens (*Clostridium difficile* and *E. coli*) (Kozlowicz *et al.,* 2006). This allows for the movement of large quantities of genetic information to and from VRE based on little more than spatial location. Such an example would be the identification of a (*vanA*) vancomycin resistance gene with identical gene sequences to an *Enterococcus* determinant in a pathogenic *S. aureus* MRSA strain (Magi *et al.,* 2003; Périchon and Courvalin, 2009).

Enterococci are known for their unique sex pheromone conjugation system. Enterococci use sex pheromone peptides along with quorum sensing to aggregate potential donor strains to allow greater efficiency of horizontal gene transfer (as illustrated in figure1.3) (Palmer *et al.,* 2010; Clewell, 2011). All enterococci, produce a specific pheromone that induces the expression of aggregation substance, a cells surface protein, from the *PrgB* aggregation gene located on all sex pheromone inducible plasmids (as illustrated in figure 1.4) (Palmer *et al.,* 2010). Bacteria that contain a sex pheromone responsive plasmid have their own pheromone production inhibited by a plasmid produced binding protein (the inhibitor – iCF10) (Kozlowicz *et al.,* 2006; Palmer *et al.,* 2010; Clewell, 2011). The machinery for the production of the inhibitor is also encoded on the plasmid. The inhibitor interacts with the pheromone receptor in the same way as the pheromone peptide acting in a competitive nature. This mechanism can be overcome by the presence of un-inhibited pheromone at a median concentration 80 fold higher than the inhibitor, produced by a plasmid free *Enterococcus* when it reaches a certain cell density through quorum sensing (Hirt *et al.,* 2002; Lysakowska *et al.,* 2012). Once the inhibition system has been successfully out competed, downstream signalling activates the production of aggregation substance causing the clumping of the donor strain, making it competent for conjugation

(figure 1.4) (Clewell, 2011; Lysakowska *et al.*, 2012). This allows the *E. faecalis* strain to conjugate with the donor at efficiencies up to 10^{-1} transconjugants per donor (Hirt *et al.,* 2002; Donelli *et al.,* 2004). They have also been previously instigated in two directional interspecies HGT of antibiotic resistance to other enterococci, staphylococci and streptococci (Palmer *et al*., 2010; Gomez *et al*., 2011)

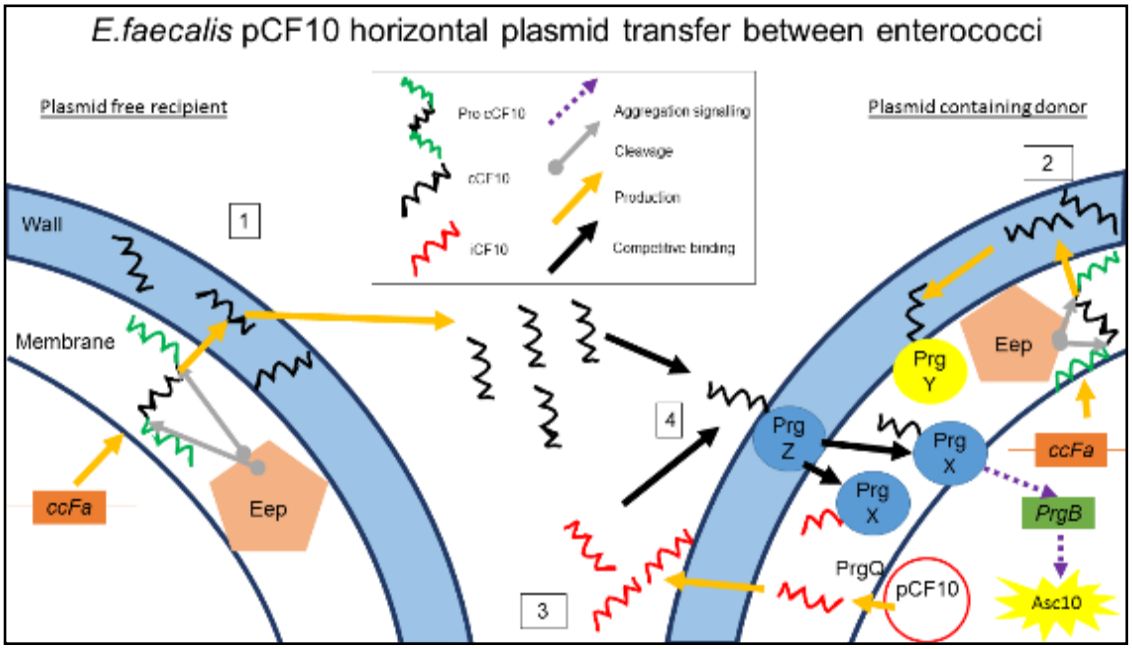


Figure 1.4. transfer of enterococcal plasmid pCF10. The first fully characterised plasmid harbouring tetracycline resistance, created for illustration of the mechanism of sex pheromone conjugation in this thesis. 1 – recipient cells secrete 8 amino acid long hydrophobic sex pheromones bound by a plasmid encoded binding protein that is cleaved by enhanced expression of pheromone (*Eep*). 2 – The plasmid pCF10 containing donor also possess the same machinery but is inhibited by production of *PrgY* transcribed from the plasmid pCF10 to prevent auto aggregation. 3 – The plasmid pCF10 also produces an inhibitor inert molecule to competitively bind *PrgZ/X*. 4 – When the extracellular concentration of the cleaved pheromone reaches a threshold level, competitive binding with the inhibitor is outperformed and the pheromone is taken into the donor cell and releases a transcriptional block of the *Tra* family of repressors. Internalisation of the pheromone induces the transcription of Asc10 the aggregation protein which allows for tight physical contact between donor and recipient and subsequent conjugation. Created with information from Dunny and Berntsson, (2013).

1.12.2. Enterococcal biofilm formation capability

Biofilms are an essential element of enterococcal nosocomial infection, by providing a location for the attachment of a population of bacteria to an abiotic surface, such as the heart valves during endocarditis (O'Toole *et al.*, 2000). Enterococcal biofilms can establish successful chronic colonies causing bacteraemia and culminating into life-threatening systemic infections (septicaemia) (Deligianni *et al.*, 2010). These biofilms can form when a particularly virulent strain, such as VRE V583 or V586, permanently attaches to an abiotic surface on or around indwelling catheters Thus, gaining a foothold on internal human tissues and even obtaining access to the vascular system through potential catheter related injuries (Sahm *et al.*, 1989; O'Toole *et al.*, 2000). The initial colonising isolate produces anchoring sites through excretion of DNA and polymeric substances, paving the way for later additions of new members using the DNA as an attachment site (Mohamed and Huang, 2007; O'Toole *et al.*, 2000). The step wise addition of new members to the microcolony biofilm means that late adaptors can consist of bacteria that could never form biofilm at the specific site due to issues with nutrient availability and oxygen saturation (Whitchurch *et al.*, 2002).

Having several attachment sites may allow more pathogenic strains of bacteria to enter the biofilm, creating continual seeding sites for chronic, systemic infection (O'Toole *et al.*, 2000; Gill *et al.*, 2005; Vuong *et al.*, 2004). Mature biofilms are not completely defined by specific quantity or variation in bacterial species, they are however usually harder to eradicate due to increased surface area of attachment, variation in species of bacteria and formation characteristics of the biofilm itself (Boles *et al.*, 2004; Rochex *et al.*, 2008). Where enterococci are concerned, the expression of the enterococcal surface protein (ESP) cell wall-associated protein has been shown improve adhesion and therefore biofilm formation (Toledo-Arana *et al.*, 2001). Studies by Kristich *et al.* (2004)

and Tendolkar *et al.* (2004) concluded that ESP must act in coordination with various factors involved in enterococcal biofilm formation and its presence can improve formation. Hence, enterococcal biofilm is now described as multifactorial in nature (Dunny *et al.*, 2014; Garg *et al.*, 2017) Additionally, enterococcal gelatinase contributes to biofilm during infection by hydrolysing host tissues (collagen, fibrinogen, fibrin) into derivatives (gelatin, various peptides, amino acids) and is recognised as a key virulence factor (Hancock and Perego, 2004). Mediated through the Fsr quorum response, gelatinase provides both nutrients and anchoring sites for the development of biofilm through aiding in the production of aggregation substance (Fisher and Phillips, 2009; Thurlow *et al.*,, 2010).

As previously stated, multispecies biofilms are generally harder to eradicate due to variations in tolerance to biocides amongst the inter-biofilm species. Enterococcal infection originating in a biofilm has been shown to have significantly higher tolerance to biocides, including antibiotics, rendering many ineffective (ECDC, 2013). This is the main advantage to the thick polymeric matrices completely encasing the community of bacteria (O'Toole *et al.*, 2000).

Multispecies biofilms are in general more advanced in terms of structural integrity and spatial organisation of its members compared to single species biofilms. Indirect evidence for previous HGT events was detected by Schwartz *et al.* (2003), when they identified the enterococcal vancomycin *vanA* gene in wastewater and drinking water biofilms, with no detectable presence of enterococci. It was postulated that enterococci were once present in this localised source of biofilm, either through leaching from another biofilm or introduction of genetic material from various enterococcal sources. They also detected the *mecA* methicillin resistance gene found in staphylococci in hospital wastewater

biofilms further bolstering the notion of undescribed natural HGT processes within biofilm.

Direct experimental evidence for enterococcal conjugation in biofilm has only been identified once. Cook *et al.* (2011) performed biofilm mediated enterococcal conjugation with genetically modified, characterised plasmids on laboratory strains of enterococci, but it was carried out in a manner that highlighted a limitation in their experimental design. The possibility of planktonic conjugation was not accounted for in their methodology, particularly as the enterococcal plasmid pCF10 can be transferred through planktonic conjugation (Christie and Dunny, 1986).

1.13. Potential for human infection with enterococci sourced from the natural environment

The Republic of Ireland has the highest percentage of vancomycin resistant enterococci in Europe (ECDC, 2015). Surveillance for the EU/EEA in 2015 reported that *E. faecium* with resistance to vancomycin was at 45.8% of all isolates tested. The second and third highest incidence rates of vancomycin resistance in *E. faecium* was in Cyprus at 28.6% and Croatia at 25.8%. The EU/EEA population weighted mean was 8.3% and, in general, the vancomycin resistance incidence rates are increasing in hospital acquired infections.

The effects observed in nosocomial niches can be postulated to be attributed to the natural environment, as hospital effluents drain into these environments. Enterococcal resistance propagation and infection has been described as a foodborne zoonosis and global importance has been placed on Enterococci due to their high levels of antibiotic resistance and conjugation abilities (Nilsson, 2012). The European commission has set a motion to prosecute authorities in Ireland over severe continual breaches of wastewater treatment

standards set under EU council directive 91/271/EEC; whereby 38 agglomerations were in breach of standards including the detection of *E. faecalis*. These rules were put in place in the year 2000, and 13 years later infringement of Irish water standards were continuing (European commission, 2012).

There exists clear evidence of persistent contamination of communal waterways by a bacterium which is known to form biofilm in this environment, harbours significant AMR gene traits and is competent at conjugation of these genes. This is a model example of re/introducing potentially augmented, infective bacteria to humans.

1.14. Conclusion

As antibiotic resistance continues to prevail, identification of antibiotic resistance genes in bacterial pathogens with similar gene sequence identity to other organisms is important. Bacterial pathogens efficiently pass on antimicrobial resistance genes through contact mediated horizontal gene transfer. Resistant members of the enterococcal family can easily form biofilm and conjugate antibiotic resistance genes, such as vancomycin determinants. A lack of knowledge regarding this potential environmental source of AMR could be helping to perpetuate the antibiotic resistance crisis globally.

1.15. Hypothesis and Aims

General Hypothesis:

Environmentally isolated *E. faecalis* and *E. faecium* form natural biofilms which facilitate the transfer of antibiotic resistance genes.

Aims:

- Characterise environmentally isolated *E. faecalis* and *E. faecium* for their ability to carry out horizontal gene transfer.
- Determine the biofilm production capabilities of enterococci and define the unique characteristics contained within their own biofilm.
- Demonstrate conjugation in *E. faecalis* using traditional laboratory assays and any extensions to the methodology that can be carried out to mimic the environment from which they were isolated.

Chapter 2

General Materials and Methods

2.1. Isolation of enterococci

The *E. faecalis* and *E. faecium* used in this study were previously isolated from various sources feeding into river headwaters in the Blackwater catchment in County Monaghan, Republic of Ireland (Daniels, 2011). Isolation sites are detailed in table 3.4.1. Briefly, water was collected and filtered using the Millipore Microfil membrane filtration system with 0.45µm filters (Merck Millipore, Hertfordshire, UK). Volumes of 1-50ml of water samples diluted in maximum recovery diluent (Oxoid, Hampshire, UK) were filtered and grown on Slanetz and Bartley agar (Oxoid). Plates were incubated for four hours at 37°C and 44 hours at 42°C respectively.

2.2. Confirmation of enterococci

Phenotypic identification of isolates as *Enterococcus* was previously carried out using: aesculin hydrolysis, PhenePlate™ analysis, Gram staining, catalase activity, PYRase and azide tests (Kuhn *et al.,* 2000; Ahmed *et al.,* 2005; Daniels, 2011). A selection of these tests were used to ensure no contamination of the stock cultures.

2.2.1. Growth in azide dextrose

An enterococcal universal tube suspension in sterilised azide dextrose broth (Oxoid – CM0868) was created using a nichrome wire loop, taking a single colony of bacteria. Cultures were incubated at 37^{o}C for 24 hours. An increase in turbidity indicated bacterial growth and therefore successful growth in the presence of azide (Devriese *et al.,* 1992).

2.2.2. Aesculin hydrolysis

Enterococcal isolates were spread on bile aesculin agar (Oxoid – CM0888) and incubated at 37^{o}C for 24 hours. Positive hydrolysis was indicated with a colour change from straw yellow to black (Ruoff *et al.,* 1990).

2.2.3. Catalase test

Enterococcal isolates were smeared on a glass slide, air dried and hydrogen peroxide (3% vol/vol) was dropped directly onto the smear. A positive reaction was indicated by the presence of bubbles of oxygen created by the reaction (Taylor and Achanzar, 1972). A negative reaction confirmed enterococci.

2.2.4. Gram staining

Standard Gram staining was employed using safranin as the counterstain (as discussed in Beveridge, 2001). Indications of enterococci are confirmed by visualisation of Gram positive cocci.

2.2.5. Growth at 45^{o}C

Tryptone soy broth (TSB) (Oxoid – CM0129) bacterial suspensions were created in universal tubes and incubated at 45^{o}C for 24 hours. A change in turbidity confirmed growth. Enterococci can grow at 45^{o}C (Martínez *et al.,* 2003).

2.3. Storage of isolates and standard growth conditions

Isolates to be used in experiments were taken from the deep freezers (-80^{o}C) using a sterile toothpick and transferred onto an appropriate growth medium. All bacteria were maintained on tryptone soy agar (TSA) slants, (Oxoid – CM0131) at 4^{o}C throughout each experimental time-period. Fresh cultures were thawed after each experiment. All growth and experiments, unless specifically mentioned, were carried out on TSB, or TSA at 37^{o}C for 24 hours incubated statically under aerobic conditions. All chemicals and antibiotics used were obtained from Sigma-Aldrich (Dorset, UK), unless otherwise stated.

2.4. Growth curves

Growth curves were calculated using the viable plate count method (Meynell and Meynell, 1965). Each *Enterococcus* isolate to be counted was serially diluted in phosphate buffered saline (PBS) (Oxoid – BR0014) from a single colony grown overnight (16 hours) statically in TSB, under aerobic conditions. The serial dilutions were measured using a spectrophotometer (600nm). The serially diluted enterococci were spread on TSA at a volume of 0.1ml and incubated at 37^{o}C overnight (16 hours). Colonies were counted (between 30-300) and colony forming units (CFU) were calculated ($CFUml^{-1}$ = Colonies counted/volume plated x dilution factor) and growth curves were constructed using CFU and turbidity measurements over time.

2.5. Antimicrobial disc diffusion assay

Enterococcal isolates were tested for the presence of antimicrobial resistance phenotypes using the disc diffusion assay under European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (EUCAST 2016). Enterococci were selected for testing based on results from the clumping assay identifying them either as potential conjugation recipients or donors. An 18-hour culture of bacteria grown in TSA was re-suspended in PBS to a MacFarland 0.5 standard. The bacterial suspension was swabbed and spread over the surface of a dried Iso Sensitest plate. Antibiotic discs (Oxoid) were stamped on the plates using a disc dispenser (Oxoid). Antibiotics used were ampicillin (10µg), amoxycillin (25µg), cephalothin (30µg), ciprofloxacin (5µg), erythromycin (30µg), gentamicin (120µg), imipenem (10µg), linezolid (10µg), neomycin (30µg), oxytetracycline (30µg), quinupristin/dalfopristin (15µg), streptomycin (25µg), trimethoprim (5µg), trimethoprim/Sulfamethoxazole (25µg), tetracycline (30µg), teicoplanin (30µg), and vancomycin (30µg). Plates were incubated for 24 hr at 37^{o}C. Zones were measured (mm) and compared to EUCAST guidelines. *E. faecalis* ATCC 29212 was used as a susceptibility control.

Table 2.1 Antibiotic susceptibility breakpoints for enterococci relative to disc concentration

Antibiotic	Disc concentration (µg)	Zone diameter Breakpoint (mm)	
		S ≥	R <
Ampicillin	10	10	8
Amoxicillin	25	10	8
Cephalothin	30	18	14
Ciprofloxacin	5	15	15
Erythromycin	30	23	13
Gentamicin	120	8	8
Imipenem	10	21	18
Linezolid	10	19	19
Neomycin	30	18	15
Oxytetracycline	30	19	14
Quinupristin/Dalfopristin	15	22	20
Streptomycin	25	14	14
Sulfamethoxazole/Trimethoprim	1.25 + 23.75	21	21
Trimethoprim	5	50	21
Teicoplanin	30	16	16
Tetracycline	30	18	15
Vancomycin	30	12	12

2.6. Antimicrobial minimum inhibitory concentrations

Minimum inhibitory concentrations were determined using the broth microdilution protocol outlined by Wiegand *et al.* (2008) with the addition of p-Iodonitrotetrazolium as described by Amir *et al*. (2013) as a colorimetric indicator to confirm naked eye observations of inhibition. Antibiotics used were: vancomycin 512-1µg/ml, erythromycin 1024-0.25µg/ml, streptomycin 1024-2µg/ml, tetracycline 256-0.5µg/ml, trimethoprim 16-0.008µg/ml, teicoplanin 32-0.25µg/ml, rifampicin 512-1µg/ml, kanamycin 2048-1µg/ml (Gibco-ThermoFisher), chloramphenicol 1024-1µg/ml and gentamycin 1024-1µg/ml. All experiments were carried out using Muller Hinton broth and iso-sensitest broth for trimethoprim. Resistance/susceptibility was determined according to the EUCAST breakpoint publications version 6 (EUCAST, 2016).

Table 2.2. Antibiotic minimum inhibitory concentration breakpoints (24 hours) for enterococci.

Antibiotic	MIC (µg/ml) S ≥	R <
Ampicillin	4	8
Amoxicillin	4	8
Ciprofloxacin	4	4
Erythromycin	4	4
Gentamicin	128	128
Impenem	4	8
Kanamycin	1024	1024
Linezolid	4	4
Quinupristin/Dalfopristin	1	4
Streptomycin	512	512
Trimethoprim	0.03	1
Tetracycline	4	4
Teicoplanin	2	2
Vancomycin	4	4

Breakpoints determined from the EUCAST breakpoint publications version 6 (EUCAST, 2016).

2.7. Generation of rifampicin resistant strains

Due to the relative antimicrobial sensitivity of all recipients identified and in order to perform counter-selection in conjugation experiments, rifampicin resistance was generated. The rifampicin sensitive pheromone-producing isolates were grown in sub-minimum inhibitory concentrations of the antibiotic to log phase (eight hours at 37ºC in TSB). Successive generations were sub-cultured daily in increasing two-fold concentrations of the antibiotic until isolates could grow in at least 32 µg/ml (seven days of culturing) of rifampicin starting from 0.5µg/ml up to a maximum of 512µg/ml. All donor isolates in this study were susceptible to rifampicin excluding MW02102, MW03025 and MW03051 which displayed an MIC of 8 µg/ml. All recipients were resistant to rifampicin (denoted as ^{Rif}) as compared to the donors.

2.8. Stability of resistance under no selection

E. faecalis MF06036 and MF06035 with established vancomycin resistance phenotypes were subjected to weekly sub-culture on TSA under zero antimicrobial stress for six months. Incrementally, isolates were spread on TSA plates containing 10µg/ml of vancomycin and incubated for 24 hours at 37ºC and viable counts were determined.

2.9. NCBI gene analysis and primer design from published enterococcal MGEs

Antimicrobial resistance genes which have been shown to be located on mobile elements were analysed. This was accomplished by systematically going through deposited partial and complete plasmid sequences with identified genes in the NCBI gene database. All entries associated with antimicrobial resistance and plasmid/mobile element genes were collated to create a database of all genes of interest. Their associated plasmid and

enterococcal species were also recorded. Nucleotide sequence information for each gene was recorded and collated in FASTA format. These sequences were aligned in ClustalX (Larkin *et al.,* 2007) to highlight sequence identity between sequence variations of the same genes. Sections of identical sequence between gene variants of the same gene were inputted to the NCBI primer – BLAST programme to create a single set of primers. This approach ensured selection of mobile antibiotic resistance phenotypes using one primer set per gene, across all tested enterococci where possible. This information allowed published primers to be cross checked for the precise amplification location on the gene of interest.

2.10. Creation and acquisition of PCR primers and FISH probes

All primers used in this study were screened and acquired in the same way. Published primer/probe sequences were subjected to BLAST against a consortium of all published specific gene sequences for *E. faecalis* and *E. faecium* to confirm specificity. Primers/probes that were created were first subjected to primer-BLAST. Primers/probes were cross checked for specificity to all genes in the gene database. After confirmation of specificity, primers/probes were analysed for stability using the PCR primer stats tool in the sequence manipulation suite (Stothard, 2000). All primers/probes were acquired through the ThermoFisher custom DNA primers and, where specifically stated, Integrated DNA Technologies primer creation tools. Antibiotic resistance gene primers were controlled with *E. faecalis* ATCC 29212. *Staphylococcus aureus* ATTC 43300 was used as a non-specific control for *Enterococcus* probes.

Table 2.3 Primer sequences used in this study

Gene	Primer	Sequence 5' to 3'	Size (bp)	Reference
tetK	tetK F	TTAGGTGAAGGGTTAGGTCC	718	Aarestrup, 2000
	tetK R	GCAAACTCATTCCAGAAGCA		
tetL	tetL F	ATAAATTGTTTCGGGTCGGTAAT	1077	Trzcinski *et al.*, 2000
	tetL R	AACCAGCCAACTAATGACAATGAT		
tetM	tetM F	GTTAAATAGTGTTCTTGGAG	657	Aarestrup, 2000
	tetM R	CTAAGATATGGCTCTAACAA		
tetO	tetO F	GATGGCATACAGGCACAGAC	614	Choi and Woo, 2015
	tetO R	CAATATCACCAGAGCAGGCT		
tetS	tetS F	TGGAACGCCAGAGAGGTATT	660	Choi and Woo, 2015
	tetS R	ACATAGACAAGCCGTTGACC		
tetT	tetT F	AAGGTTTATTATATAAAAGTG	169	Aminov *et al.*, 2001
	tetT R	AGGTGTATCTATGATATTTAC		
tetW	tetW F	GAGAGCCTGCTATATGCCAGC	168	Choi and Woo, 2015
	tetW R	GGGCGTATCCACAATGTTAAC		
ermB	ermB L	AGGGTTGCTCTTGCACACTC	119	This study
	ermB R	CTGTGGTATGGCGGGTAAGT		
vanA	vanA L	CTACTCCCGCCTTTTGGGTT	109	This study
	vanA R	TTCACACCGAAGGATGAGCC		
E. faecalis	FL1	ACTTATGTGACTAACTTAACC	360	Jackson *et al.*, 2004
16s rRNA	FL2	TAATGGTGAATCTTGGTTTGG		

Table 2.4 Probes used for FISH

Target	Probe	Sequence 5' to 3'	Reference
Enterococcus vanA	vanA 1	GCAAGTCAGGTGAAGATGGA	This study
	vanA 2	AGGAGCATGACGTATCGGTA	This study
E. faecalis 16S rRNA	ENF 191	GAAAGCGCCTTTCACTCTTATGC	Wellinghausen *et al.*, 2007
E. faecium 23S rRNA	ENU 1470	GACTCCTTCAGACTTACTGCTTGG	Wellinghausen *et al.*, 2007
Bacteria 16S rRNA	EUB 338	GCTGCCTCCCGTAGGAGT	Amann *et al.*, 1990

2.11. Plasmid extractions

The alkaline lysis method for large plasmids was used (Sambrook and Russell 2001). Overnight (16 hours) cultures of enterococci in TSB were diluted 1/10 and propagated for four hours at 37ºC. Up to 1.5 ml of bacterial suspension was taken in an Eppendorf tube and centrifuged at 13,000 x g for 30 seconds. Samples were aspirated to dry, re-suspended in 100 µl ALS1 (10 mM EDTA pH 8.0, 50 mM glucose and 25 mM Tris-Cl pH 8.0) containing lysozyme (30 mg/ml), and incubated for 10 minutes at 40ºC. Fresh ALS2 (0.2 N NaOH, 10% SDS) at 200µl was added to the tube and inverted gently five times. Fresh ALS3 (100ml stock made from: 5 M potassium acetate (60 ml), glacial acetic acid (11.5 ml) and H_2O (28.5 ml)) at 150µl was then added and inverted a further five times and stored at 4ºC for 16 hours. Upon completion tubes were brought to room temperature and centrifuged for five minutes at 13,000 x g. Supernatant was transferred to a fresh tube and precipitated with 2 volumes of ethanol and inverted once. Tubes were centrifuged for five minutes at 13,000 x g. Samples were gently aspirated; 1ml of ethanol was added and centrifuged for two minutes at 13,000 x g. Tubes were again aspirated and re-suspend with 50µl of TE (20µg/ml RNase). Sufficient time for precipitate to dissolve was required (24-96 hours at 4ºC). Extractions were analysed on a 0.75% agarose gel for 3.5 hours at 100 volts. Other methods tested included the Kado and Liu method (1981), the Anderson and McKay method (1983) and the Williams method (2006).

2.12. Clumping assay

To test if an isolate could be a pheromone producing recipient or a pheromone responsive donor, cells were grown overnight (16 hours) in 20 ml of TSB statically. Cells were pelleted by centrifugation (10,000 x g) for 15 minutes at 4^{o}C. Supernatant was removed and filter sterilised (Millipore 0.22 micron filters) providing pheromone-enriched broth. The clumping assay consisted of 500µl pheromone-enriched broth, 500µl fresh TSB, and 20µl of an overnight culture (16 hours) added in a 1.5ml Eppendorf tube (Eppendorf, Stevenage, UK) and incubated for four hours (37^{o}C rotating at 150 rpm). From the final suspension, 20µl was dropped on a glass slide and a coverslip (22mm) was applied. Clumping was determined by microscopy. Isolates that induced clumping but did not clump in the presence of other supernatants were deemed potential recipients. Isolates that readily clumped were characterised as potential donors. Cells were imaged using phase contrast with 100x (Nikon plan fluor 1.3 oil ph3 DLL) on a Nikon eclipse E400 with a Nikon DS-fi1c. Images were captured with NIS-elements and processed in imageJ (NIH).

2.13. Liquid phase conjugation methodology

Selected conjugation partners were grown overnight (16 hours) in TSB at 37^{o}C. Cell free supernatant, containing pheromone from a presumptive recipient isolate, was added to the presumptive donor and incubated for four hours at 37^{o}C, allowing time for the aggregation of the presumptive donor. The donor was added in equal volume (500µl) to the recipient in an Eppendorf and incubated for 20 minutes to 24 hours at 37^{o}C statically. Finally, the conjugation reaction was spread on a double selection TSA plate (vancomycin (10µg/ml), cephalothin (30µg/ml)) and incubated for 24 hours at 37^{o}C. Colonies were counted and conjugation efficiency was calculated (number of

transconjugant colonies/number of donor bacteria added to conjugation reaction). Controls consisted of streaking the conjugation partners individually onto a double selection TSA plate.

2.14. Solid phase conjugation methodology

Solid phase conjugation experiments were carried out using the solid agar mating method described by Cook *et al*. (2011). Potential donor and recipient isolates (after a 90-minute growth in fresh TSB from a 1/10 dilution from an overnight (16 hour) culture in TSB) were added together at a ratio of 1:9 onto a non-selective TSA plate and allowed to conjugate for 24 hours. The resulting lawn was re-suspended in 1ml of PBS, diluted (1/50-1/250) and spread onto TSA selection plates containing appropriate antibiotic combinations. Plates were incubated for a further 24 hours and transfer efficiencies were calculated (number of donors per transconjugant). Selection plates were comprised of TSA with rifampicin (100µg/ml) and either vancomycin (10µg/ml); erythromycin (50µg/ml); tetracycline (16µg/ml); or kanamycin (512µg/ml). Antibiotic free TSA was used as a control. All conjugation reactions were performed at 37ºC.

2.15. Detection of antibiotic resistance genes in transconjugants and their donors

Antibiotic resistance genes were detected using colony PCR (Tsuchizaki *et al.,* 2000). Template DNA was added at a final concentration of 200 ng (in 1µl) to 19µl of mastermix at a final concentration of 1.5mM of Mg^{2+} total (2µl of 1x PCR buffer and 0.6µl of separate $MgCl_2$), 0.2mm dNTP's each, 0.5µM forward and reverse primer and 1.0U of Taq polymerase. Primer sequences (Table 2.2) were selected based on antibiotic genes of interest as determined by transferred MIC phenotypes. All PCR reactions were run for 30

cycles with a final extension of five minutes. Samples were analysed by electrophoresis with Tris-Borate EDTA (TBE), in 1.5% agarose with ethidium bromide (final concentration 0.5µg/ml) at 100 volts and visualised on an Alpha Imager (Cell biosciences Heidelberg, Germany).

2.16. Sponge preparation for incubation with enterococci

Sponge gemmules were treated with 1% H_2O_2 for 10 mins (Rasmont 1970; Elliott & Leys 2007) and stored in deionised water at -20ºC until needed. Multiple gemmules were hatched into Universal tubes with 20 ml ultraviolet (UV) treated (10 mins at 254 nm) mineral water. Once sponges had hatched, they were fed 500 µl of food mixture (Interpet: Nutrifry no. 1 mixed in autoclaved distilled water, concentration of 0.4 mg/ml). Sponges were fed for four days prior to experimentation. The size of the sponges ranged from 5 - 36 mm^2 for *S. lacustris* and 0.5 - 48 mm^2 for *E. fluviatilis*.

2.17. Enterococcal sponge binding assay

One millilitre of *E. faecalis* MF06036 ($5x10^5$ CFU/ml) was added to a universal tube of sterile water containing a single *Spongilla lacustris* (five days' post hatching, 5-36 mm^2) and incubated for 24 hours at 20ºC. The sponge was removed from the tube, washed in sterile PBS, cut in half and stained with 1% crystal violet for 15 minutes in the well of a 6-well plate. The sponge was dry mounted on a glass slide and imaged on an OLYMPUS CX21 bright field microscope with 40x/0.65 and 100x/1.25 oil, plan objectives. Sponge sections were examined for evidence of enterococcal binding to tissue.

2.18. *Enterococcus* Conjugation in the presence of sponge

Conjugation partners were grown individually in TSB ($2\pm0.5x10^9$ CFU/ml approximately). They were then diluted 1/10 and grown for 90 minutes before being mixed. Universal tubes containing 19 ml of sterile water and sponge were inoculated with one ml of the mixed cells. The tubes were incubated at 20ºC for 24 hours. After incubation, the tubes were agitated and the water removed. To all of the tubes, 100µl of autoclaved deionised water was added and all biological material collected at the bottom of the tubes was homogenised. If the tube contained a sponge, this was also homogenised. All homogenates were plated on double selection plates and incubated at 37 °C for 48 hours before colony counts were performed. Controls consisted of sponge incubated under the same conditions with a single conjugation partner; or without any conjugation partners; or conjugation partners without sponge in the tube.

2.19. Conjugation within a biofilm

A biofilm-forming enterococcal conjugation partner was added (30µl of TSB 1% glucose with enterococcal cells ($2.5x10^9$CFU/ml)) to a gene frame on a 0.75% gelatin coated slide, sealed and incubated at 37ºC for 24 hours. The plastic seal was removed under sterile conditions in a cell culture hood, and biofilm was washed in sterile PBS to remove planktonic cells. The second conjugation partner was added (30µl) to the pre-established biofilm, sealed and incubated for 24 hours at 37ºC. The gene frame was removed, biofilm was washed with PBS, and the biofilm was dried in a cell culture hood. Biofilm was scraped and homogenised using a scalpel and Dimethyl sulfoxide (DMSO) (30µl). The homogenate was added to double selection plates and incubated for 24 hours at 37ºC before CFU's were counted. Starved biofilms were created using stationary phase cells in spent TSB media.

2.20. Biofilm examination techniques

2.20.1. Biofilm production in enterococci

Enterococci were grown overnight (16 hours) in TSB in round bottomed 96-well plates (ThermoFisher, Lutterworth, UK). Each culture was transferred (100µl) to a new 96-well plate with eight replicates and incubated for 48 hours at 37 ^{o}C. Planktonic cells were removed by washing three times with PBS and 100µl of 1% crystal violet was added to each well and incubated for 15 minutes. Plates were again washed with PBS three times, inverted and air dried in a fume hood. Glacial ethanol (200µl) was used to solubilise the dried crystal violet for 15 minutes before 125µl was transferred to a fresh plate (Flat bottomed – ThermoFisher) to be read on a VersaMax microplate reader (Molecular Devices, Berkshire, UK) at 570nm.

2.20.2. Enterococcal biofilm growth

Enterococcal biofilm formation involved the use of TSB (1% glucose) grown for 24-48 hours statically at 37^{o}C.

2.20.3. Biofilm formation in static microplates visualised with crystal violet

Biofilms were grown in 96-well polystyrene microplates for 48 hours, dried, washed three times with sterile PBS and stained with 1% crystal violet for 15 minutes. Crystal violet was removed, plates were gently washed with PBS and air dried. Crystal violet was solubilised with 200µl of 95% ethanol in each biofilm coated well for 15 minutes and absorbance was read at 570nm.

2.20.4. Biofilm formation in polystyrene tubes

Biofilms were grown in 14 ml round bottomed polystyrene tubes (Greiner – SigmaAldarich) for 48 hours. Tubes were washed, dried, stained with 1% crystal violet for 15 minutes, washed with PBS, air dried and examined by eye.

2.20.5. Air liquid interface biofilm formation

Microplate biofilms were grown with a coverslip semi-submerged, situated transverse to the bottom of the microwell for 48 hours. Coverslips were removed, and either stained with crystal violet (1% for 15 minutes) or mounted wet/dry for phase contrast microscopy.

2.20.6. Submerged coverslip biofilm formation

Submerged coverslips were grown in the same fashion as the air liquid interface assay with the difference being that the coverslips were lying on the bottom of the well.

2.20.7. Biofilm formation on coated substrates

Glass bottomed 16-well plates were used to grow standard 24 hour biofilms with either no coating or with a gelatin or collagen surface coating. Gelatin (SigmaAldrich) was prepared as a 2% (w/v) stock in tissue grade H_2O, autoclaved and coated on the surfaces (10µl/cm^2) and dried in a tissue culture hood for two hours. Collagen type I was prepared as a 0.1% (w/v) solution in 0.1M acetic acid. Substrates were coated (10µl/cm^2) and dried overnight (16 hours) in a tissue culture hood with UV. Substrates for both gelatin and collagen were rinsed with sterile H_2O and used for biofilm formation experiments.

2.20.8. Biofilm index measurements

Microwell biofilms were grown for 24 hours. To account for variances in cell population between isolates, the optical density of the planktonic microwell content was measured with absorbance at 600nm. Wells were then treated with 0.1% safranin, washed with PBS and dried. The dried wells were solubilised in ethanol and absorbance was measured at 450nm. To get the biofilm index OD450 was divided by OD600 (Leuck *et al.,* 2014).

2.20.9. Biofilm formation in the presence of total cell lysate

A 1% final concentration of SDS (62.5µl of a 16% stock solution of SDS) was added to one millilitre of enterococci from an overnight growth (16 hours) in TSB (that was washed in PBS) and incubated for two minutes at room temperature to facilitate lysis. Cell lysate was centrifuged at 13,000 x g for five minutes at room temperature. Cell lysate supernatants were removed from pelleted tubes into fresh tubes. Normal biofilms were grown in microwells with or without the addition of total cell lysate based on a 1% vol/vol concentration of cell lysate to biofilm growth media used. Biofilms were quantified using the biofilm index and normal biofilms were used as controls.

2.20.10. Biofilm formation using DNA

Total DNA was extracted from a mid-log phase enterococcal isolate grown in TSB and incubated at 37^{o}C statically. DNA was purified and measured using a QIAamp DNA Mini Kit (Qiagen, Manchester, UK) and a NanoDrop 8000 (ThermoFisher). A 20µg/ml stock of enterococcal DNA was added to a normal biofilm formation assay at a final concentration of 0, 0.1, 1 and 10% percentage volume of total DNA by total volume of biofilm formation media. This was carried out in the same fashion as the biofilm

formation using total cell lysate. Biofilms were quantified using the biofilm index and normal biofilms were used as controls.

2.20.11. Biofilm formation using the gene frame®

Double sided adhesive Gene frames (ThermoFisher) were adhered to glass microscope slides coated with gelatin (at a concentration of 10µg/cm^2) and UV sterilised. Under a cell culture hood, normal biofilms were grown within the confines of the gene frame well and sealed using the top side adhesive and supplied coverslips. Biofilms were grown for 24 hours statically at 37^{o}C. Starved biofilms were created by incubating stationary phase enterococci with spent nutrient media under normal biofilm growth conditions

2.20.12. Elimination of parents in a conjugal biofilm

Gene frame biofilm conjugation experiments were carried out as previously described. After conjugation, had occurred, antibiotic selection was applied directly to the conjugal biofilms incubated at 37^{o}C for 24 hours. Lysozyme (2mg/ml) was then added to the conjugal biofilm (as determined with a lysozyme MIC) for an additional 24 hours at 37^{o}C. Biofilms were then subjected to LIVE/DEAD and FISH staining protocols and imaged with the fluorescence microscope.

2.21 Fluorescent imaging of enterococci

Hoechst 33342 DNA stain was prepared in stocks of 10µg/ml in DMSO, and was optimised for detecting enterococci at working concentrations (diluted in PBS) of 0.1µg/ml in planktonic suspensions, 2.5µg/ml in biofilm and 3µg/ml in sponge. All Hoechst incubation times were 15 minutes at room temperature.

Concanavalin A (conA) Texas red conjugate (Invitrogen, Renfrew, UK) was used as a novel biofilm stain for enterococcal biofilm and was made into a stock solution of 1mg/ml in 0.1M sodium bicarbonate (pH 8.3). When long-term storage was required in solution, 2mM sodium azide was added allowing aliquots to be frozen or refrigerated. Working concentrations of conA were 25-50µg/ml and incubation times were 30-60 minutes at room temperature.

The LIVE/DEAD *Bac*Light bacterial viability kit L7012 (ThermoFisher) was used for live dead staining of enterococci. Stock solution preparation was followed as per instructions in the kit. Cell suspensions were centrifuged at 10,000 x g for 15 minutes and washed with deionised H_2O and re-suspended in 0.85 NaCl. Component A and component B were mixed in equal volumes thoroughly. The stain was added at 3µl to each millilitre of bacteria cell suspension and incubated at room temperature for 15 minutes (20 minutes for biofilm). A 5µl sample of stained bacteria was placed between a coverslip for fluorescent imaging, and for biofilms, stains were washed off with PBS, dried and mounted using mounting medium (Vectashield) for fluorescence microscopy. Microscopy was carried out with a 100x objective on a Nikon eclipse E400 with a Nikon DS-fi1c using a G2-A and UV filter set. Images were captured with (NIS-elements and image j (NIH)).

2.22. Fluorescent *in situ* hybridisation (FISH) techniques

2.22.1. FISH protocol for visualisation of planktonic enterococci

Experimentation was completed by smearing enterococcal cells from an overnight growth (16 hours) in TSB onto a gelatin coated slide. The protocol was adapted from Warr *et al*. (2005). Cell smears were fixed in 50% ethanol/PBS for 15 minutes. Cells were permeabilised in 20µl of lysozyme (0.5mg/ml, 0.1M Tris-HCl, 0.05M EDTA for 65 minutes at 37ºC in a prewarmed humidified chamber. Lysozyme was washed off with PBS and cells were dehydrated to ethanol at increments of 50%, 70% and 100% for three minutes in each stage. Volumes of 10-20µl of FISH probes were used at concentrations between 5-10ng/µl in hybridization buffer (0.9M NaCl, 20mM Tris-HCl (pH 7.5) and 0.1% (w/v) SDS) and were incubated and optimised in temperatures of 45-55ºC from 15 minutes to 24 hours with formamide concentrations from 0-60%. Cells were washed (3x5 minutes) in wash solution (0.9M NaCl, 20Mm Tris-HCl (Ph 7.5) at room temperature, air dried and mounted in vectashield hardset antifade for fluorescent microscopy (Vector laboratories, Cambridgeshire, UK). Slides were imaged with a 100x objective on a Nikon eclipse E400 with a Nikon DS-fi1c using a G2-A and UV filter set. Images were captured with (NIS-elements and image j (NIH)).

2.22.2. FISH protocol for visualisation of enterococcal cells in biofilm

Working from the method described above (section 2.25.1): Enterococci were grown in tryptone soy broth (TSB) to approximately $2.5x10^9$ CFU/ml. Cells were harvested, re-suspended and diluted 1:200 in TSB (1% glucose) and were inoculated into gene frames, adhered to 0.75% gelatin-coated microscope slides. Biofilms were developed on the slides which were incubated at 37ºC for 24hrs. Biofilms were washed with PBS and fixed

in 97% ethanol for five minutes. Fixed biofilms were permeabilised with lysozyme (1mg/ml) for 30 minutes at 37^{o}C and probed (table 2.3) with either ENF 191, ENU 1470 or EUB 338 in 10% formamide for 24hrs at 50^{o}C. Cells were washed (3x5 minutes) in wash solution (0.9M NaCl, 20Mm Tris-HCl (Ph 7.5) at room temperature and air dried. Slides were mounted with vectashield mounting medium for fluorescence microscopy. Slides were imaged with a 100x objective on a Nikon eclipse E400 with a Nikon DS-fi1c using a G2-A and UV filter set. Images were captured with (NIS-elements and image j (NIH)).

2.22.3. FISH protocol for visualisation of vancomycin *vanA* in enterococcal biofilms

Vancomycin resistant *E. faecalis* (VRE) were grown in tryptone soy broth (TSB) to approximately 2.5x10^{9} CFU/ml. Cells were harvested, re-suspended and diluted 1:200 in PBS, TSB or TSB (vancomycin 10µg/ml). VRE were inoculated into 25µl gene frames, adhered to 0.75% gelatin-coated microscope slides. Biofilms were developed on the slides which were incubated at 37^{o}C for 24hrs. Biofilms were washed with PBS and fixed in 97% ethanol for five minutes. Fixed biofilms were incubated with two distinct fluorescein labelled FISH probes specific to *vanA* for 2-24hrs at 50^{o}C in 10% formamide. An Alexa fluor 594 probe targeted to *E. faecalis* 16S rRNA was used as a control. Probes are listed in table 2.3. Slides were mounted with vectashield mounting medium for fluorescence microscopy. Slides were imaged with a 100x objective on a Nikon eclipse E400 with a Nikon DS-fi1c using a G2-A and UV filter set. Images were captured with (NIS-elements and image j (NIH)).

2.22.4. FISH on sponge frozen sections from *Enterococcus* conjugation experimentation

After conjugation experimentation in the presence of sponge, sponges were fixed in 50% ethanol PBS for 24hrs at -20°C for crysosectioning. Ten micron sections were cut through whole sponges and deposited on 0.75% gelatin coated slides. ENF 191 with a 5' Texas red fluorophore was hybridised to sponge sections for 90 minutes in a hybridisation chamber at 10 ng/µl as described previously (Waar *et al.*, 2005). Hoechst was added for 15 minutes at room temperature (0.5µg/ml). Sudan black B was used to reduce tissue autofluorescence in sponge tissue. Fresh Sudan black at a concentration of 0.1% diluted in 70% ethanol was added at the end of fluorescent staining for five minutes at room temperature (Sun *et al.,* 2011). Slides were mounted with vectashield hardset antifade for fluorescent microscopy. Slides were imaged with a 100x objective on a Nikon eclipse E400 with a Nikon DS-fi1c using a G2-A and UV filter set. Images were captured with (NIS-elements and image j (NIH)).

2.23. Statistics

Plate based assays such as the clumping and biofilm assays were performed at least three times with eight biological repeats each time. Assays performed on the gene frame biofilm apparatus were performed five times with six biological repeats. Microscopy based statistics came from 10 regions of interest with three independent repeats. Averages were taken and when appropriate standard error of the mean is displayed. Significance was computed using GraphPad prism 6 t-test function, one way and two-way analysis of variance.

Chapter 3

Characterisation of environmentally isolated enterococci for their ability to carry out horizontal gene transfer

3.1. INTRODUCTION

3.1.1 Antibiotic resistant “superbugs”

The 2010’s marked a major shift in the global understanding of antimicrobial resistant (AMR) infection. The mass media broadcast of the growing ineffectiveness of antibiotic therapy has proliferated across the globe, and is beginning to reach a critical status. Conglomerating google searches of news reports from 2016 has highlighted, over 100,000 articles referencing ‘antibiotic resistance’. Comparing this statistic to the previous decade of stable numbers of around 16,000, clearly AMR has come to the forefront of the general public’s understanding of the 21st century disease. The term ‘superbug’ was used to describe bacteria that were genetically engineered for useful purposes; however, this name no longer bears positive merit and has become synonymous with antibiotic resistant pathogenic bacteria (Horikoshi and Grant, 1991; Alum and Obuba 2015). The term has evolved in the last decade to include an ever-expanding list of bacteria. Almost 20 bacterial species are now commonly identified as AMR superbugs, with the compounding description as ‘multi’ drug resistant (Control and Prevention, 2013). The overuse of antibiotics has undoubtedly caused the rapid proliferation of these AMR trends. Recent research has shown that some of these deadly pathogens such as Methicillin Resistant *Staphylococcus aureus* (MRSA) are developing acquired resistances from other superbugs, such as Vancomycin Resistant *Enterococcus faecalis* (VRE) (Ray *et al.*, 2003; Weigel *et al.*, 2003; Zhu *et al.*, 2010). Therefore, it is of utmost importance to understand when, where and how these interactions can take place *in situ*, in order to limit the spread of current AMR phenotypes as well as preventing novel proliferation.

3.1.2 Enterococci in the extra-enteric environment

Environments where humans and domesticated animals share resources has shown cross colonisation of enterococci from various origins, including VRE and pathogenic strains of *E. faecium* (Witte, 2000). Enterococci isolated from pigs was shown to be highly similar to human isolates when using Multilocus Sequence Typing (MLST) isolate characterisation profiles as well as antimicrobial resistance phenotypes (Hasman *et al.,* 2005). Enterococci are used to monitor mammalian faecal contamination in the wider environment (Raisanen *et al.,* 2007; Purnell *et al.,* 2011). From this monitoring activity, it has been noted that enterococci can be capable survivors in the harsh *ex vivo* environment. There is no direct evidence to suggest that enterococci can grow in oligotrophic environmental waterways; however, they have been shown to grow in beach sands and water supplemented with kelp (Yamahara *et al.,* 2009; Imamura *et al.,* 2011). The possibility for enterococci to grow in environmental waterways has been stipulated from successful experiments conducted on *E. coli* O157:H7 (Vital *et al.,* 2008). Lleo *et al*. (2005) demonstrated that *E. faecalis* and *E. faecium* could enter a starvation state or the viable but non culturable (VBNC) state when introduced to extended oligotrophic conditions. They remained viable for 40 days in sterilised lake water at 4^{o}C without direct illumination, and up to 2 months at room temperature under the same conditions.

3.1.3 Antibiotic resistance in enterococci

As antibiotic resistant enterococci can possess so many AMR genes, identification of resistance phenotypes can take up to five days in many health clinics. Treatment varies from a single antibiotic targeted to susceptible phenotypes; to complicated dosing regimens with combinations of antibiotics because, extensive resistance phenotypes are present (Drews *et al.,* 2006; Habib *et al.,* 2009). Enterococci can possess several intrinsic

resistance phenotypes; such as resistance to penicillins, aminoglycosides, and cephalosporins; as well as many acquired AMR genes. The extensive resistance phenotypes associated with enterococci can be explained by their natural ecology (Bonten *et al.,* 1998; Fisher and Phillips, 2009). Enterococci are commensals of human GI and would therefore have been exposed to antibiotics. This selective pressure with antimicrobial therapy and the current examples of enterococcal AMR observed can be linked as cause and effect (Gilmore *et al.,* 2014).

Enterococci possess several complex mechanisms of AMR. Most noted are the glycopeptide resistance mechanisms to vancomycin/teicoplanin. Glycopeptides are primarily effective against Gram positive bacteria as the drugs target the D-Ala D-Ala peptide residues of peptidoglycan during cell wall synthesis, thereby destabilising cell wall integrity through impaired enzyme mediated transglycosylation (Arthur and Courvalin, 1993; Courvalin, 2006). Resistance mechanisms are underpinned simply by modification of the D-Ala D-Ala binding site to D-Ala D-Ser therefore impairing drug binding affinity and overcoming any bactericidal effects (Courvalin, 2006). Aminoglycosides inhibit protein synthesis through binding to ribosomal 16S rRNA and enterococci are intermediately resistant to aminoglycosides. Generally, uptake of these antibiotics is limited by their facultative anaerobic metabolism (Aslangul *et al.,* 2006). Additionally, drug uptake bottlenecks include aph(2'')-I phosphotransferase that uses ATP to phosphorylate a hydroxyl group on the antibiotic; and AAC(6')Ie-APH(2'')-Ia acetyltransferase that uses acetyl-CoA to acetylate an amino group on the antibiotic. These aminoglycoside enzymes are commonly located on MGE's (Kak *et al.,* 2000; Galloway-Pena *et al.,* 2012). Rifampicin acts by binding to the beta subunit of RNA polymerase, halting transcription (Wehrli *et al.,* 1968). General resistance to rifampicin

comes through point mutations in the *rpoB* gene which reduces binding affinity of the drug (Enne *et al.*, 2004).

3.1.4 Horizontal gene transfer in enterococci

A significant factor for the rise in prominence of enterococcal infections is extensive multidrug resistance (MDR), another factor, which in many ways is more important in the long term is: proficient gene transfer mechanisms. When the genome of *E. faecalis* OG1X, one of the first and most studied 'benign' isolates was sequenced, no foreign DNA, indicative of horizontal gene transfer was detected (Bourgogne *et al.*, 2008). When examining the NCBI gene databases, it was clear that MDR enterococci possess significant (>25%) quantities of acquired genetic material. Most enterococcal antimicrobial resistance genes are on mobile genetic elements, including plasmids. These invasive enterococci now possess complex mechanisms which can efficiently transfer these AMR genes (Thomas and Nielsen, 2005).

One of the most interesting and studied mechanisms of horizontal gene transfer in enterococci is sex pheromone mediated plasmid transfer in *E. faecalis* (Panesso *et al.*, 2005). There are many mobile element variants across *E. faecalis* and *E. faecium* with a range of AMR genes (Kristich *et al.*, 2014). The AMR genes associated with pheromone mediated plasmids transfer with high efficiency (Hirt *et al.*, 2002). These plasmids are sensitive to specific short chain peptides produced by plasmid deficient members, which when successfully bound to 'donor' members induce aggregation substance production (Waters and Dunny, 2001; Waters *et al.*, 2003). Aggregation substance induces clumping of donors containing pheromone plasmid, significantly increasing efficiency of bacterial plasmid conjugation as seen in figure 1.4 (Yagi *et al.*, 1983). This process of horizontal

gene transfer occurs primarily amongst *E. faecalis* species, but interspecies transfer has been recorded (vancomycin (*vanA*) resistance from *E. faecium* to *E. faecalis*) (Heaton *et al.,* 1996). Tetracycline resistance transfer has been extensively demonstrated on the pheromone responsive plasmid pCF10 (Christie *et al.,* 1987). These efficient pheromone responsive plasmids have thus far not been shown to replicate outside the *Enterococcus* genus (Kristich *et al.,* 2014). However, enterococci have several other ways of accomplishing horizontal gene transfer. They possess other plasmids (Inc18) containing AMR genes that can transfer to members outside their genus (*Staphylococcus* and *Streptococcus*) through conjugation (Zhu *et al.,* 2010). Although this method is less efficient than sex pheromone plasmid transfers, both mechanisms involve the seeding of undesirable AMR phenotypes (tetracycline, gentamycin, erythromycin, beta lactamase and streptomycin genes) to pathogenic strains (Ray *et al.,* 2003). The final method of gene exchange utilised by enterococci is the transfer of transposons encoding AMR genes.

Transposons are important genetic inclusions in the strain identity of many enterococci, often responsible for strain specific virulence and resistance phenotypes (Kristich *et al.,* 2010). The main categories of enterococcal transposon are: - composite, Tn*3* family and conjugative transposons. Transposons discussed here are summarised in table 3.1. The most relevant composite transposon regarding AMR would be Tn*5281,* which contains the aac -6'/ aph – 2'' aminoglycoside modifying enzyme responsible for resistance to all aminoglycosides except streptomycin (Kristich *et al.,* 2010). Tn*1547* encodes variable resistance to vancomycin (Rand *et al.,* 2007). Tn*3* – family transposons include Tn917 which encodes resistance to macrolides, lincosamides and streptogramin B and shares 100% identity to the Tn*551* macrolide resistance transposon in *S. aureus* (Wu *et al.,* 1999). No Tn*3* – family transposon can transfer, however the most significant Tn*3*

transposon with relevance to AMR is Tn*1546* which contains the *vanA*, vancomycin resistance cluster, and can be located in Inc18 plasmids and therefore transferred through conjugation (Arthur *et al.,* 1993). The *vanA* subtype results in the highest AMR to vancomycin as well as teicoplanin and has been shown to transfer into *S. aureus* on an Inc18 plasmid, demonstrating how vancomycin can make its way from VRE into MRSA. Finally, conjugative transposons include Tn*916* which encodes resistance to tetracycline and shows identity to elements in pneumococcal strains and they can transfer whole elements through site excision, transfer and integration through intercellular transfer (Shaw and Clewell, 1985).

Table 3.1. Transposons of interest within the mobilome of enterococci.

Transposon	Categorisation	Function (genotype)	Host range
Tn*5281*	composite	Gentamycin (aac - 6'/ aph – 2'')	*Enterococcus, Staphylococcus. aureus, Streptococcus agalactiae, Mycoplasma*
Tn1547	composite	vancomycin (*vanB1*)	*Enterococcus,*
Tn917	Tn3	Erythromycin (*ErmB*)	*Enterococcus, Staphylococcus, Streptococcus, Lactococcus,, Bacillus, Listeria, Paenibacillus*
Tn1546	Tn3	vancomycin (*vanA*)	*Enterococcus, Bacillus, Staphylococcus, Oeskorvia, Streptococcus, Rhodococcus, Arcanobacterium haemolyticum, Paenibacillus*
Tn916	Conjugative	Tetracycline (*TetM*)	*Enterococcus, Staphylococcus, Streptococcus, Lactococcus, Lactobacillus, Bacillus, Clostridium, Leuconostoc, Listeria, Mycoplasma, Actinobacillus, Acholeplasma, Acinetobacter, Alcaligenes, Butyrivibria, Citrobacter, Erysipelothrix, Escherichia, Fusobacterium, Granulicatella, Haemophilus, Neisseria, Pseudomonas, Thermus, Ureaplasma, Veillonella, anaerobes*

3.2 Aim and Objectives

This chapter aimed to demonstrate the compatibility of enterococci to conjugate in the extra-enteric environment.

Objectives:

- Test the antibiotic resistance phenotypes of *Enterococcus faecalis* and *Enterococcus faecium*
- Test the compatibility of these isolates to commit to horizontal gene transfer of antibiotic resistance
- Examine the ability of the isolates to form biofilm using standardised protocols

3.3 MATERIALS AND METHODS

All the materials and methods carried out in this chapter are detailed in chapter two.

3.4 RESULTS

3.4.1 Selection of *Enterococcus faecalis* and *Enterococcus faecium* with the potential to undergo horizontal gene transfer

The outline of the process of carrying out characterisation tests on selected enterococci is detailed below. Selection of enterococci involved testing the compatibility of isolates to: conjugate, have antimicrobial resistance, and biofilm formation ability. By fulfilling these criteria, all bacteria would have the highest possibility of passing on AMR genes through HGT. The candidate isolates were chosen from 665 total enterococci. These enterococci had previous characterisation data by Daniels, (2011) aiding in rapid selection of a manageable number if isolates. There were 23 candidates partially characterised in the previous study to contain AMR genes and demonstrate phenotypic clumping (Daniels, 2011). These isolates consist of 14 *E. faecalis* and 9 *E. faecium*, a good spread of clinically relevant species. The enterococcal isolate nomenclature was devised previously (Daniels, 2011)

Figure 3.4.1. Flow Diagram outlining selection criteria used to identify a subgroup of isolates compatible for horizontal gene transfer.

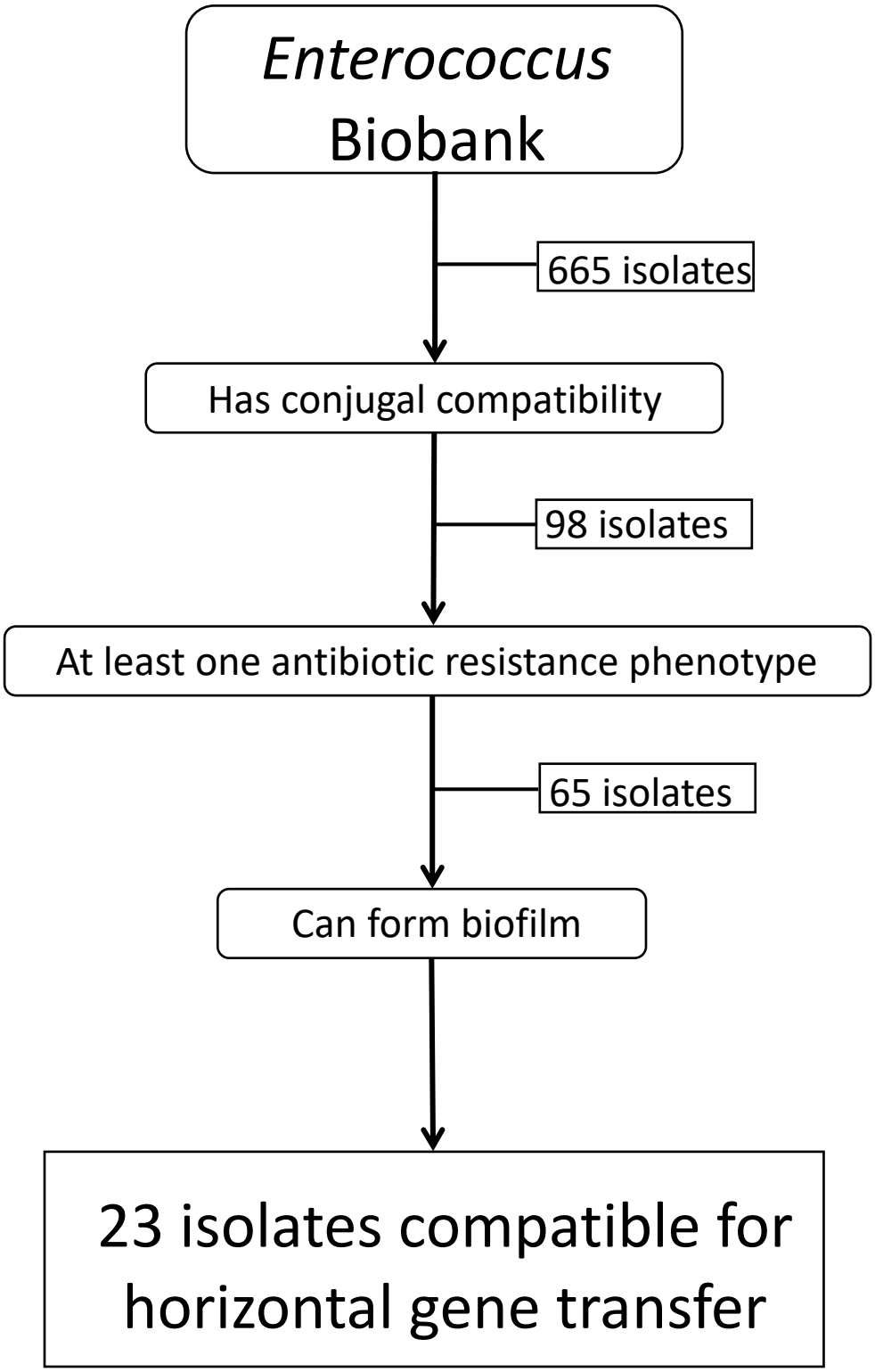


Table 3.4.1. Candidate enterococci selected for compatibility testing. From Daniels, (2011).

Isolate	Species	Isolation source	Diversity index from source	PhP type	Clustering strength
MF04019	*E. faecium*	Farm 4	0.97	si	***
MF04010	*E. faecalis*	Farm 4	0.97	si	***
MF06035	*E. faecalis*	Farm 6	0.95	6	***
MF06036	*E. faecalis*	Farm 6	0.95	6	***
MF06019	*E. faecium*	Farm 6	0.95	si	-
MF06030	*E. faecium*	Farm 6	0.95	5	***
MF07008	*E. faecium*	Farm 7	No information		
ST01053	*E. faecium*	Storm 1	0.92	si	***
ST01063	*E. faecium*	Storm 1	0.92	3	***
ST01109	*E. faecium*	Storm 1	0.92	2	**
ST02011	*E. faecalis*	Storm 2	0.476	1	**
ST02227	*E. faecalis*	Storm 2	0.476	1	**
MW01038	*E. faecalis*	Water site 1	0.712	3	***
MW01021	*E. faecalis*	Water site 1	0.712	si	**
MW01105	*E. faecalis*	Water site 1	0.712	si	**
MW02102	*E. faecalis*	Water site 2	0.712	si	***
MW02077	*E. faecium*	Water site 2	0.961	2	***
MW02087	*E. faecalis*	Water site 2	0.712	2	**
MW02043	*E. faecalis*	Water site 2	0.712	2	***
MW03020	*E. faecalis*	Water site 3	0.712	2	**
MW03025	*E. faecalis*	Water site 3	0.712	3	***
MW03051	*E. faecalis*	Water site 3	0.712	4	***
MW03061	*E. faecium*	Water site 3	0.961	2	***

List of Enterococci detailing isolate name, species, isolation site and the diversity index from the isolation source. Diversity index calculated using Simpsons Diversity Index. PhP type details the grouping of relevant isolates from their unique source site. Two isolates with the same PhP type from the same isolation source share the same type of PhenePlate biochemical fingerprint. Clustering strength indicates the fit of each isolate within its PhP type: *** strong, ** intermediate, * weak, - unreliable information. Si indicates that the isolate is of a single unique PhP type. Information tabulated from Daniels, (2011).

3.4.2 Characterisation of candidate enterococci for conjugation studies – general antimicrobial susceptibility

The first compatibility assay applied to the 23 selected enterococci was the disc diffusion assay. There were 19 antibiotics used in this assay, most of which have been used historically to treat enterococcal infection. Some antibiotics (Trimethoprim and sulphonamides) are not clinically relevant but were used in this assay as a control. The least effective antibiotics on the group, in descending order of total number of resistant isolates (indicated with parentheses) were: trimethoprim (23), ampicillin (17), streptomycin (16), amoxicillin (16), sulfamethoxazole + trimethoprim (15), tetracycline (13), cephalothin (13), and erythromycin (10). The most effective antibiotics were ciprofloxacin, neomycin and linezolid with total susceptibility for all isolates tested. Gentamycin and imipenem both had one resistant isolate, oxytetracycline and teicoplanin had 2 resistant isolates, quinupristin/dalfopristin had 3 and vancomycin resistance was displayed by 8 isolates.

Isolate MF06035 and MF06036 were the most multiresistant with resistance to 11 of the 19 antibiotics tested. ST01109 and MF06019 were resistant to 10 antibiotics. MW01038, MW03020, ST02011, MF04010 and MW03051 were resistant to nine antibiotics. MW02102, MW03025, MF06030, MW02043 and MW01105 were resistant to eight antibiotics. MW02077 and MW03061 were resistant to seven antibiotics and ST02227 was resistant to five. ST01063 and ST01053 were resistant to four antibiotics. Finally, MF07008, MF04019, MW01021 and MW02087 were resistant to three antibiotics. The most resistant isolates of enterococci were isolated from poultry litter apart from MF04019. The single isolate from the septic tank (ST01109) was the third most resistant isolates. The single bovine isolate had one of the lowest resistance profiles.

Table 3.4.2. Disc diffusion antimicrobial resistance profiles of selected enterococci.

Enterococcal isolate	Resistance																
	AMP	VAN	E	S	TET	CIP	Q,D	TRI	TEI	GEN	AMO	IMP	OXY	S,T	N	CEP	LIN
MF06035	R	R	R	R	S	S	S	R	R	S	R	S	S	R	S	R	S
MF07008	R	S	S	R	R	S	S	I	S	S	S	S	S	S	S	S	S
MF06036	R	R	R	R	R	S	S	R	R	S	R	S	S	R	S	S	S
MW01038	R	R	S	R	S	S	S	R	S	S	R	S	S	R	S	R	S
ST01053	S	S	R	S	R	S	R	I	S	S	S	S	S	S	S	S	S
MW02102	R	R	S	R	S	S	S	R	S	S	R	S	S	R	S	R	S
MW03020	R	R	S	R	S	S	S	R	S	S	R	S	S	R	S	R	S
MW03025	R	R	S	R	S	S	S	R	S	S	R	S	S	R	S	R	S
MW03051	R	R	S	R	S	S	S	R	S	S	R	S	S	R	S	R	S
ST02011	R	R	R	R	S	S	S	R	S	S	R	S	S	R	S	R	S
ST01063	S	S	R	S	R	S	R	R	S	S	S	S	S	S	S	S	S
MF04019	S	S	R	S	R	S	S	I	S	S	S	S	S	S	S	S	S
MW01105	R	S	S	R	S	S	S	R	S	S	R	S	S	R	S	R	S
MW02077	R	S	R	R	S	S	R	R	S	S	R	R	S	S	S	S	S
MW02043	R	S	S	R	S	S	S	R	S	S	R	S	S	R	S	R	S
ST01109	R	S	S	R	R	S	S	R	S	S	R	S	R	R	S	R	S
MW01021	S	S	S	R	R	S	S	I	S	S	S	S	S	S	S	S	S
MF06019	R	S	R	S	R	S	S	R	S	S	R	S	R	R	S	R	S
MF04010	R	S	S	R	R	S	S	R	S	S	R	S	R	R	S	R	S
ST02227	S	S	R	R	R	S	S	R	S	R	S	S	S	S	S	S	S
MF06030	R	S	S	S	R	S	S	R	S	S	R	S	S	R	S	R	S
MW03061	R	S	S	S	R	S	S	R	S	S	R	S	S	R	S	S	S
MW02087	S	S	R	S	R	S	S	I	S	S	S	S	S	S	S	S	S
ATCC29212	S	S	S	S	S	S	S	I	S	S	S	S	S	S	S	S	S

AMP - Ampicillin, VAN - vancomycin, E - erythromycin, S - streptomycin, TET - tetracycline, CIP - ciprofloxacin, Q,D - quinupristin/dalfopristin, TRI - trimethoprim, TEI – teicoplanin, GEN - gentamycin, AMO - amoxicillin, IMP - imipenem, OXY - oxytetracycline, S,T - sulfamethoxazole + trimethoprim, N - neomycin, CEP - cephalothin, LIN - linezolid. * R – resistant; S – susceptible; I – Intermediately susceptible

3.4.3 Characterisation of candidate enterococci for conjugation studies – Biofilm formation and clumping

The second criterion for strain selection was based on the ability to form biofilm. This method provided information on the ability of the 23 enterococcal isolates to form biofilm using the 96-well crystal violet absorbance assay. The assay is well characterised and has delineations of biofilm formation quantity. Two thirds of the tested isolates formed biofilm using this method and half of those were strong biofilm producers. There was a good representation of biofilm forming ability present among the isolates, combining this data with the antimicrobial data allowed for examination of the final characterisation assay for compatibility amongst the isolates for conjugation studies.

The third compatibility assay was designed as the crucial step in highlighting any relationship between any two isolates to carry out horizontal gene transfer. The clumping assay would visually demonstrate compatibility of two isolates with a phenotypic aggregation of the cells of one isolate with the supernatant of the other. Figure 3.4.3. demonstrates the range of clumping intensity observed across all tested isolates ranging from 1 to 6 (devised in this study). Isolates that produced no reaction were labelled as 1. Isolates that produced a weak reaction were labelled as 2. Isolates that produced either a weak reaction with 1 high density clump or less than 5 high density clumps were labelled as 3. Isolates that produced 5 to 20 high density clumps were labelled as 4. Isolates that contained over 20 high density clumps were labelled as 5. Finally, any isolate that produced a reaction which clumped the entire visual field contained within the well (reducing the opacity of the TSB) were labelled as 6.

Table 3.4.3. details the output of the clumping assay tested on all isolates, the assay revealed that MW01105, ST01109 and MW02043 were the best pheromone producing enterococci. They could not clump in the presence of the supernatant of other isolates, yet

they could induce the clumping reaction in those isolates. In general, the most effective potential donor isolates at clumping to all isolates were MF04010, MF06030, MF04019, MF06019 MF06035, MW02087, MW03025 and MW03020 in descending order of general clumping intensity. Potential recipient isolate MW01105 induced the highest number of clumping reactions, followed by MW02043. Recipient isolate ST01109 induced the lowest clumping intensity including five results scoring 1 on the clumping scale.

To understand how clumping affected cells at the microscopic level, all isolates were examined with phase contrast microscopy. This tool was invaluable when defining weak clumping, as defined by the clumping scale: clumping intensities of 1 and 2 (no reaction and weak reaction respectively). All potential donor isolates of enterococci were exposed to supernatant from each of the three pheromone producers for four hours in a 24-well microplate at 37^{o}C. Figure 3.3.4.(a) illustrates the typical reaction observed when aggregation signalling is activated. Figure 3.3.4.(b) is a high-resolution micrograph of a high density clumping reaction. Figure 3.3.4.(c) was the largest recorded clumping reaction (clumping in the entire field of view) and occurred between *E. faecalis* MF06035 and the supernatant of MW01105. Figure 3.3.4.(d) was a control illustrating the absence of clumping that occurred when MF06036 supernatant was added to MW01105 in a reverse reaction.

The final step required for categorisation of the candidate isolates of enterococci involved extensively screening the NCBI (https://www.ncbi.nlm.nih.gov/gene) gene database for all published mobile genetic elements as well as AMR genes (table 3.4.4) and conjugation genes (table 3.4.5) present contained within the elements for *E. faecalis* and *E. faecium*. All information was collated in a database listing all genes of interest as well as any variations in homology of the same gene. Descriptions of the genes and their aliases are

detailed in tables 3.4.4. and 3.4.5. These genes were used to select antimicrobials to use in MIC testing on the selected enterococci as well as any PCR tests to confirm genes and any further gene transfer events.

Figure 3.4.2. Ability of selected enterococci to form biofilm in 96-microwell plates stained with crystal violet.

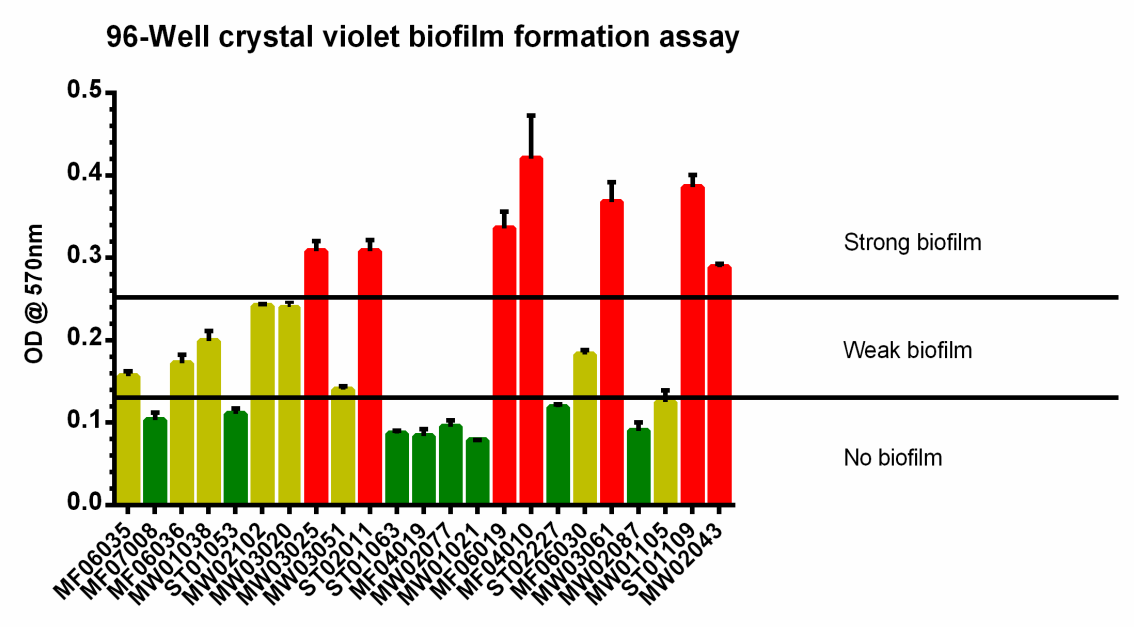
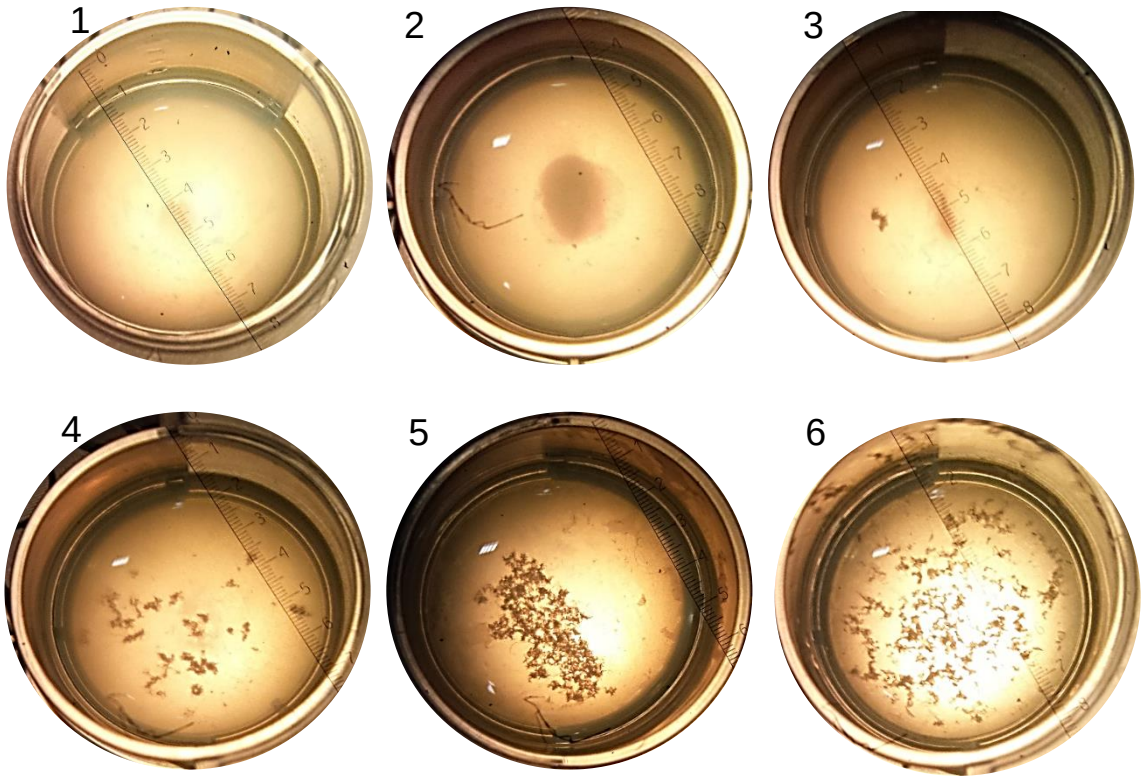


Figure 3.4.2 Crystal violet absorbance at 570nm after the crystal violet 96 well 24-hour biofilm formation assay on enterococci using tryptone soy broth incubated at 37ºC statically under aerobic conditions. Optical density correlates to relative biofilm strength: Strong biofilm - ≥0.241, weak biofilm 0.121-0.24, non-biofilm ≤0.120. Seven isolates produced strong biofilm. Eight isolates produced weak biofilm. Eight isolates did not produce biofilm (MF07008, ST01053, ST01063, MF04019, MW02077, MW01021, ST02227 and MW02087). Data representative of eight biological repeats, independently repeated three times. Using multiple comparison analysis of variance, p values for each strong biofilm compared against weak biofilm and no biofilm producers is <0.0001. each isolate had eight biological controlS, independently repeated three times.

Figure 3.4.3. Bright field macroscopy outlining the degree of variation of clumping ability of selected enterococci.



Low powered bright field microscopy detailing variation of inter-isolate clumping proficiency. Clumping reactions occur when the pheromone from one of two compatible isolates is added to the other in planktonic conditions. Clumping intensity was graded from 1-6. 1 – no reaction, 2 – weak reaction, 3 – weak reaction and/or up to five of high density clumps, 4 – five to 20 high density clumps, 5 – more than 20 high density clumps, 6 – confluence of high density clumps spanning entire well.

Table 3.4.3. Identification of pheromone producing ‘recipient’ isolates vs clumping ‘donor’ isolates expressed as clumping ability based on guidelines from 3.4.5.

Clumping of enterococci in the presence of cell free supernatant pheromone

Enterococcal isolate	Clumping intensity from macroscopic control ranges (0-6)		
	MW01105	ST01109	MW02043
MF06035	6	5	4
MF07008	4	1	4
MF06036	4	4	3
MW01038	4	2	5
ST01053	3	1	5
MW02102	4	2	3
MW03020	3	3	4
MW03025	4	3	4
MW03051	3	3	4
ST02011	3	4	2
ST01063	4	1	5
MF04019	2	1	1
MW02077	1	2	1
MW01021	4	1	5
MF06019	5	3	4
MF04010	6	4	4
ST02227	4	1	4
MF06030	5	3	5
MW03061	4	3	3
MW02087	4	2	1
ATCC29212	0	0	0

Table 3.4.3. MW01105, ST01109 and MW02043 induced clumping in the other candidate isolates but could not be clumped themselves, rendering them as free pheromone producing potential ‘recipient’ isolates. Their pheromone was added to the other candidate isolates (potential ‘donors’) and the subsequent clumping reactions were graded using a scale of 1-6 relative to intensity of clumping using macroscopic visualisation assays

Figure 3.4.4. Phase contrast microscopy of donor enterococci induced into clumping with the addition of recipient pheromone.

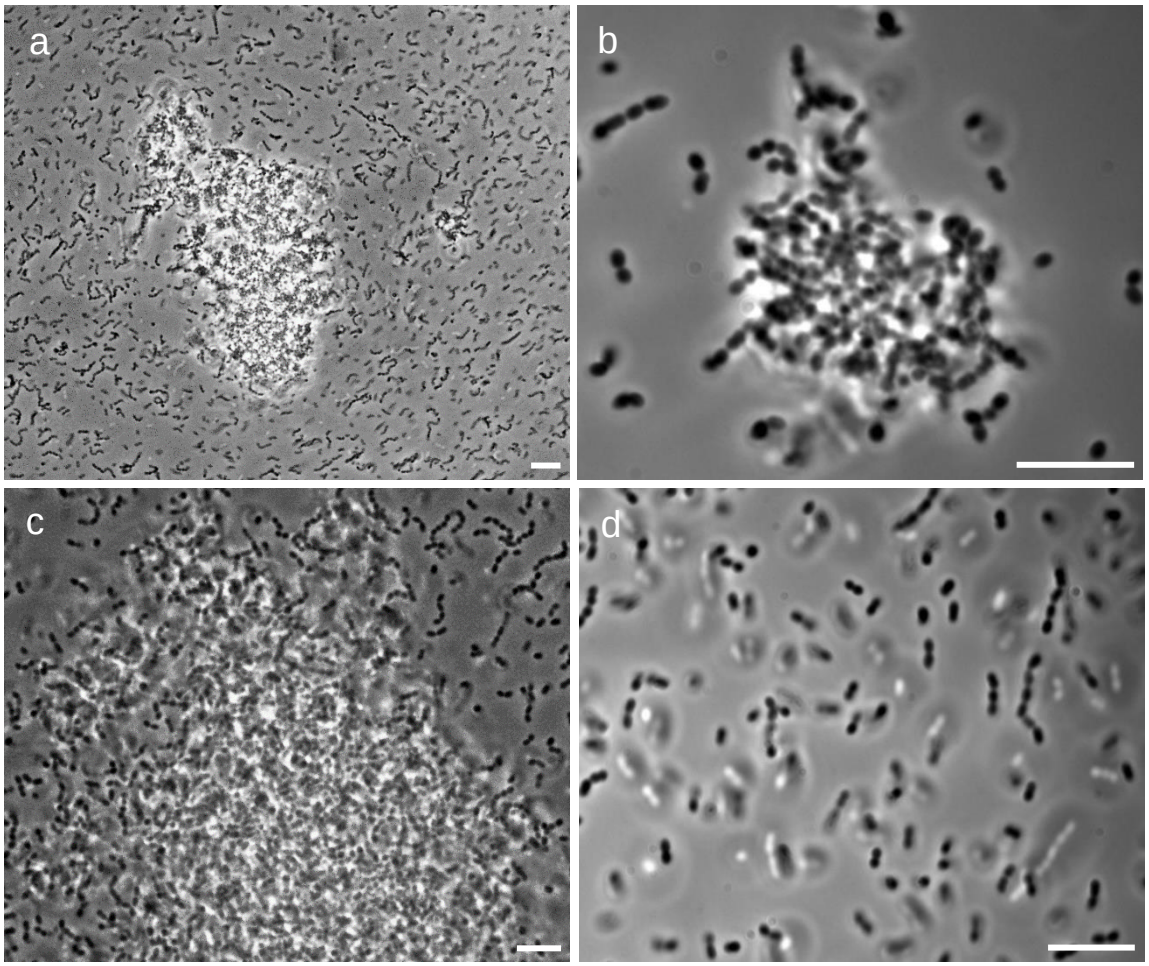


Figure 3.4.4. Phenotypic clumping micrographs of enterococci, examined with phase contrast. (A) 20x micrograph of *E. faecalis* MF06036 with the supernatant of *E. faecalis* MW01105, (Control and Prevention, 2013), the typical reaction observed when aggregation signalling is activated. (B) 100x micrograph of MF06036 with the supernatant from *E. faecium* ST01109. (C) 40x image of a large reaction between *E. faecalis* MF06035 and the supernatant of MW01105. (D) 100x Negative clumping reaction of MF06036 supernatant added to MW01105. Red arrow indicates microscopic clumping; scale bars represent ten microns.

Figure 3.4.5. Establishment of a gene database based on Antimicrobial resistance genes and horizontal transfer genes.

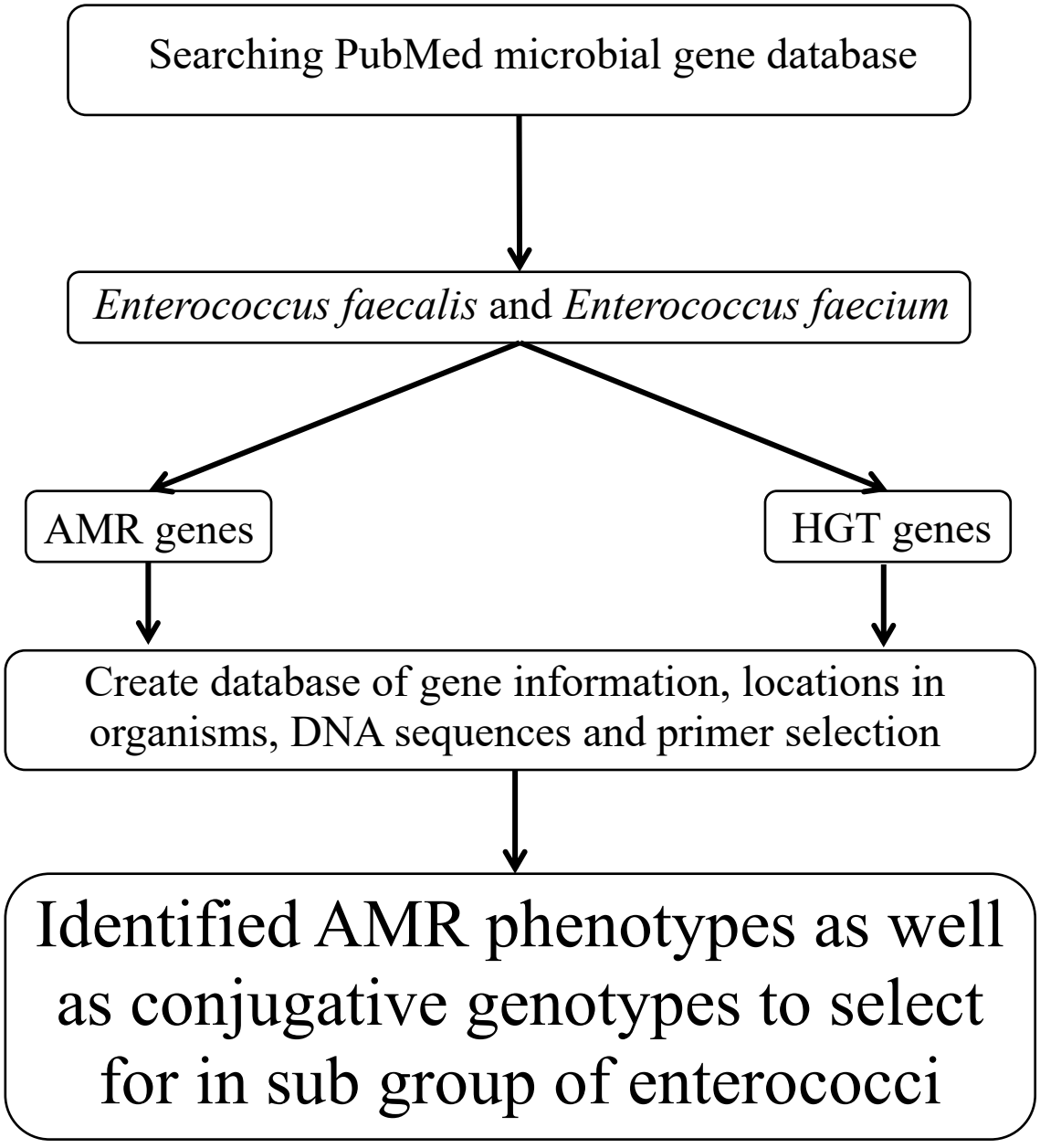


Table 3.4.4. Collation of antimicrobial resistance genes identified from PubMed gene database analysis in *E. faecalis* and *E. faecium*.

Gene name	Phenotype	Identified resistance	Mobile element
vanA	D-Ala-D-Lac ligase	Vancomycin	pS177, pWZ1668, pWZ1668, pTW9, pWZ7140, pWZ909, pF856, p5753cA, pZB18
vanB	D-alanine--D-lactate ligase	Vancomycin	pVEF1, pVEF3, pIP816, EF2294, pMG2200 pVEF2
vanZ	Teicoplanin resistance protein	Teicoplanin	pDO2, pS177, pWZ1668, pTW9, pWZ7140 pWZ909, pF856, pVEF1, pVEF3, pIP816 p5753cA
aadE	Aminoglycoside 6-adenylyltransferase	Streptomycin	pDO2, pS177, pF856, pEF418
ermB	rRNA adenine N-6-methyltransferase	Erythromycin	pS177, pWZ1668, pTW9, pWZ7140, pWZ909, pF856, pRUM
aphA	Aminoglycoside 3'-phosphotransferase	Kanamycin	pDO2, pS177, pF856, pCoo
pRE25	Aminoglycoside phosphotransferase type III	aminoglycosides	pDO2, pRE25
cat	Chloramphenicol acetyltransferase	chloramphenicol	pDO2, pRE25, pCPPF5, pRUM, pEF-01
tetL	MFS family major facilitator transporter, tetracycline: cation symporter	Tetracycline	pDO1, pM7M2, pAMalpha1
tetM	Tetracycline resistance protein	Tetracycline	pM7M2, p5753cB, pCF10
tetP	Tetracycline resistance protein	Tetracycline	pDO1
sace	Streptothricin acetyltransferase	Streptothricin	pDO2

Table 3.4.5. Collation of conjugation genes identified from PubMed gene database analysis in *E. faecalis* and *E. faecium*.

Conjugation genes

Gene name	Identified phenotype	Aliases
prgB	Aggregation substance	pCF10, pMG2200, pAM373, pTW9, pTEF1, pTEF2,
prgQ	Pheromone peptide inhibitor	pCF10, pMG2200, pTEF1,
prgX	Pheromone receptor	pCF10, pMG2200, pBEE99,
traA	Pheromone binding protein	PZB18, pHTbeta, pTW9, pMG1, pAM373
traB	Pheromone shutdown protein	pCF10, pMG2200, PZB18, pTW9
traE1	Regulator for conjugation	pHTbeta, pAM373
traG	Conjugation protein	pCF10, pMG2200, pTW9, pMG1, pAM373, pDO3, PZB18, pBEE99

3.4.4 Shortlisting potential conjugation partners based on antibiotic resistance profiles, biofilm formation, phenotypic clumping and phenotypes of interest from gene database analysis

There were 15 of the 23 candidate enterococci chosen with strong potential to carry out HGT (table 3.4.5). These isolates had the highest levels of clumping intensity with the three pheromone producing isolates, their AMR phenotypes were diverse and each isolate contained at least one testable phenotype that was shown to be contained on MGE's. Finally, these isolates all produced biofilm. The isolates that were eliminated (MF07008, ST01053, ST01063, MF04019, MW02077, MW01021, ST02227 and MW02087) did not produce biofilm under selection conditions and had the lowest compatibility when examined using the clumping assay. These isolates had useable AMR profiles however, they were eliminated to reduce the population to a more workable number based on the other two criteria.

Table 3.4.6. Shortlist of potential conjugation isolates of enterococci based on selection criteria outlined in figure 3.4.1.

Enterococcal name, conjugation role and mobile antimicrobial resistance phenotypes

Isolate	Species	Role	Antimicrobial resistance phenotypes
MF06035	*E. faecalis*	Donor	van, ery, str, tri, tei
MF06036	*E. faecalis*	Donor	van, ery, str, tet, tri, tei
MW01038	*E. faecalis*	Donor	van, str, tri
MW02102	*E. faecalis*	Donor	van, str, tri
MW03020	*E. faecalis*	Donor	van, str, tri
MW03025	*E. faecalis*	Donor	van, str, tri
MW03051	*E. faecalis*	Donor	van, str, tri
ST02011	*E. faecalis*	Donor	van, str, tri
MF06019	*E. faecium*	Donor	ery, tet
MF04010	*E. faecalis*	Donor	str, tet, tri
MF06030	*E. faecium*	Donor	tri
MW03061	*E. faecium*	Donor	tri
MW01105	*E. faecalis*	Recipient	str, tri
MW02043	*E. faecalis*	Recipient	str, tri
ST01109	*E. faecium*	Recipient	str, tet, tri

van - vancomycin, ery - erythromycin, str - streptomycin, tri - trimethoprim, tei - teicoplanin,

3.4.5 Determination of antibiotic minimum inhibitory concentrations of the shortlist of enterococci

Once the 15 isolates were shortlisted (table 3.4.6), the next step was to determine their MIC's to all antimicrobials present on MGE's. MIC's were required to establish selection criteria in subsequent conjugation attempts as well as indicating potential for genotyping AMR genes based on resistance phenotype. Of all the isolates tested, most demonstrated at least two antibiotic resistance phenotypes (Table 3.4.7.). MW01105 was resistant to streptomycin (at least 4 times more resistant than the others) and gentamycin (twice as high as other donors). MW02043 was resistant to kanamycin. ST01109 only demonstrated intermediate resistance to trimethoprim. MF06035 was resistant to six antibiotics (vancomycin, erythromycin, streptomycin, trimethoprim, teicoplanin and gentamycin) demonstrating the highest resistance to gentamycin of all isolates. Isolate MF06036 was similarly resistant to six antibiotics (vancomycin, erythromycin, streptomycin, tetracycline trimethoprim, and teicoplanin) displaying the highest resistance to vancomycin.

The isolates (MW01038-ST02011) appeared to show a typical susceptibility to vancomycin in broth microdilution assays up to 24 hours. However, exposing the bacteria to longer exposure times showed a limited increase in resistance, making the isolates heteroresistant (denoted in figure 3.4.6.). Additionally, ST02011 was resistant to kanamycin (at least x4 higher than the other isolates). MF06019 was resistant to erythromycin (x32 higher compared to EUCAST breakpoints) and kanamycin. MF04010 was one of two isolates that was resistant to tetracycline (x8 higher than the general resistance phenotype found in the laboratory strains) as well as demonstrating resistance to gentamycin. MF06030 and MF03061 were both resistant to tetracycline and kanamycin.

Table 3.4.7. Minimum Inhibitory Concentration profiles of enterococci to antibiotics.

Enterococcal isolate	MIC (µg/ml) at 24 hours								
	VA	E	SM	TET	TMP	TE	KAN	CHL	GEN
E. faecalis MW01105	1	0.5	>1024^{R}	<0.5	0.13^{I}	2	128	4	64^{R}
E. faecalis MW02043	0.5	<0.25	32	<0.5	0.06	<0.25	>1024^{R}	4	32
E. faecium ST01109	2	8	128	<0.5	0.5^{I}	<0.25	256	4	32
E. faecalis MF06035	256^{R}	>1024^{R}	1024^{R}	8	>16^{R}	>32^{R}	64	8	128^{R}
E. faecalis MF06036	>512^{R}	>1024^{R}	>1024^{R}	128^{R}	>16^{R}	>32^{R}	64	8	16
E. faecalis MW01038	4^{H}	<0.25	256	<0.5	>16^{R}	0.5	256	8	32
E. faecalis MW02102	4^{H}	<0.25	256	<0.5	>16^{R}	1	64	8	32
E. faecalis MW03020	4^{H}	<0.25	256	64^{R}	0.25^{I}	<0.25	64	4	32
E. faecalis MW03025	8$^{R/H}$	<0.25	256	1	1^{I}	<0.25	64	8	32
E. faecalis MW03051	4^{H}	<0.25	256	1	0.5^{I}	1	64	4	32
E. faecalis ST02011	4^{H}	32^{R}	128	<0.5	0.25^{I}	<0.25	1024^{R}	8	32
E. faecium MF06019	1	>1024^{R}	64	<0.5	0.06	<0.25	>1024^{R}	8	16
E. faecalis MF04010	1	<0.25	1024^{R}	128^{R}	0.13^{I}	<0.25	256	4	64^{R}
E. faecalis MF06030	0.5	<0.25	64	16^{R}	0.13^{I}	<0.25	>1024^{R}	4	32
E. faecium MF03061	1	1	32	32^{R}	0.06	<0.25	1024^{R}	4	32

*VA, vancomycin; E, erythromycin; SM, streptomycin; TET, tetracycline; TMP, trimethoprim; TE, teicoplanin; RIF, rifampicin; KAN, kanamycin; ^{R}, resistant; ^{I}, intermediately resistant; ^{H}, heteroresistant

3.4.6 Generation of rifampicin resistance in susceptible 'recipient' isolates

The data in table 3.4.2, combined with table 3.4.7, highlighted that the designated recipient isolates were either completely susceptible to the antibiotics of interest in this study (ST01109) or they carried the same antibiotic resistance phenotypes as the donors (MW01105 and MW02043). Capture of future horizontal gene transfer attempts required double antibiotic selection plates. The double selection would contain two antibiotics; each inhibiting the growth of a specific conjugation partner. Growth on these plates would only occur if a transconjugant was created from the conjugation partners with resistance to both antibiotics. Therefore, these recipient isolates needed a unique AMR phenotype. Propagation on doubling concentrations of rifampicin was carried out until the three potential recipient isolates became uniquely resistant. From this point, any reference to these isolates contains the suffix (^{Rif}).

MW01105^{Rif} had an MIC >1024µg/ml, MW0204^{Rif} had an MIC of 64µg/ml, ST01109^{Rif} had an MIC >1024µg/ml. The EUCAST environmental cut-off (ECOFF) MIC for rifampicin is 4µg/ml, making these potential recipients at least 16 times as resistant as the cut-off value. The potential donors had their MIC for rifampicin determined: MF06035, MW01038, MW03020, ST02011, MF06019, MF06035, MW03061 had an MIC <0.5µg/ml. MF06036 had an MIC of 2µg/ml, MF04010 had an MIC of 4µg/ml. MW02102, MW03025, MW03051 had an MIC of 8µg/ml, making them the only potential donors to have an ECOFF resistance to rifampicin.

3.4.7 Vancomycin ‘heteroresistance’ phenotypes in enterococci

When examining AMR using the disc diffusion assay, isolates MW01038, MW02102, MW03051 and ST02011 tested positive for vancomycin resistance. However, they tested susceptible using the MIC assay as per the 24-hour incubation time outlined in EUCAST susceptibility testing for vancomycin resistance. Upon testing susceptibility for greater lengths of time it was noted that the four isolates mentioned here grew colonies in vancomycin selection plates containing double the vancomycin MIC at 8µg/ml. When the isolates were tested for the *vanA* and *vanB* genes it was noted that only MF06035 and MF06036 tested positive. This result is reflected in each isolates MIC. The vancomycin “heteroresistant” isolates and the low-level resistance isolate (MW03025) did not test positive for *vanA* and *vanB*. These isolates were labelled as presumptively “heteroresistant” as their phenotype does not reflect the absolute resistance demonstrated by MF06035 and MF06036 but they can grow beyond the MIC value for vancomycin after longer incubation times.

Figure 3.4.6. Assessing vancomycin (*vanA*) resistance amongst the donor isolates.

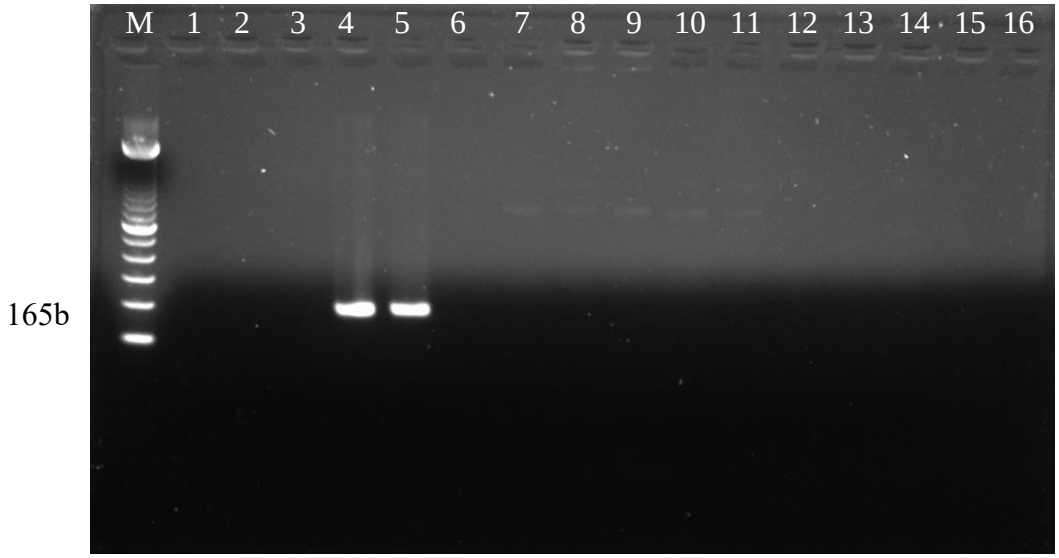


Figure 3.4.6. Gel electrophoresis of vancomycin *vanA* PCR from candidate enterococci growing from a six-hour growth in TSB. Gel concentration was 1.5% and was ran in TBE at 100 volts for 35 minutes. The gel was created with ethidium bromide at a final concentration of 0.5µg/ml.

Lane M – 100bp ladder

Lanes 1-10 – *vanA* PCR result for isolates MW01105^{Rif} (1), MW02043^{Rif} (2), ST01109^{Rif} (3), MF06035 (4), MF06036 (5), MW01038 (6), MW02102 (7), MW03020 (8), MW03025 (9) MW03051 (10), ST02011 (11), MF06019 (12), MF04010 (13), MF06030 (14), MW03061 (15), Negative control (16).

3.4.8 Mobile genetic element characterisation on enterococci with strong antibiotic resistance phenotypes

Alkaline lysis plasmid extraction assays were carried out to identify plasmids in the donor isolates. The Sambrook and Russell alkaline lysis plasmid extraction protocols were tested extensively as described in the general materials and methods. This protocol was effective at isolating plasmid content from *E. coli*, however even with extensive literature searches and specially modified protocols for Gram positive bacteria including '*coccus*' specific adaptions, the agarose gel shown in figure 3.4.7 was the only result with banding in the anticipated locations. Additionally, The Kado and Liu, Anderson and McKay, and the Williams methods were tested using the entire eluate of each extraction with up to 96-hour resuspension times from the alkaline lysis protocol on the four *E. faecalis* donors. Each protocol was carried out independently twenty times, with modifications to electrophoresis voltage (50-200 Volts) and time (90-300 minutes), concentration of agarose in the gel (0.5-1.75%) with little success. Figure 3.4.7 had high molecular weight bands indicative of plasmid content (indicated with red arrows) in MF04010 with 2 distinct bands on and above the 23kb marker. Isolates MF06036 displayed at least one band at the same location on the gel as the upper band from MF04010. It appeared that MF06035 may also have a band in the same region however it is too faint to confirm banding.

Due to the equivocal results of plasmid extractions, a secondary testing assay was devised to provide data highlighting the phenotype of plasmid persistence. This assay would test the stability of antimicrobial resistance from a resistance gene (only shown in MGE's) over a period of time (figure 3.4.8). Therefore MF06035 and MF06036 were propagated in TSB only or in TSB with vancomycin selection

at 10 µg/ml. The isolates were tested at regular intervals over the course of 25 weeks. Under no selection both isolates remained resistant to vancomycin at the conclusion of the experiment.

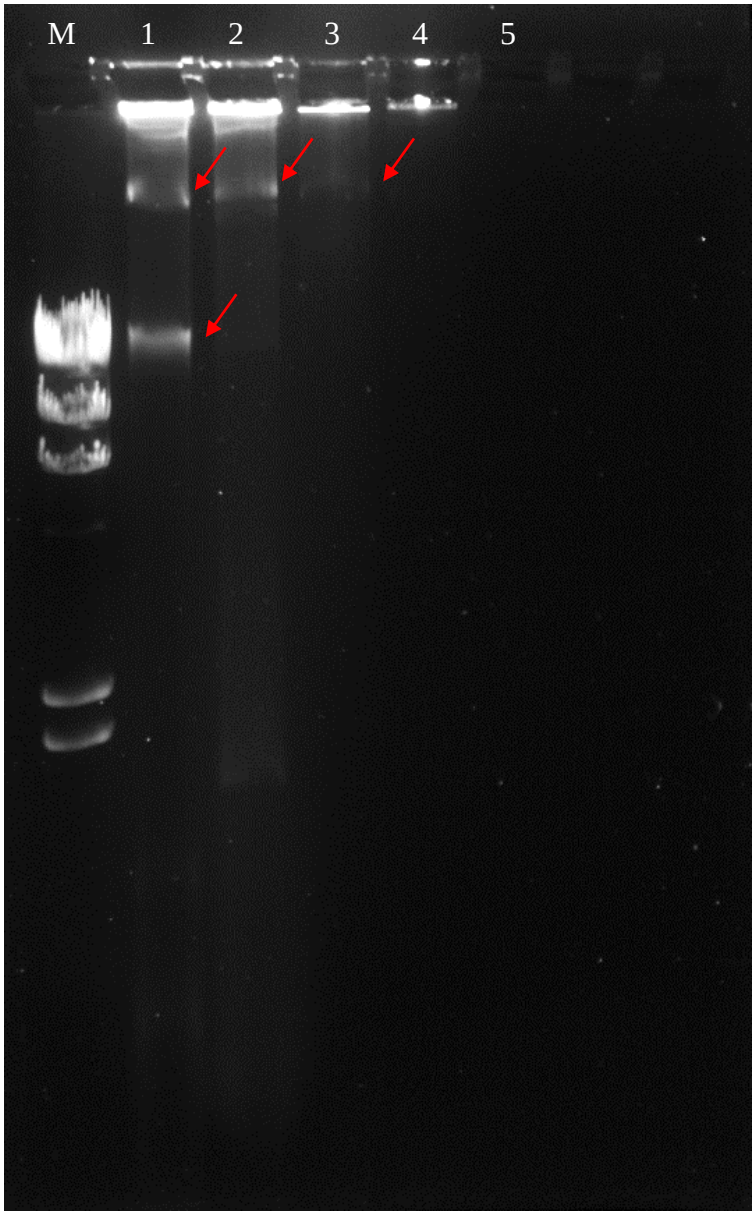


Figure 3.4.7. Agarose gel electrophoresis of plasmid extraction from *E. faecalis* isolates using the alkaline lysis method for large plasmids.

Gel electrophoresis of ALS plasmid extraction from *E. faecalis* growing from a six-hour growth in TSB. Gel concentration was 0.75% and was ran in TBE at 80 volts for four hours. The gel was created with ethidium bromide at a final concentration of 0.5µg/ml. Red arrows indicate high molecular weight banding.

Lane M – HindIII digest of lambda DNA

Lanes 1-4 – ALS plasmid extraction for isolates MF04010 (1), MF06036 (2), MF06035 (3), MF06030 (4) Negative control (5).

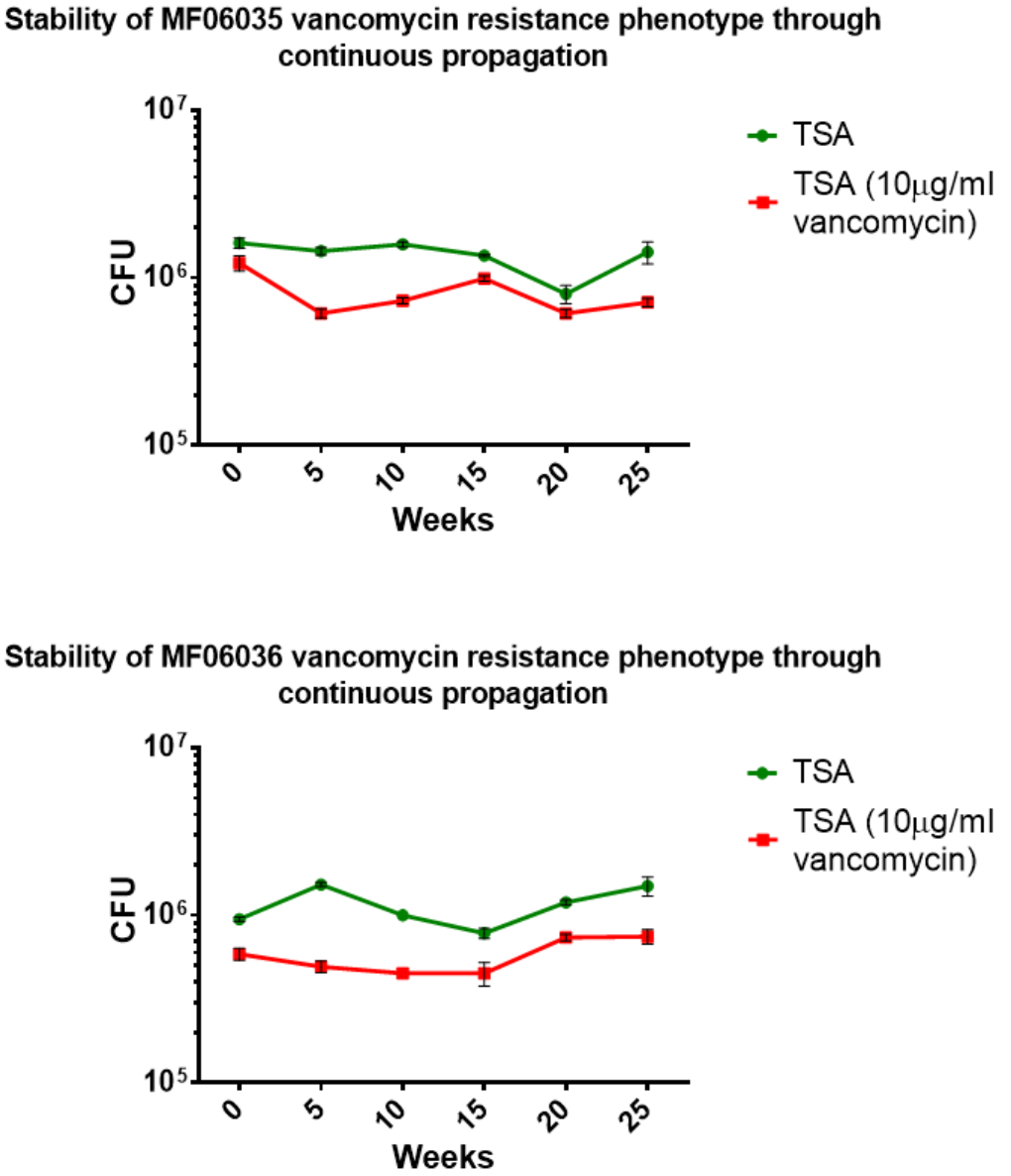


Figure 3.4.8. Stability of MF06035 and MF06036 *vanA* vancomycin resistant *E. faecalis* isolates. Both *E. faecalis* isolates were propagated in and out of vancomycin selection. At each time point isolates were subjected to a standardised CFU count to examine loss of resistance phenotype. After 25 weeks of propagation there was no drop off in resistance phenotype. Maintenance in vancomycin produced CFU counts lower than those that were propagated in TSA only.

3.5. DISCUSSION

Enterococcal isolates were taken from a biobank containing environmentally isolated bacteria. They were characterised for traits suitable to conjugate mobile elements containing prolific antimicrobial resistance genes, and having biofilm forming ability.

3.5.1. Selection of Enterococci from biobank

Understanding the dynamics of how *E. faecalis* and *E. faecium* carry out horizontal gene transfer highlighted the importance of selecting candidates that would meet specific requirements phenotypically. As horizontal gene transfer, can involve pheromone responsive plasmids, Inc18 plasmids and conjugative transposons; all having the ability to transfer the same gene types, selection criteria had to account for all possibilities (Figure 3.4.1.). Antimicrobial resistance phenotype was chosen as it is the most well-regarded of all traits relevant to human colonisation from enterococci, and is the main concern for treatment of nosocomial pathogens. It is also the most straightforward acquired trait to examine when analysing horizontal gene transfer. The second selection criterion involved clear demonstration of phenotypic aggregation amongst the candidates and was vital for pairing isolates for horizontal gene transfer events. Testing compatibility using the clumping assay, any positive results would demonstrate the potential for successful transfer of genetic material from one ‘donor’ bacterial isolate to another ‘recipient’ isolate. The final characteristic desired for this study was the ability of each candidate to form biofilm. Isolates that can form biofilm would allow for testing internal conjugation of AMR.

Screening the biobank of 655 partially characterised enterococci produced 23 isolates which could be tested for their ability to harbour AMR phenotypes, conjugation possibility and biofilm formation capability.

The list of 23 enterococci (Table 3.4.1.) included *E. faecalis* and *E. faecium* only. These species were selected as they are the most frequently isolated, commensal and pathogenic enterococci in human infection. The sources of these isolates are shown in table 3.4.1 and they vary from animal faeces to storm water run-off into waterways of an agrarian environment (Daniels 2011).

3.5.2. Characterisation of enterococci – General antimicrobial susceptibility

The first trait to be examined in the 23 isolates of enterococci was the phenotypic presentation of AMR. Isolates were selected on the prerequisite of having at least one resistance phenotype. As can be observed in table 3.4.2. the isolates demonstrate a broad AMR profile across the 19 antibiotics: with identifiable resistance in all tested antibiotics, excluding ciprofloxacin, neomycin and linezolid. Ciprofloxacin is usually therapeutically effective against *E. faecalis* infection and is commonly used to treat secondary endocarditis and primary non-resistant UTI (Carlier and Courvalin, 1990). It acts on DNA gyrase and topoisomerase IV working on protein synthesis. However, there are isolates of *E. faecium* that possess resistance to quinolones (MIC90 >128µg/ml) and therefore ciprofloxacin. There does seem to be a link to vancomycin (*vanA*) resistance and increased resistance of ciprofloxacin (MIC90 >128µg/ml) in *E. faecium* but this phenomenon is not observed in *E. faecalis vanA* resistance (MIC90 2µg/ml) (Information on relationship between vancomycin (*vanA*) resistance and increased resistance of ciprofloxacin accessed through The Antimicrobial index (TAMI): accessed through http://antibiotics.toku-e.com, and discussed by Amirkia and Qiubao, (2011)).

Neomycin resistance in enterococci is accomplished through an acquired gene encoding 4',4''-aminoglycoside nucleotidyltransferase (Carlier and Courvalin, 1990). The other aminoglycosides tested on the 23 isolates (gentamycin and streptomycin) demonstrate opposing susceptibilities; gentamycin testing revealed susceptibility in 22 of the 23 enterococci; whereas streptomycin testing revealed resistance in 16 of the 23 enterococci. It was evident that these enterococci demonstrated intrinsic resistance to streptomycin, something that is generally observed in many isolated strains. Streptomycin resistance generally presents with the highest degree of resistance among all aminoglycosides. With reference to streptomycin: where resistance presents itself usually the same isolates of enterococci have high tolerance to beta lactam antimicrobials. This can be observed in table 3.4.2. where seven enterococci display resistance to three out of four (ampicillin, amoxicillin, cephalothin) beta lactam antimicrobials when resistant to streptomycin; inversely there are 4 enterococci that display total susceptibility to the same beta lactams and streptomycin. There was only a single instance where isolate ST02227 displayed resistance to streptomycin and susceptibility to all beta lactams tested (ampicillin, amoxicillin, imipenem, cephalothin).

Despite a majority resistance to tested beta lactams there was only one isolate (MW02077) resistant to the carbapenem, imipenem, the drug most commonly used in for the treatment of susceptible enterococcal infections. It acts only as a bacteriostatic agent and is never used in clinical treatment of infection as a monotherapy as a result. The observed tolerance to cell wall inhibitors does appear to directly influence whether enterococci display intrinsic resistance to streptomycin (uptake limited efficacy). The general resistance to streptomycin and susceptibility to gentamycin among the tested isolates matches the phenotype expressed by the bifunctional enzyme, Aac(6′)-Ie-Aph(2″)-Ia. This enzyme provides resistance to gentamycin amongst other

aminoglycosides but not streptomycin, which fits the antimicrobial phenotypes profiles observed in table 3.4.2. and could be functional within the isolates (Chow, 2000).

Linezolid works well as a bacteriostatic antimicrobial against persistent *E. faecalis* and *E. faecium* infections, as it acts through inhibition of the 30S ribosome initiation complex (Shinabarger *et al.,* 1997). This specific mechanism of action significantly reduces rates of resistance in *E. faecalis* and *E. faecium* and acquiring resistance usually involves mutations in the majority of its 23S rRNA gene copies, as compared to rifampicin resistance only requiring a single point mutation. There were 11 enterococci resistant to tetracycline, and three of those bacteria were also resistant to oxytetracycline. Tetracycline resistances were first described on mobile elements in enterococci and are the most studied in conjugative transfer events. Tetracycline resistance was one of the earliest resistances (1961) identified in enterococci and with increased selective pressures, has had a sufficiently long time to propagate on mobile elements (van Schaik *et al.*, 2010). Therefore, these tetracycline resistances would be suitable markers to examine horizontal gene transfer in these selected enterococci.

Vancomycin resistance was observed in eight of the 23 selected enterococci and was of interest for future conjugation assays. It is the unique identifying resistance of enterococci (VRE), is situated exclusively on mobile elements and has significant relevance to clinical treatment of AMR pathogenic infections: in many countries vancomycin was a controlled drug, used as a last stand antibiotic, yet resistance still developed in human strains. Two vancomycin resistant isolates (MF06035 and MF06036) were the only isolates to demonstrate resistance to teicoplanin. Interestingly, published enterococcal mobile genetic element data shows a link between vancomycin resistance and teicoplanin resistance on mobile elements (Tn*1546*). Two forms of folate pathway inhibition antimicrobials were used on the 23 selected isolates in folate deficient media (trimethoprim, and sulfamethoxazole & trimethoprim,). Fifteen isolates demonstrated

total resistance to the folate antimicrobials. Five enterococcal isolates demonstrated intermediate susceptibility to trimethoprim. However, they demonstrated susceptibility when tested on sulfamethoxazole & trimethoprim as a potential synergistic interaction. The increasing trend of susceptibility with complexity of mode of action of these folate pathway inhibition antimicrobials (as they target several steps in the folate metabolism within enterococci) is interesting. However, the fact that enterococci can absorb exogeneous folate, reversing the efficacy of folate pathway drugs such as trimethoprim questions the effectiveness of using this group of drugs (Wisell *et al.*, 2008; Silverstein and Hopper, 2014). This was confirmed in this study when repeating the disc diffusion assay on media containing folate (Muller Hinton) as opposed to Iso-sensitest. All folate pathway antimicrobials were completely ineffective on the enterococci.

Quinupristin/dalfopristin proved relatively effective against the selected enterococci with only three isolates demonstrating resistance. This compound antimicrobial is only used in selective cases of persistent enterococcal infection due to toxicity contraindications, and resistance is only beginning to emerge. Typically, quinupristin/dalfopristin is ineffective against *E. faecalis* due to intrinsic resistance (uptake limitations and efflux pumps), however in this study the only isolates resistant were in fact *E. faecium* (Linden *et al.*, 2001). Finally, there were eight isolates that demonstrated resistance to erythromycin. Erythromycin resistance has been linked to a pheromone responsive plasmids (pLG2) and conjugative transposons (Tn*917* and Tn*3871* Tn*1545* Tn*5385*) and are of importance for attempts to conjugate AMR genes in this study.

3.5.3. Characterisation of enterococci –Biofilm formation

With varied antimicrobial resistance profiles, many of which are potentially mobile, the second trait examined in the 23 enterococcal isolates was their ability to form a biofilm.

The biofilm protocol was carried out according to protocols described in Daniels, (2011) and is regarded as the standard biofilm assay. This assay has been used extensively for batch testing biofilm formation capabilities of many microorganisms *in vitro*. The objective at this stage was to establish if any enterococcal isolates produced biofilm in any quantity. The results of this assay are outlined in figure 3.4.2. There is a clear variation amongst the selected isolates for their relative biofilm formation capability. Seven isolates presented with strong biofilm, eight isolates formed weak biofilm and eight isolates were produced no biofilm. Whilst this method uses optical density to quantitate values from relative absorbance at 570nm, these results are still quite subjective. However, it was clear that some isolates produced biofilm as they were visible to the naked eye. Seven out of the eight vancomycin resistant isolates formed biofilm. At this stage, these results further select candidate isolates for future horizontal gene assays.

3.5.4. Characterisation of enterococci – Clumping assay

The final trait tested was a direct analysis of the 23 enterococci for their compatibility to carry out successful horizontal gene transfer. Compatibility was identified using the well-established clumping assay. When two isolates are compatible and the cell free supernatant containing pheromone from one is introduced to a liquid suspension of the other, a clumping reaction occurs which can be visible to the naked eye. Figure 3.4.3. contains macrographs demonstrating the variation in clumping intensity of the isolates from no reaction 3.4.3(1) to highly reactive 3.4.3(6). There appears to be little difference between 3.4.3(1) and 3.4.3 2) apart from a size difference between the mass of cells in the centre of each well. In figure 3.4.3(1) the relatively small concentrating of cells occurred due to the rotational force applied from the orbital incubator that the experiments were incubated in. In figure 3.4.3(2) the central mass of cells is much larger. When examined under phase contrast microscopy, the cells formed small clumps containing on

average 6-10 chains as exemplified in figure 3.4.4 (a – red arrow). These small dense chains concentrated in the centre of the well but took up more space resulting in a larger central mass. Figure 3.4.3(3-6) visibly show an increase in the number of macroscopic clumps observed with the naked eye. These individual clumps can have lengths up to 1 centimetre and represent high compatibility of the pheromone contained in the supernatant to the activation of aggregation signalling in the subject isolate. The microscopic visualisation of clumping in the enterococci in this thesis were comparable to the observed clumping in the literature (Donelli *et al.,* 2004). Table 3.4.3 is the output of the clumping assay when applying the control ranges outlined in figure 3.4.3.

The first significant findings from the clumping assay was the identification of 'donor' isolates and 'recipient' isolates. Recipient isolates (MW01105, ST01109 and MW02043) were identified based on their cell free supernatants inducing clumping reactions in the other 20 isolates but not themselves: applying cell free supernatant from the now labelled 'donor' isolates produced no clumping on the 'recipient' isolates. It is clear from table 3.4.3. that there is strong compatibility to conjugate between the donor and recipient isolates with most reactions being categorised as 4-6 in intensity. Clumping response in the literature tend to be graded qualitatively (Franz *et al.,* 2001). The physical characteristics of a weak clumping reaction can be difficult to differentiate from typical microadhesions at play with plasmid-free isolates (Muscholl-Silberhorn, 1993). Daniels, (2011) described weak clumping reactions that could only be visible under prolonged microscopy. In an attempt to include microscopic clumping phenotypes and clumping reactions visible to the naked eye, grading from 1-6 was employed in this thesis. The grading was aimed to provide more detailed quantification of clumping strength than strong or weak, as described by Donelli *et al*. (2004). Examples of clumping reactions are shown microscopically under phase contrast in figure 3.4.4. Enterococci do not naturally exist in clumps *in vitro* so demonstration of large bodies of 'clumped' cells surrounded

by a soup of free floating diplococcus cells, often in chains demonstrates the effect of the phenomena on donor isolates.

3.5.5. Composition of enterococcal MGE associated genes of interest

Before selecting final candidate isolates the NCBI microbial gene database was searched for AMR and conjugation genes against *E. faecalis* and *E. faecium,* verified to be on mobile genetic elements. A database was created listing all queries of AMR genes and conjugation genes. This database contained gene name information, alias names, where appropriate - descriptions of genes, locations on named mobile elements, accession numbers and FASTA sequences. This information database was used to identify AMR phenotypes of interest as well as conjugative possibilities to select for in the final subgroup of enterococci. Over 20 AMR and 18 conjugation genes were identified and all genes with identical aliases had their sequences aligned using clustal X. These sequences were used to generate universal primer sequences to search for genes of interest related to AMR and HGT in both *E. faecalis* and *E. faecium*. All AMR gene types that were identified through searching the microbial gene database (Accessed 01/2014-03/2014) were collated and are summarised in tables 3.4.4. and 3.4.5. Searching the microbial gene database guaranteed that all genes selected at the time of accession were confirmed with access to FASTA sequences and gene variants present in other enterococcal MGE's. Selected genes were only chosen based on their presence on plasmids and transposons. All mobile elements identified containing the specific published genes of interest with confirmed functions are listed under aliases in tables 3.4.4 and 3.4.5.

This protocol was undertaken to ensure genes of interest are in fact limited to MGE's and when creating or purchasing published primers that they would universally amplify all homologues of the same gene. There was an unforeseen consequence of this method of

ensuring primers could be designed to universally amplify a gene, taking account of single nucleotide polymorphisms (SNP's): There was an issue within enterococcal gene nomenclature. Genes that are described in publications can often not be located in the published NCBI gene database. Additionally, primers used in the literature for enterococcal research, listed as amplifying unique genes, do in fact amplify the same gene. This was clear upon further analysis of the 'aggregation genes': - *agg*, *prgB*, and *asa1*. When searching the NCBI gene database there was no entry for the *agg* gene, additionally *prgB* and *asa1* share significant homology (96% identity) and carry out the same function. A paper in question has primer sets for each aggregation 'gene' and lists each as a unique virulence factor and separate gene (Choi and Woo, 2015). However, when these primers were aligned in clustal it was found that all three can amplify the *prgB* and *asa1* genes. This is an example of ensuring diligence when analysing conjugative traits in enterococci and indeed in all aspects of gene analysis.

3.5.6. Minimum inhibitory concentrations of donor and recipient enterococci

When 15 isolates had been shown to exhibit all the hallmarks of carrying out HGT of AMR, further characterisation experiments were required in order to start testing conjugation assays on pairs of enterococci. Minimum inhibitory concentration testing was carried out using vancomycin, erythromycin, streptomycin, tetracycline, trimethoprim, teicoplanin, kanamycin, chloramphenicol and gentamycin in accordance with the mobile AMR genes shown previously. Testing the isolates using MIC revealed new resistance profiles specific to enterococci that were not identified using the disc diffusion assay. Firstly, there were eight isolates that tested positive for vancomycin resistance in the disc diffusion assay, when tested in the MIC assay revealed that only MF06035 and MF06036 were resistant to vancomycin.

Regarding the other isolates labelled in table 3.4.7 as heteroresistant: five produced MIC's on the susceptibility cut off value of 4µg/ml; whereas MW03025 technically tested resistant at 8µg/ml. These tests were recorded after incubation at 37^{o}C for 24 hours, as is the standard procedure for vancomycin testing in enterococci. Interestingly the MIC values and the disc diffusion values contrast for these isolates, highlighting issues with disc diffusion as a standalone assay for identifying resistance phenotypes in bacteria. Other discrepancies with the disc diffusion assay highlighted the failure to capture gentamycin resistance in MW01105 and MF06035; tetracycline resistance in MW02102; susceptibility in ST01109 as compared to the MIC. The disc diffusion assay labelled MW02043, MW01038, MW03020, MW03025 and MW03051 as resistant to streptomycin whilst the MIC's were not resistant (in accordance with EUCAST MIC's) however enterococcal, low level aminoglycoside resistance is recognised as having a range of 4-256 µg/ml in general (Chow, 2000).

Whilst disc diffusion assays are commonly used, their interpretation can lead to misdiagnosis of susceptibility (Sabol *et al.,* 2005). MIC testing relies on absolute resistance to specific concentrations of antimicrobials and is therefore much more accurate in the interpretation of susceptibility results. As there are several standard testing protocols (CLSI & EUCAST) utilising different materials for testing as well as a myriad of associated physical errors (agar surface moisture, drying conditions, formulations of media and antimicrobials) which are continuously monitored and updated, it can be easy for breakpoints from only a year or two to jump categories of susceptibility and resistance (Swenson *et al.,* 1989; Liu *et al.,* 2012; Palavecino and Burnell, 2013). Therefore, reliance on previous data using disc diffusion assay as a mono-susceptibility assay is unreliable. The broth microdilution MIC testing also utilised iodonitrotetrazolium chloride (INT), which changes from straw/yellow to pink/red when metabolised by bacteria (Kuete *et al*.,

2010). The addition of INT ensured accurate readouts of MIC in the broth microdilution assay (Onajole *et al*., 2011).

The six isolates were labelled as heteroresistant, as when the MIC plates were further incubated for another 12-48 hours they began to grow considerably. Changing the INT colour from straw to pink in vancomycin concentrations up to a maximum of 16µg/ml (Resistance >4µg/ml). This phenomenon was not deemed as a reduction in antimicrobial efficacy over time, as vancomycin is bactericidal not bacteriostatic. This phenomenon of heteroresistance did not occur in any of the other nine isolates at these time points. Upon the further analysis of the disc diffusion results, adhering to the EUCAST breakpoint guidelines for vancomycin action on enterococci, these isolates demonstrated colonies inside zones <12mm with fuzzy edges, and were therefore deemed resistant. This can commonly cause heteroresistance as there was clearly a sub population resistance phenotype present. All isolates were tested by PCR for the mobile vancomycin resistance genes (figure 3.4.6) and as can be seen, only MF06036 and MF06035 tested positive for the gene. All other isolates were negative, including the heteroresistant isolates.

The PCR testing in combination with resistance phenotypes confirms these isolates display heteroresistance to vancomycin and may in fact possess one of the low level non-mobile vancomycin gene clusters (Courvalin, 2006). Vancomycin heteroresistance has been reported in *E. faecium* previously (Alam *et al.*, 2001) whereby isolates from a patient were passaged in vancomycin to increase their resistance profiles. Unlike the enterococci in this thesis, the isolates from Alam *et al*. (2001), including the susceptible population tested positive for *vanA* gene. They also could only isolate the heteroresistant subpopulation through E-test, however their method of discovery of heteroresistance through treatments in vancomycin was the same as in this thesis. It appears that this is the first time that vancomycin heteroresistance has been observed in *E. faecalis* and as such

this is an area with potential for future investigation (Alam *et al.,* 2001; Hsueh *et al.,* 2005; Fitzgibbons *et al.,* 2011; Klare *et al.,* 2012).

The final use of the MIC table was to establish a list of priority partnering for attempts at transfer of AMR genes. Through analysis of table 3.4.7 some designated donor isolates stood out as the most likely candidates to pass on antimicrobial resistance genes. Donor isolates MF06035 and MF06036 were of most interest as they were resistant to six antimicrobials each, having MIC values greater than the tested maximum concentration for three (erythromycin, streptomycin and trimethoprim) and five antibiotics (vancomycin, erythromycin, streptomycin, trimethoprim and teicoplanin) respectively. Published scientific reports outlining donor isolates transferring AMR genes typically present with one or two resistance phenotypes. Vignaroli *et al*. (2011) had isolated enterococci from similar sources to the ones tested in this thesis and subjected them to conjugation. They captured a maximum of two antibiotic resistance determinants (*vanA* and *ermB*). Having two donor isolates with six resistance phenotypes linked to mobile resistance genes would give a high chance of success. Donor isolates ST02011 and MF04010 were also of interest due to the strength of their compatibilities to the recipient isolates, and having two and three resistance phenotypes that could transfer with high probability during conjugation.

3.5.7. Susceptible recipients and the subsequent generation of rifampicin mutants

An identified issue that arose after the MIC and disc diffusion assays, was that none of the designated recipient isolates contained unique resistance determinants that could be exploited for counter-selection against donor strains. The most straightforward approach to isolating a unique antimicrobial resistance phenotype amongst the recipient isolates was to create one. As stated previously, rifampicin resistance can be generated with ease

in enterococci (Kristich and Little., 2012). In their study, enterococci were grown at 200µg/ml (rifampicin) in agar plates for 24 hours to isolate rifampicin resistant mutants. These mutants were discovered as an unexpected side effect on testing intrinsic cephalosporin resistance in enterococci. The mutants were derived from the *E. faecalis* OG1 strain and carried a mutation in the *rpoB* gene. Spontaneous rifampicin mutants are often used as recipient for enterococcal conjugation assays (Gilmore *et al*., 2014). It's an antimicrobial not commonly used in the treatment of enterococcal infections and chances of resistance in the selected donor isolates would be low as resistance emerges from hospital acquired strains exposed to specific antimicrobial stress (Poole, 2012; Munita and Arias, 2016). MIC tests were carried out on all isolates after the rifampicin resistance generation assay as can be seen in table 3.4.7. MW01105, MW02043 and ST01109 were now resistant to rifampicin and displayed much higher MIC's than the donor isolates.

3.5.8. Qualitative MGE characterisation assays on donor isolates

Several protocols for the extraction of whole plasmid content were carried out on the enterococcal donor isolates of interest. The majority of all extractions were fruitless, however on a single occasion there were concentrated bands of DNA present at molecular weights consistent with mobile elements previously studied (Clewell *et al.,* 2014). However, when these bands were excised from the gels for purification and with a commercial gel purification kit, plasmid recovery was too low for sequencing. The low recovery and resolution from gel electrophoresis could be attributed to a large number of plasmids present within each isolate. The plasmid extraction work carried out in this thesis built on what was carried out previously (Daniels, 2011) and provided successful plasmid bands, giving additional credence to the success of forthcoming conjugation attempts. Future investigations could focus on midi or maxi preps to gather more plasmid DNA using the successful protocol that was tested in this thesis. They could also focus

on more advanced plasmid extractions, such as pulse field gel electrophoresis (Freitas *et al*., 2011).

A final phenotypic identification test to establish the presence of mobile elements in the enterococcal donor isolates was to establish antimicrobial resistance stability. Many plasmids introduced into an organism will not stability replicate or disperse during partitioning inside the host without selective pressure (Peeters *et al.,* 1988; Kiewiet *et al.,* 1993; Summers *et al.,* 1993). Most enterococcal plasmids that contain virulence and AMR genes are traditionally low copy number (2-10 copies) and are relatively large (over 100kb) (Clewell *et al.,* 2014). These plasmids cannot actively pass through cellular membranes and rely on successful host replication (Hayes and Van Melderen, 2011). They can include accessory partition mechanisms which either instigate a killing mechanism in unsuccessful plasmid replication during cellular replication or they guarantee the equal movement of plasmid copies during cytokinesis (Hayes and Barilla, 2006; Schumacher, 2012).

Many enterococcal MGE's have replication components, including the incompatibility Inc18 plasmids, whose name has often been disassociated with identical replication functions (Novick, 1987). In enterococci this term refers to plasmids with replication initiators that have high sequence homology to the plasmids originally described for the Inc18 incompatibility plasmids (Brantl *et al.,* 1990). These low copy number MGE's can help explain the difficulty in isolating high molecular weight DNA in the ALS plasmid extraction protocols discussed previously.

MF06035 and MF06036 were selected to assess the stability of vancomycin resistance through continuous propagation. This assay involved subculturing the two isolates in and out of selection to establish any drop off in bacterial numbers associated with failure of maintenance of MGE's responsible for vancomycin resistance. Typically, unstable or

incompatible plasmids will yield to plasmid free progeny through continuous subculturing (Scott, 1984; Novick, 1987; Ryan and Parulekar, 1991). As can be seen in figure 3.4.8 there is no appreciable drop-off, of MGE AMR containing progeny (TSA containing vancomycin) versus all progeny (Liu *et al.*, 2011). There are however differences in total cellular count versus MGE containing progeny, indicating that these MGE's containing vancomycin resistance do not fully segregate upon fission. This example of incomplete stability transfer could explain how vancomycin heteroresistance can occur.

Chapter 4

Environmentally isolated enterococci can transfer antimicrobial resistance genes to one and another *in vitro* and in an environmental *in vivo* model.

4.1. INTRODUCTION

The prokaryotic world is one where survival is a constant challenge, and therefore necessitates continuous adaptation (Hacker and Carniel., 2001). With the ever changing milieus created by human presence, a great number of bacterial cell populations have adapted to survive in environments with significant selection against them (Ley *et al.*, 2006). Some of the main survival adaptations of bacteria are located in plasmids, transposons, insertion sequences and integrons (Frost *et al.,* 2005). These, often transmissible elements contain virulence, pathogenicity and survival genes which have permitted the adaptation of a plethora of bacterial species (Shapiro, 2012). Plasmids are ubiquitous in enterococci, especially the conjugative plasmids found in *Enterococcus faecalis* and the Inc18 plasmids found in *Enterococcus faecium* (Fisher and Phillips, 2009). Whilst these plasmids vary enormously in size and copy number per cell, they frequently carry antimicrobial resistance genes (Noble *et al.,* 1992). Enterococci have evolved to harbour these AMR (antimicrobial resistance) genes on mobile elements, and as such the majority of all antimicrobial resistance traits observed in the genus are contained within them. The large quantity of extrachromosomal DNA within enterococci can be attributed to its commonality in many environmental niches (Paulsen *et al.*, 2003). This can be used to theorise how they exist now as human GI (gastro intestinal) commensals. With their ability to acquire survival qualities, enterococci are organisms that are hard to eradicate. They can pass on a plethora of their survival traits in a single instance through contact with another plasmid free bacterium, in environments with dense cell populations such as the gut (Palmer *et al.,* 2010; Byappanahalli *et al.,* 2012). Survival traits such as vancomycin resistance (*vanB*), aggregation substance, bacteriocin production and ultraviolet resistance have been transferred on the pheromone responsive plasmid, pMG2200 (Zheng *et al*., 2009). Enterococci are found in many places outside

the GI tract, and it was hypothesised that these strains will conjugate as observed in the literature and on the evidence collated in chapter three.

4.1.1. Horizontal gene transfer

Horizontal gene transfer (HGT) is the translocation of genetic elements from one bacterium to another (Zhaxybayeva and Doolittle, 2011). Conjugation is the primary method of horizontal gene transfer amongst the *Enterococcus* genus (Clewell and Dunny, 2002). Viral transduction is emerging as a means of HGT and transformation has yet to be demonstrated within enterococci (Mazaheri Nezhad Fard *et al.,* 2011; Gilmore *et al.,* 2014). Enterococci have been shown to conjugate both on solid surfaces and in suspension (Lampkowska *et al.,* 2008). Plasmids such as pCF10 and pAD1 were demonstrated as mobile, through planktonic enterococcal conjugation (Christie *et al.,* 1987; Clewell and Weaver, 1989). It was noted that these plasmids transfer with the use of a sex pheromone signalling pathway, allowing for efficient gene transfer at maximum rates of 10^{-1} transconjugants to donors for pCF10 and pAD1 (Clewell *et al*., 1982; Christie *et al*., 1987). There are plasmids such as pAMβ1 which transfer well under solid surface conditions, but have low transfer efficiency under planktonic conditions (Vescovo *et al.,* 1983). Conjugative transposons are present within enterococci, chromosomally bound, with the ability to be excised into a circular non-replicable intermediate that can conjugate to other bacteria and insert into its genome (Clewell *et al.,* 1995). The majority of these elements have a broad host range and carry many antimicrobial resistance genes (Kristich *et al.,* 2014). Significant steps in molecular genetics in the last few years, has allowed for the discovery of varied and extensive genomic islands of horizontal transfer origins (Lam *et al.,* 2012). As such there are now integrative conjugative elements (ICE's) which have been shown to be mobile, such as the *Tn916* family (Roberts and Mullany, 2009). There are also pathogenicity islands, so called due to the extent of virulence and antimicrobial

resistance traits located within them (Dobrindt *et al.,* 2004; Gilmore *et al.,* 2014). As more studies are published on the enterococcal genome, an emerging "mobilome" has been described, listing all the conjugative elements as well as horizontally transferred sequences (Mikalsen *et al.,* 2015). It has become clear only recently, the huge extent of the role that the enterococcal genus has played on horizontal gene transfer events, with particular reference to antimicrobial resistance (Jain *et al.,* 2016).

4.1.2. *In vitro* and *in vivo* horizontal gene transfer amongst bacteria

Most studies on horizontal gene transfer amongst enterococci and other bacteria, are tested through *in vitro* analyses of the organism with conditions favourable to the bacteria (Ray and Nielsen, 2005). Most tests either involve conditions conducive for the transfer of traits either in broth or on a solid phase *in situ* (Lampkowska *et al.,* 2008). These investigations have been crucial for the understanding of the roles played by bacteria in the process of HGT, and they have highlighted the adaptability and host range of the genus *Enterococcus*. The limitation with the action of *in vitro* examinations across the board are that bacteria either live communally *in vivo*, or in suspension/biofilm in the *ex vivo* environment (Lorenz and Wackernagel, 1994; Pacio *et al.,* 2003). Many studies have been published on the occurrence of HGT *in vivo* (Gilmore *et al.,* 2014). With specific reference to enterococci: examples of *in vivo* transfer of plasmids from *Lactobacillus reuteri* and *Lactococcus lactis* into *Enterococcus faecalis* was demonstrated in the faecal content of mice and rats, with variations of efficiency based on sub therapeutic concentrations of appropriate antimicrobials (Morelli *et al.,* 1988). Dahl *et al.* (2007) demonstrated that *in vivo* conjugation was not only more efficient, it allowed for the continued survival of transconjugants post transfer. Lester *et al.* (2006) demonstrated how isolates of enterococci from animal origin can transfer AMR genes into human isolates within human GI tracts. These studies have formed the basis of what is known with

regards to potential source origins of new AMR gene propagation into the commensals/pathogens of humans causing serious human infection.

4.1.3. Sources of bacterial horizontal gene transfer in the environment

The human GI tract is a large, diverse reservoir for the containment of bacterial species with prolific antimicrobial resistance traits, and increased selective pressure in a confined space (Salyers *et al.,* 2004; Bäckhed *et al.,* 2005). This increased pressure would therefore perpetuate horizontal gene transfer; however, it is worth noting the possibility of environmental sources of horizontal gene transfer, such as soils, sands, stagnant water sources as well as stationary host organisms that exist in aquatic ecosystems (Allen *et al.,* 2010; Davies and Davies, 2010). Horizontal gene transfer has been demonstrated in the environment previously. *Bacillus thuringiensis* were shown to transfer *cry1Ac* (encoding an insecticidal protein) between strains in un-supplemented soil with a conjugation frequency of 10^{-5} and in infected *Anticarsia gemmatalis* larvae with a conjugation frequency of 10^{-1} (Vilas-Bôas *et al.,* 1998). Dahlberg *et al*. (1998) demonstrated interspecies conjugation of the plasmids pB7, pBF1 and pB9 from the soil bacterium *Pseudomonas putida* into several species of recipients. This included the aquatic bacterium *Aeromonas hydrophila* (conjugation frequencies of 10^{-2}) and the human associated pathogen *Serratia marcescens* (conjugation frequencies of 10^{-4}). Henschke and Schmidt (1990) showed *in situ* transfer of plasmid pFL67-2 from *E. coli* into soil microbiota, where transconjugants were phenotypically characterised as *P. fluorescens*. Van Elsas *et al*. (1988) demonstrated bacterial conjugation of plasmid RP4 from *Pseudomonas sp*., on the rhizosphere of wheat germs at maximum transfer frequencies of 10^{-2}.

4.1.4. Enterococcal presence in the environment: Faecal contamination

Enterococci are found in human and animal faeces in abundance, however they only represent <1% of all bacteria present in the GI tract (Salyers *et al.*, 2004). Historically enterococci have been used as an indicator of human faecal contamination in the environment and in places of human presence (Hussain *et al.*, 2007; Colford *et al.*, 2012). Enterococci can be found in a varied number of places, including soil, water sources, and plant fauna (Teixeira and Merquior, 2013). Using enterococcal identification tests as a means for faecal contamination can prove difficult due to the range in locations that they can be found (Field and Samadpour, 2007). Additionally, studies have begun to demonstrate that enterococci may survive in these *ex vivo* environments with specific ecological compositions such as sand, and waters rich in seaweed/plankton (Gilmore *et al.*, 2014). It is clear that enterococci are equipped to deal with the extra-enteric environment and as a result they may be able to remain competent for HGT for significant periods of time.

4.1.5. Enterococcal presence in aquatic ecosystems

The rise of nosocomial pathogens harbouring horizontally transferred AMR genes is of great concern; however, it raises questions regarding the importance of human exposure to AMR bacteria from sources beyond the hospital care setting. Detection of horizontally transferred AMR genes in clinically isolated bacteria may have origins in the natural environment (Canton, 2009). Wastewater treatment and drinking water facilities have been identified as hotspots for the identification of such bacteria (Rizzo *et al.*, 2013). These observations have yielded a hypothesis that aquatic environments may function as conduits for the propagation of AMR, increasing baseline infective and resistive traits of bacteria. These aquatic environments act as sinks for many sources of microorganisms, maintaining them and in the case of enterococci could allow them to thrive if not treated

correctly. This provides opportunity for cell mediated contact thus facilitating HGT. However, our understanding of environmental factors and processes with a potential of contributing to increased AMR transfer is limited (Berendonk *et al.,* 2015).

4.1.6. Potential substrates for HGT in the environment

Experimental and analytical evidence from water systems mainly suggests that aquatic biofilms may be hotspots of horizontally transferred AMR genes (Balcázar *et al.,* 2015). Conjugative plasmid transfers in biofilms have been observed in flow chambers (Christensen *et al.,* 1998) and biofilms in a drinking water treatment plants have been identified as a reservoir for AMR genes (Farkas *et al.,* 2013). While it has been documented regarding soils, that a wide range of biotic factors have an impact on conjugative AMR transfer (Aminov, 2011), this research area has remained largely unexplored for aquatic environments. For example, potential *in vivo* facilitation of conjugative AMR transfer by aquatic filter feeders has been suggested by Lupo et al. (2012), but evidence is still outstanding. Hence there is a need for a greater understanding of the potential for organisms in aquatic environments to facilitate bacterial horizontal gene transfer. An example of a potential 'substrate' organism that could be utilised by enterococci would be freshwater sponges.

There are 14 species of freshwater sponges found in Europe with most of these having a wide geographic distribution (Økland & Økland 1996). *Ephydatia fluviatilis* and *Spongilla lacustris* are the most widely found in rivers and lakes on hard or soft sediment in Europe, North America and Asia (Annandale 1911; Poirrier 1969; Økland & Økland 1996). They are active in water temperatures between 5 and 30ºC producing gemmules outside of this range (Poirrier 1969; Økland & Økland 1996). Gemmules are protective bodies capable of reforming the sponge when favourable conditions return. The majority

of sponge tissue is comprised of a collagenous matrix with a broadly similar structure to type IV collagen found in mammals. *Ephydatia fluviatilis* and *Spongilla lacustris* were found actively growing in the same regions that the enterococci in this study were isolated from.

4.2. Aim and Objectives

The primary aim of this chapter was to determine whether *E. faecalis* and *E. faecium* of environmental origin can conjugate AMR genes.

Objectives:

- Enterococci were assessed on their ability to naturally conjugate AMR phenotypes and genotypes with high efficiency
- Determine enterococcal ability to retain acquired AMR profiles when placed in a zero-stress environment
- To identify if enterococci can continue to conjugate AMR genes under environmental stresses, such as nutrient deprivation and sub-optimal temperatures
- To assess the ability of enterococci to bind to aquatic organisms such as *Ephydatia fluviatilis* and *Spongilla lacustris* and determining whether these could act as a substrate for HGT

4.3. MATERIALS AND METHODS

All the materials and methods carried out in this chapter are detailed in chapter two.

4.4. RESULTS

In this chapter 15 isolates of enterococci were tested in a series of experiments to discover conjugal transfer of AMR phenotypes. These isolates were paired in accordance with inverse susceptibility to two unique antibiotics and incubated together under various conditions to allow for any potential transfers to occur. Initial experiments were successful but transconjugants were only obtained with quadruple length incubation times (96 hours), at efficiencies much lower than published literature. Optimisation, in the form of: changing the conjugation substrate to a solid surface; changing conjugation partners to the rifampicin resistant potential recipients; changing ratios of donor to recipients introduced during conjugation to 1:9; and increasing the conjugation time to 24 hours was required. After optimisation, successful, repeatable conjugation occurred and four unique transconjugants were selected for further study. These transconjugants displayed all the characteristic AMR phenotypes and tested genotypes of both parents, transferred with high efficiency and remained stable. Additional, successful conjugation reactions were carried out under poor ambient conditions closely related to those found in the wider environment. Finally, the same parents used in the initial conjugation reactions were used to successfully conjugate on two sponge species *Spongilla lacustris* and *Ephydatia fluviatilis*.

4.4.1. Selection and testing of enterococcal conjugation partners from the candidate subset population

The 15 isolates of enterococci that were determined to be compatible for conjugation using criteria from chapter three, were arranged according to unique antimicrobial resistance phenotypes. Figure 4.4.1 summarises all possible partnering from the 15 isolates. Any isolate in this group can potentially carry out HGT with another isolate. Isolates listed in the intrinsic section (blue) can only play the role of recipient if they were chosen, as the resistance phenotypes are non-transferable. Isolates listed in the mobile resistance section (orange) can play the role of donor to isolates from the intrinsic section (blue), as well as having the potential to play either role when partnered together with another isolate from the same section. This grouping allowed for quick drafting of HGT partners to be applied to the conjugation protocols.

Conjugation reactions were carried out using an optimised Tremblay and Archambault, (2013) protocol (figure 4.4.2). MF06036 was chosen as a potential conjugation partner due to its strong resistance profile, particularly vancomycin resistance. This isolate was susceptible to cephalothin. Conjugation partners MF06030, MW01043 and MW03061 were selected as vancomycin susceptible, cephalothin resistant isolates. These isolates were tested in the liquid phase conjugation protocol (figure 4.4.2). Selection plates were incubated for 96 hours, and the MF06030 reaction had a transconjugant donor efficiency of $5.7x10^{-11}$, the MW01043 reaction had a transconjugant donor efficiency of $1.3x10^{-10}$, and the MW03061 was negative. Repeat experiments increased the conjugation time to 24 hours and the selection time to 72 hours. These changes yielded an MF06030 reaction with a transconjugant donor efficiency of $3.4x10^{-10}$. The MW01043 reaction had a transconjugant donor efficiency of $3.8x10^{-10}$, and the MW03061 reaction had a transconjugant donor efficiency of $4.1x10^{-11}$. Control selection plates with the

conjugation partners individually spread out confirmed no growth. Enterococcal phenotypic identification tests (as mentioned in chapter two) confirmed the identified colonies as enterococci.

Expected conjugation efficiencies for enterococci were x $10^{-1\text{-}5}$ for a 24-hour selection time, which was much higher than the data obtained here. The conjugation efficiencies were improved MF06030 (6 fold), MW01043 (2.5 fold) and MW03061 (positive colonies), however the changes made resulted in conjugation efficiencies that were still well behind what was observed in the literature.

Figure 4.4.1 Pie chart detailing potential *Enterococcus* conjugation partners based on mobile and intrinsic antibiotic resistance.

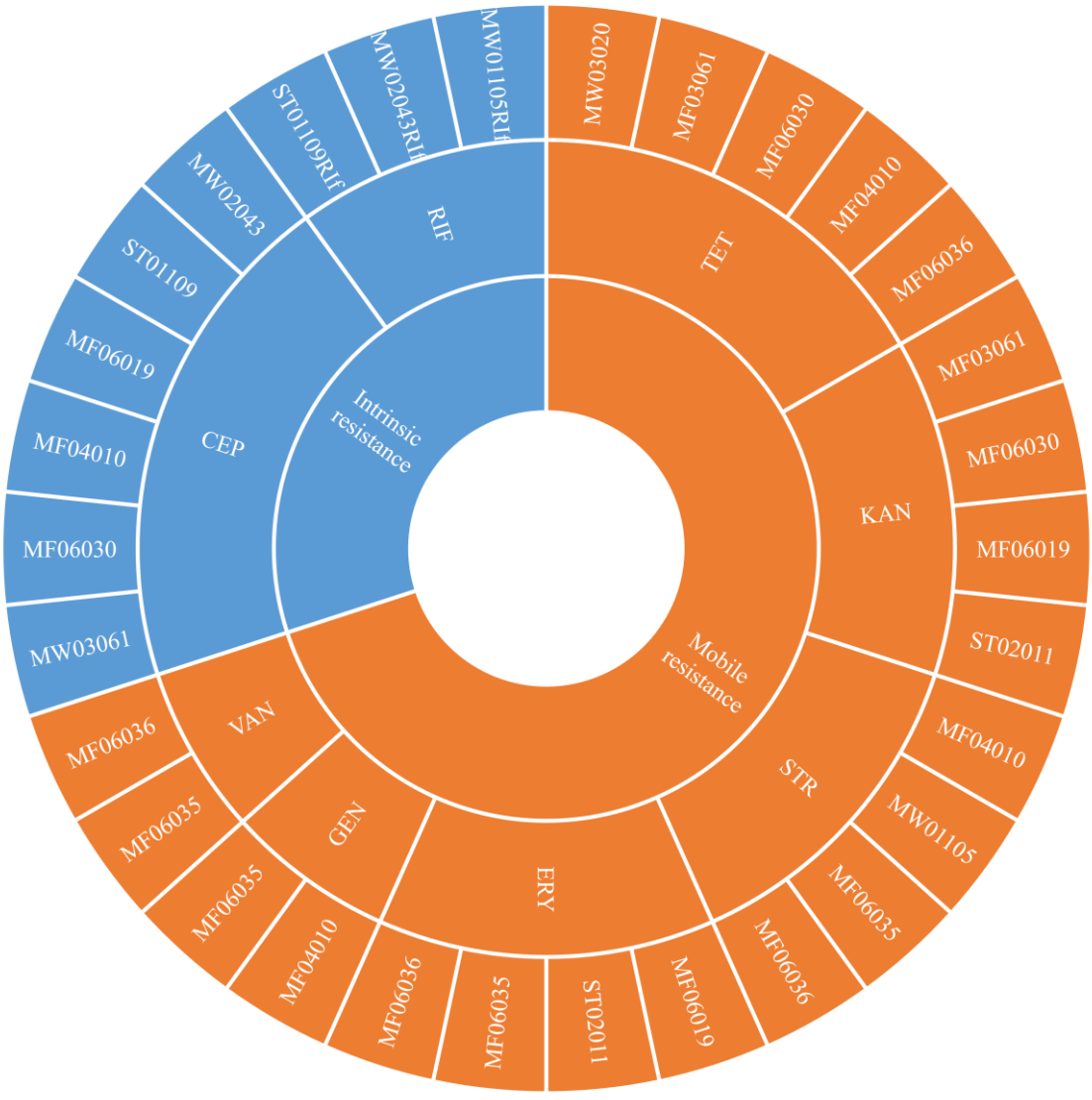


Figure 4.4.1. A pie chart detailing potential conjugation partners based on antibiotic resistance. Standardised conjugation methodologies capture the transfer of antibiotic resistance. This pie chart groups all the unique antibiotic resistance phenotypes discovered in chapter one into intrinsic or mobile resistance categories. Intrinsic resistance cannot be transferred and as such any isolate in the RIF or CEP sub-category can be utilised as a potential recipient matched with any isolates from the mobile resistance category. All isolates in the mobile resistance category can be partnered with any isolate outside their own sub category as either a potential recipient or a donor. CEP - cephalothin, RIF - rifampicin, TET - tetracycline, KAN - kanamycin, STR - streptomycin, ERY - erythromycin, GEN – gentamycin, VAN – vancomycin.

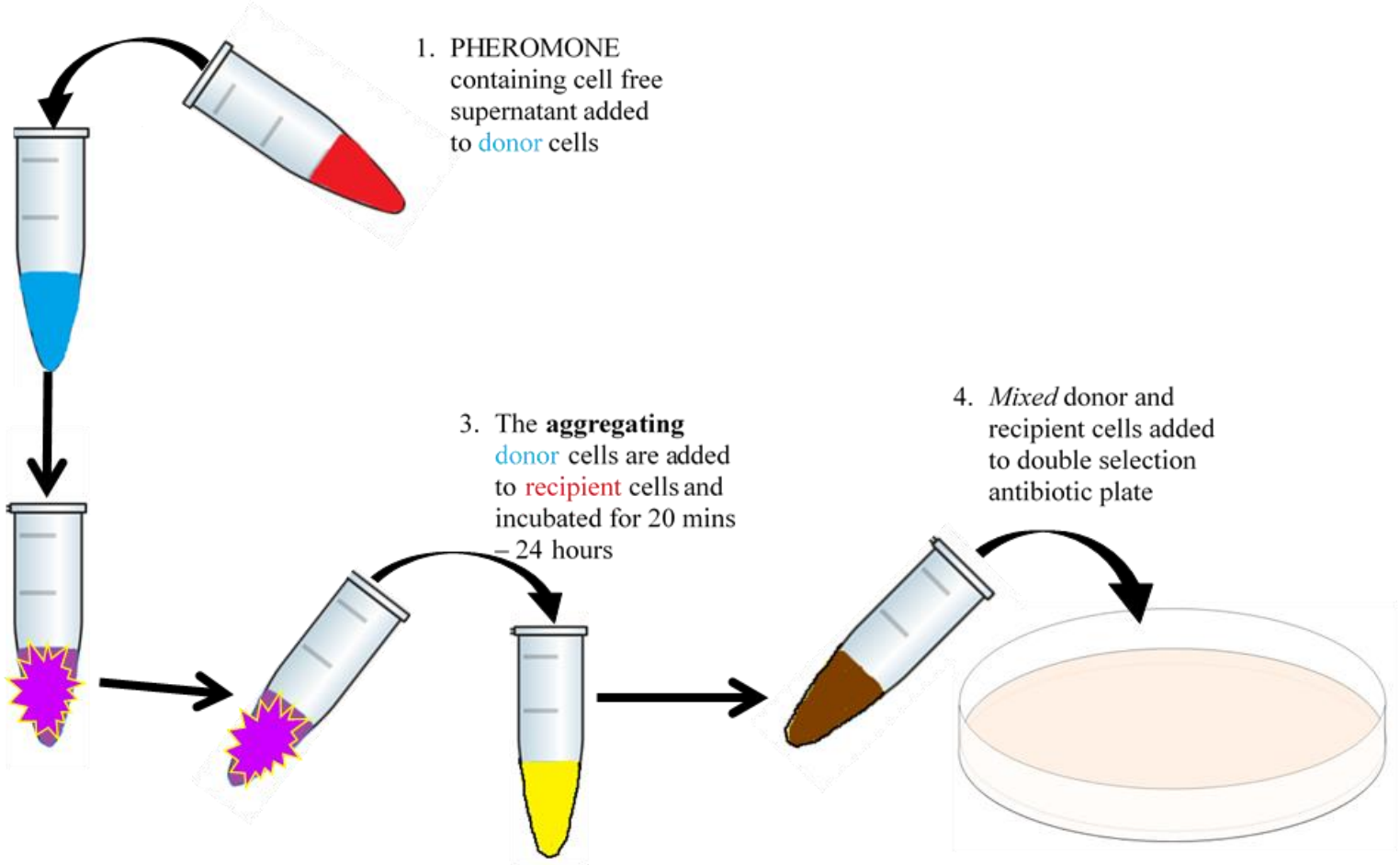


Figure 4.4.2. Liquid phase conjugation methodology. Conjugation between the enterococcal isolates was initially tested using this method. A recipient was mixed with a donor in liquid phase and allowed to conjugate for 20 minutes to 24 hours. After conjugation, the partners and potential transconjugants were plated on double antibiotic selection.

Figure 4.4.3. Liquid phase *Enterococcus* conjugation: partnering the vancomycin resistant MF06036 with the cephalothin resistant MF06036/MW01043/MW03061 plated on double vancomycin cephalothin selection.

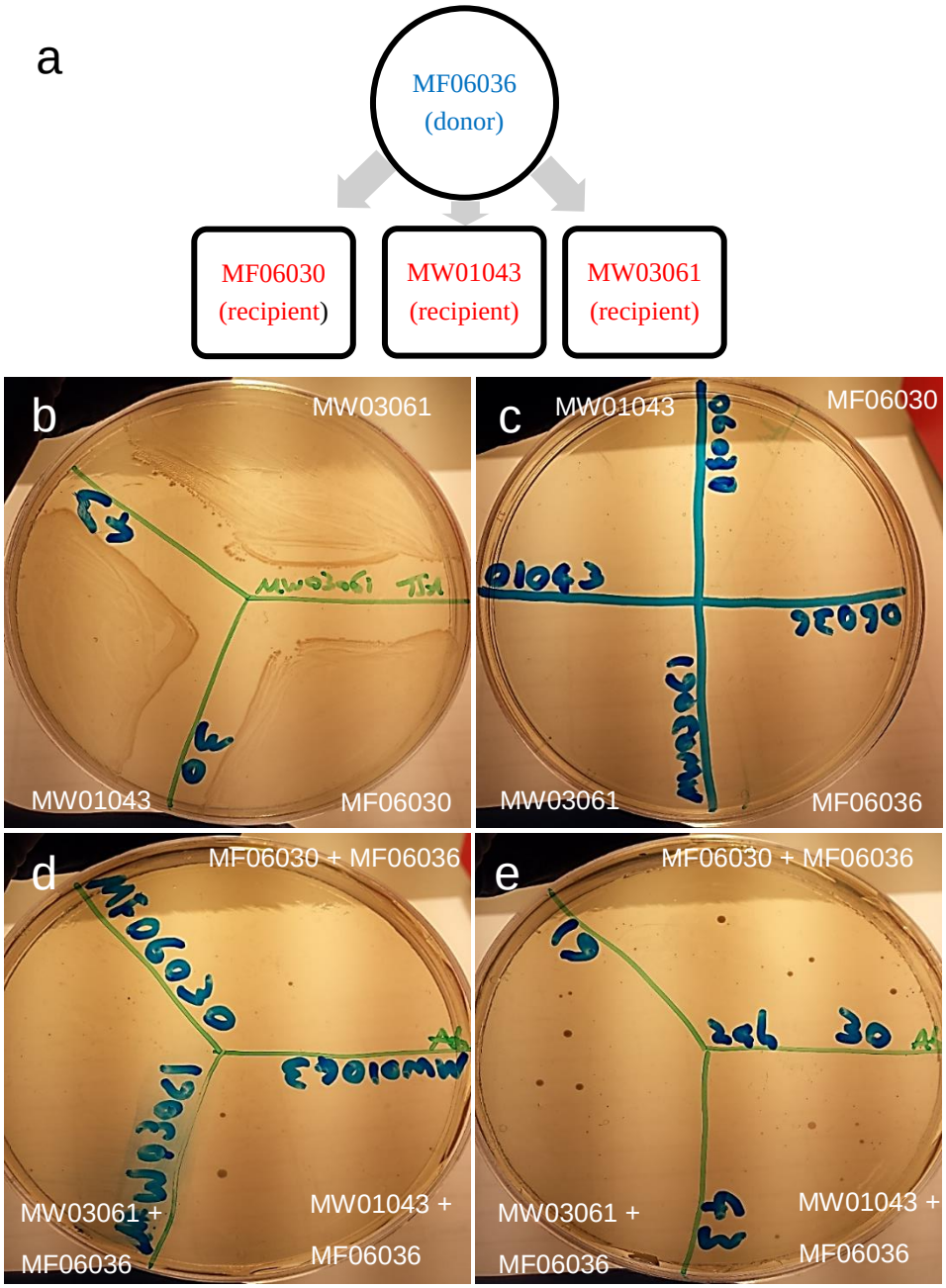


Figure 4.4.3. Liquid phase conjugation experiment. A) MF06036 acted as a donor for the potential transfer of vancomycin resistance into the three potential cephalothin resistant recipients MF06030, MW01043 and MW03061. B) Control TSA plate showing growth of the three recipient isolates. C) Double selection plate showing no growth of any of the four potential parental isolates. D) TSA double selection plate after the 20-minute conjugation incubated for 96-hours. The reaction with MF06030 had a conjugation efficiency of 5.7×10^{-11}, the reaction with MW01043 had a conjugation efficiency of 1.3×10^{-10} and the reaction with MW03061 was negative E) A repeat of experiment (D) with a 24-hour conjugation time and incubated for a further 72 hours. The reaction with MF06030 had a conjugation efficiency of 3.4×10^{-10}, the reaction with MW01043 contained had a conjugation efficiency of 3.8×10^{-10} and the reaction with MW03061 had a conjugation efficiency of 4.1×10^{-11}.

4.4.2. Improving the conjugation protocol and generating high efficiency transconjugants T1-T4

Detection of colonies on the double selection plates in the previous experiment were indicative of successful transfer of AMR phenotype, however the long incubation times and protocol efficiency indicated unreliability in the protocol and thus needed to be refined. Therefore, MW01105^{Rif} was used, as its rifampicin resistance was the highest level tested in this study as well as being the most universal recipient isolate. A change in protocol most notably using solid phase conjugation surface and longer conjugation time (figure 4.4.4) ensured successful capture of AMR transfer (figure 4.4.5).

As MF03035, MF06036 and MF04010 were strong clumping isolates and contained at least three mobile AMR phenotypes, they were selected for partnering with the three recipient isolates MW01105^{Rif}, ST01109^{Rif} and MW02043^{Rif} based on suitability requirements from figure 4.4.1. These enterococcal partnerings permitted the isolation of various transconjugants, 4 of which were selected for further analysis. Transconjugant T1 as explained in figure 4.4.5 was the result of donor MF06036 passing vancomycin resistance to MW01105^{Rif} with high efficiency ($7.8\pm0.8x10^{-3}$). Transconjugant T2 was the result of donor MF04010 passing tetracycline resistance to MW01105^{Rif}, this reaction was the least efficient at $2.3\pm0.8x10^{-5}$. Transconjugant T3 was the result of donor MF06036 passing tetracycline resistance to ST01109^{Rif}; this reaction had an efficiency of $1.8\pm0.3x10^{-4}$ and was an interspecies transfer event. Finally, transconjugant T4 was the result of donor MF06035 passing vancomycin resistance to MW01105^{Rif}; this reaction had the highest observed transfer efficiency $1.22\pm0.3x10^{-1}$. MW02043^{Rif} could not be successfully used in a conjugation reaction.

The transconjugant donor efficiencies obtained using the solid phase conjugation protocol were as expected from the literature, and were on average $1.2x10^8$ times more efficient than the observed reactions using the liquid phase conjugation protocol. Once transconjugants had been repeatedly created and phenotypically confirmed as enterococci, further examination was carried out.

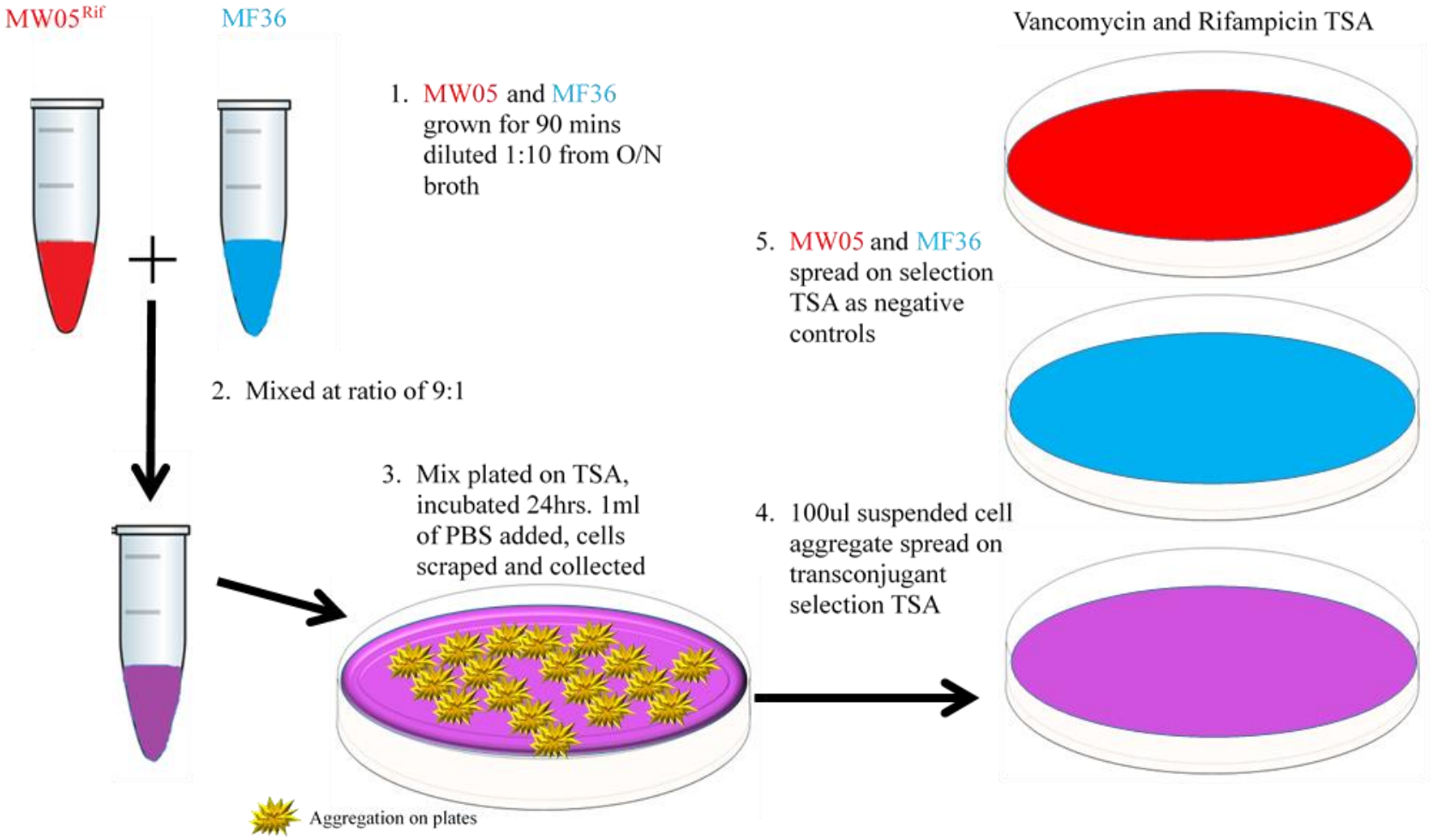


Figure 4.4.4. Solid phase conjugation methodology. Conjugation between the enterococcal isolates was revised using this method. A recipient was mixed with a donor in liquid phase at a ratio of 9:1 and immediately plated on TSA for 24 hours, aggregates were then scraped, suspended and plated on double selection for an additional 24 hours.

Figure 4.4.5. Solid phase conjugation: Isolation of T1.

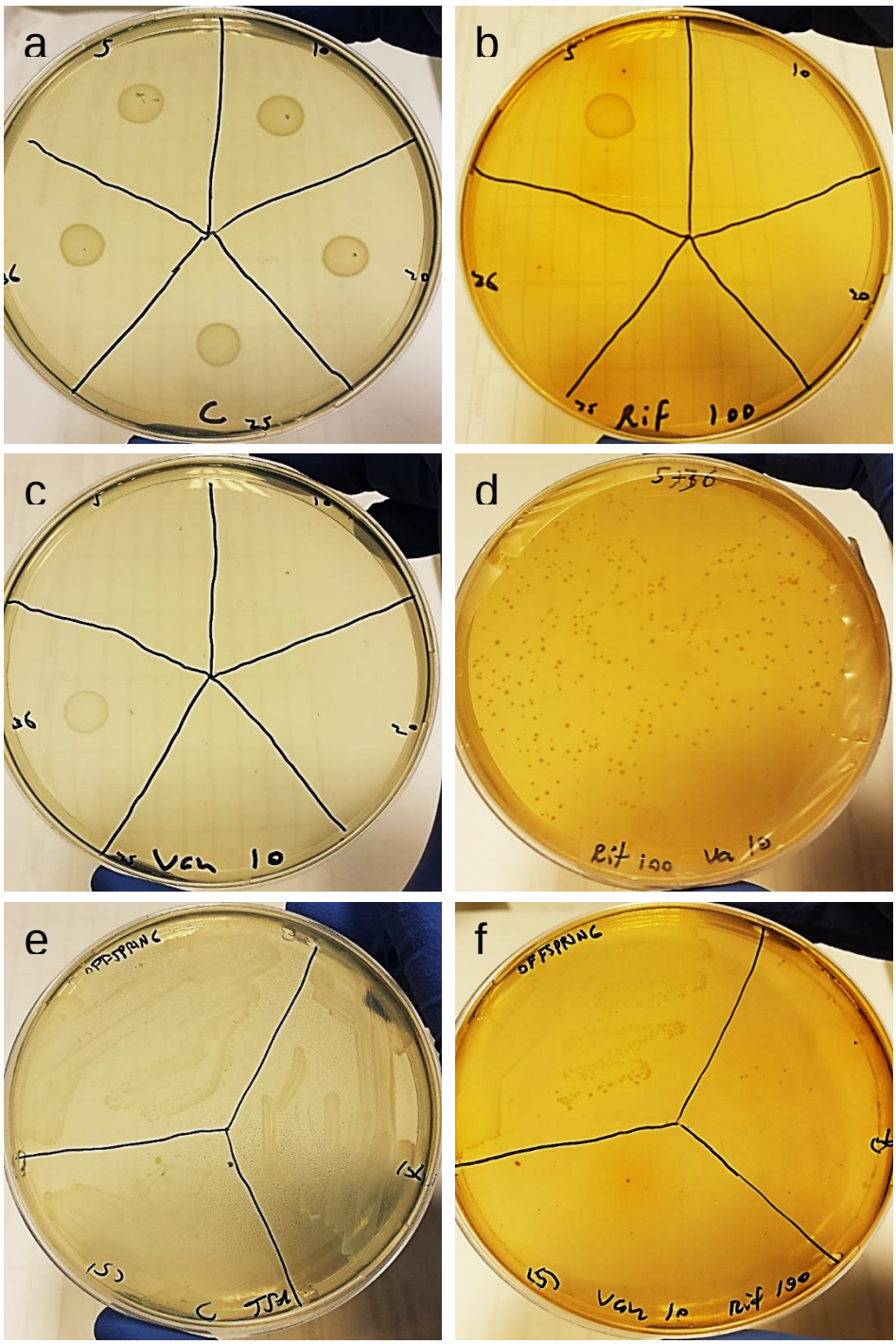


Figure 4.4.5. Successful isolation of transconjugant (T1). (A) Control TSA plate showing growth of isolates MW01105^{Rif}, MF04010, MW03020, MW03025 and MF06036. (B) Rifampicin (100 µg/ml) control TSA plate showing growth of only MW01105^{Rif}. (C) Vancomycin (10 µg/ml) control TSA plate showing growth of only MF06036. In both Figure (B) and (C) the other isolates act as negative growth controls. (D) Successful isolation of T1 from the solid phase 24-hour conjugation of MF06036 and MW01105^{Rif} incubated for 24 hours at 37^{o}C on a double selection plate (rifampicin (100 µg/ml) and vancomycin (10 µg/ml)). (E-F) Control TSA plates showing the normal growth (E) of both parents and transconjugant (T1) and double selective growth (F) of T1 only. The orange specs observed in the vancomycin and rifampicin plates are particles of rifampicin impurity that do not dissolve in TSA.

Table 4.4.1 Isolation of transconjugants T 1-4.

Transconjugants generated, parental isolates and transfer efficiencies

Transconjugant	Donor isolate	Recipient	Resistance phenotype	Efficiency
T1	MF06036	$MW1105^{Rif}$	VAN + RIF	$7.8\pm0.8x10^{-3}$
T2	MF04010	$MW1105^{Rif}$	TET + RIF	$2.3\pm0.8x10^{-5}$
T3	MF06036	$ST01109^{Rif}$	TET + RIF	$1.8\pm0.3x10^{-4}$
T4	MF06035	$MW1105^{Rif}$	VAN + RIF	$1.22\pm0.3x10^{-1}$

VAN – vancomycin, TET – tetracycline, RIF- rifampicin

4.4.3. Antibiotic resistance profiles, transferred genotypes and stability of the acquired resistance traits of the enterococcal transconjugants T1-T4

Further investigation into all possible antibiotic resistance phenotypes/genotypes, as well as the stability of the transconjugant resistance was carried out on transconjugants T1, T2, T3 and T4.

Minimum inhibitory concentrations of antimicrobials were determined for the transconjugants to see how many phenotypes transferred as well as if MIC values changed, as compared to their respective parental isolates (table 4.4.2). T1 accepted transfer of four AMR phenotypes (vancomycin, erythromycin, trimethoprim and teicoplanin) from MF06036, as well as quadrupling its MIC to tetracycline. Transconjugant T2 accepted transfer of tetracycline only from MF04010, as well as doubling its MIC to chloramphenicol. Transconjugant T3 accepted tetracycline resistance from MF06036. Interestingly T3 had a kanamycin MIC value, half that of ST01109^{Rif} and double that of MF06036, as well as halving its MIC to erythromycin. Transconjugant T4 accepted transfer of four AMR phenotypes (vancomycin, erythromycin, trimethoprim and teicoplanin) from MF06035. Interestingly T4's MIC for vancomycin was double that of MF06035.

After confirming that the four transconjugants had received AMR phenotypes from the donor isolates, PCR was carried out to confirm the presence of genes that are linked to the demonstrated phenotypes (Table 4.4.4). Initially the *E. faecalis SodA* gene (figure 4.4.6) was tested on the parents and transconjugants as a final confirmatory step in the identification of transconjugants as enterococci. *Enterococcus faecium* ST01109^{Rif} and its transconjugant T3 did not produce positive banding as expected. The *Enterococcus faecalis* ATCC29212 was used as a positive control.

As vancomycin resistance transfer was observed phenotypically, the *vanA* gene was tested for in the recipient MW01105^{Rif}, its donors MF06036 and MF06035, and the transconjugants T1 and T4. The *vanA* gene (figure 4.4.7) was identified in the donors and the transconjugants but not in the recipient and there was a similar finding for the erythromycin resistance gene *ermB* (figure 4.4.8). The recipients MW01105^{Rif} and ST01109^{Rif}, the donors MF04010 and MF06036 and the transconjugants were all investigated for the presence of seven tetracycline genes (*tetK, tetL, tetM, tetO, tetS, tetT, tetW*). The tetracycline gene *tetL* (figure 4.4.9) *and tetM* (figure 4.4.10) were found in the donors with *tetM* being transferred to both T1 and T2 with only *tetL* being transferred to T2. None of these tetracycline genes were identified in the recipients.

To assess if the transfer of AMR could be selected out of the transconjugants through propagation in negative stress, transconjugants T1, T2 and T4 were cultured daily on AMR selection; normal media then tested in AMR selection; and normal media only, for 25 days. Figure 4.4.11 illustrates that, positive selection controls have in all cases (apart from T3 day 20) lower CFU's than the negative selection control.

Table 4.4.2. Minimum inhibitory resistance profiles of parental enterococci and their transconjugants.

Enterococcal isolate	MIC (µg/ml) at 24 hours								
	VA	E	SM	TET	TMP	TE	KAN	CHL	GEN
MW01105^{Rif}	1	0.5	>1024^{R}	<0.5	0.13^{I}	2	128	4	64^{R}
ST01109^{Rif}	2	8	128	<0.5	0.5^{I}	<0.25	256	4	32
MF06035	256^{R}	>1024^{R}	1024^{R}	8	>16^{R}	>32^{R}	64	8	128^{R}
MF06036	>512^{R}	>1024^{R}	>1024^{R}	128^{R}	>16^{R}	>32^{R}	64	8	16
MF04010	1	<0.25	1024^{R}	128^{R}	0.13^{I}	<0.25	256	4	64^{R}
T1	>512^{R}	>1024^{R}	>1024^{R}	2	>16^{R}	>32^{R}	64	8	32
T2	1	<0.25	>1024^{R}	128^{R}	0.13^{I}	2	64	8	16
T3	1	<0.25	>1024^{R}	128^{R}	0.5^{I}	<0.25	128	8	32
T4	>512^{R}	>1024^{R}	>1024^{R}	8	>16^{R}	>32^{R}	128	8	32

*VA, vancomycin; E, erythromycin; SM, streptomycin; TET, tetracycline; TMP, trimethoprim; TE, teicoplanin; KAN, kanamycin; CHL, chloramphenicol; GEN, gentamycin

^{R},resistant; ^{I}, intermediately resistant

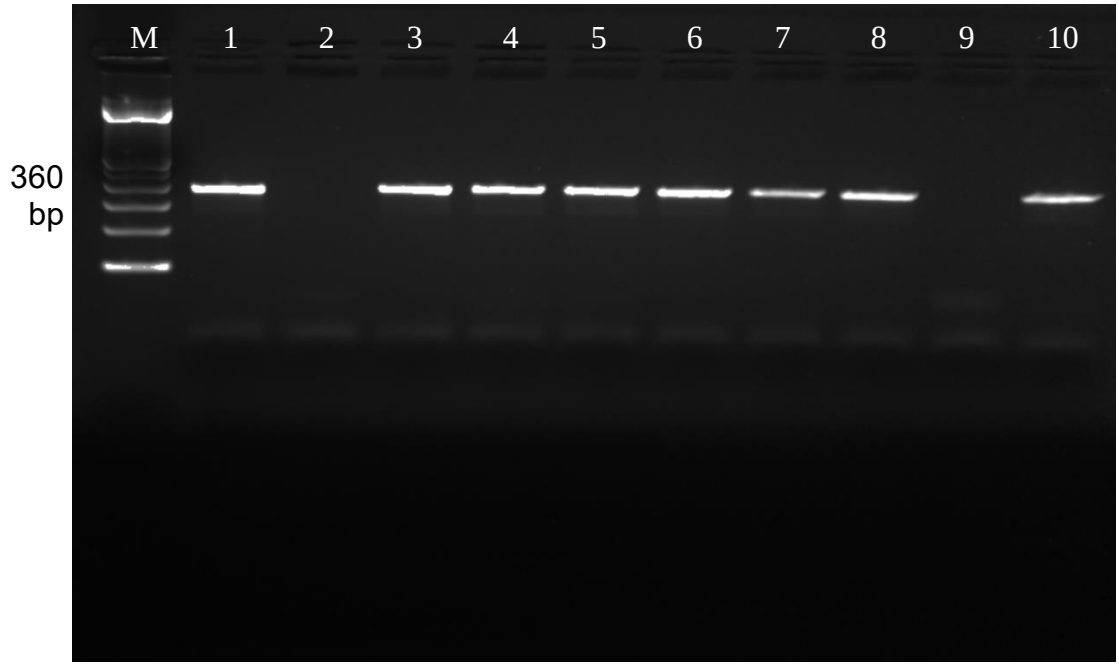


Figure 4.4.6. PCR detection of *E. faecalis SodA* genomic DNA used for species identification in parent and transconjugant enterococci.

Lane M – 100bp ladder

Lanes 1-10 – *SodA* PCR result for isolates MW01105 ^{Rif} (1), ST01109 ^{Rif} (2), MF04010 (3), MF06035 (4), MF06036 (5), T1 (6), T2 (7), T4 (8), T3 (9) ATCC 29212 (10).

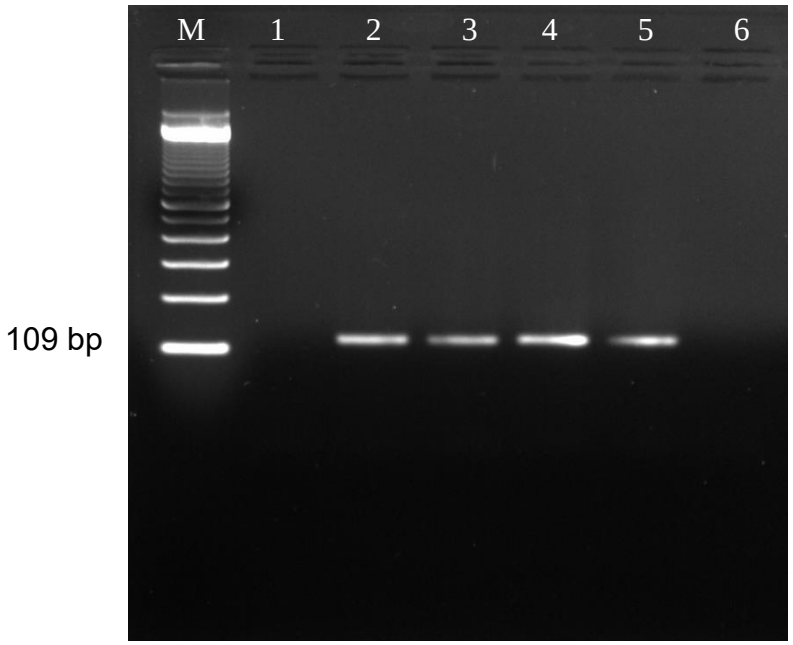


Figure 4.4.7. PCR detection of *vanA* in parent and transconjugant enterococci.

Lane M – 100bp ladder

Lanes 1-6 – *vanA* PCR result for isolates MW01105^{Rif} (1), MF06036 (2), T1 (3), MF06035 (4), T4 (5), Negative control (6).

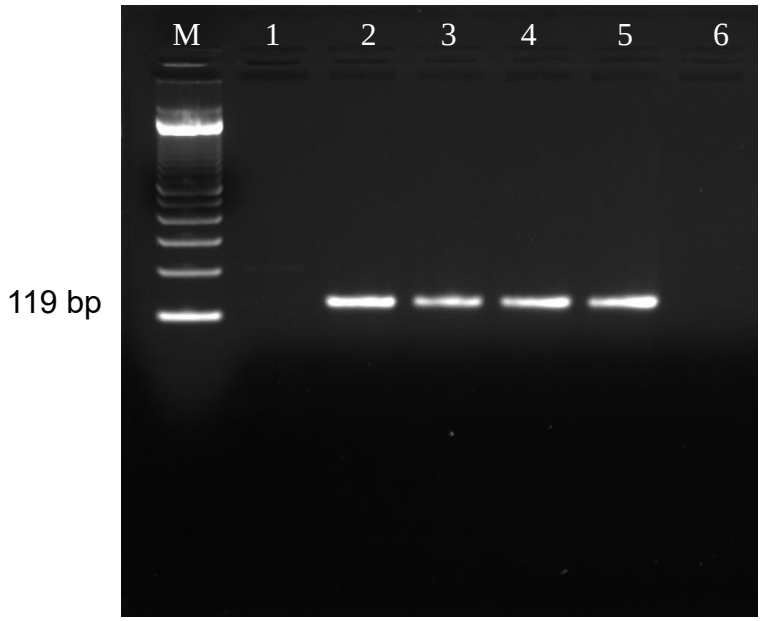


Figure 4.4.8. PCR detection of *ermB* in parent and transconjugant enterococci.

Lane M – 100bp ladder

Lanes 1-6 – *ermB* PCR result for isolates MW01105^{Rif} (1), MF06036 (2), T1 (3), MF06035 (4), T4 (5), Negative control (6).

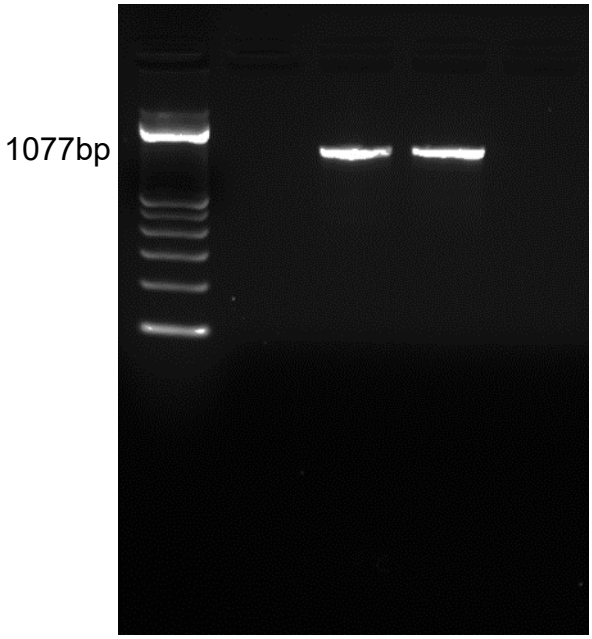


Figure 4.4.9. PCR detection of *tetL* in parent and transconjugant enterococci.

Lane M – 100bp ladder

Lanes 1-6 – *tetL* PCR result for isolates MW01105^{Rif} (1), MF04010 (2), T2 (3), Negative control (4).

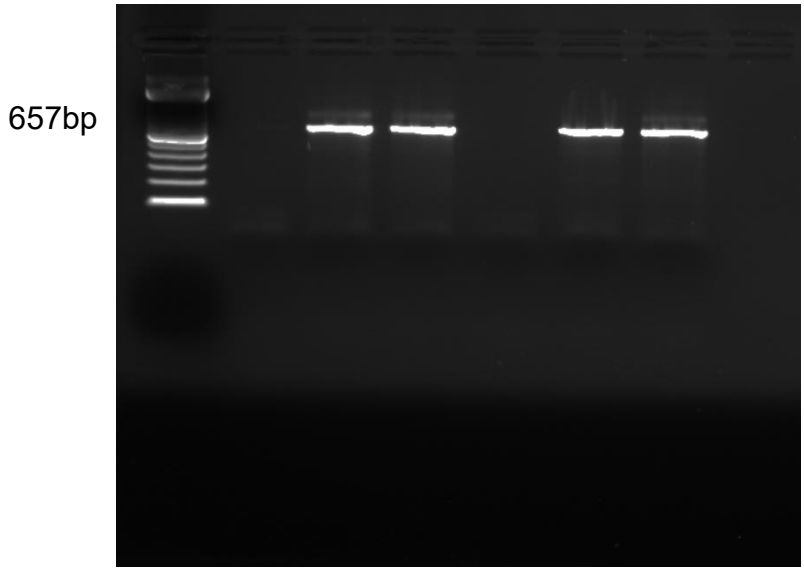


Figure 4.4.10. PCR detection of *tetM* in parent and transconjugant enterococci.

Lane M – 100bp ladder

Lanes 1-7 – *tetM* PCR result for isolates MW01105^{Rif} (1), MF04010 (2), TC2 (3), ST01109^{Rif} (4), MF06036 (5), TC3 (6), Negative control (7).

Table 4.4.3. Transferred antibiotic resistance genes identified by PCR.

Transconjugant	Genes transferred
T1	*vanA, ermB*
T2	*Tet M, Tet L*
T3	*Tet M*
T4	*vanA. ermB*

vanA – vancomycin resistance, *ermB* – erythromycin resistance, *tetM/L* – tetracycline resistance.

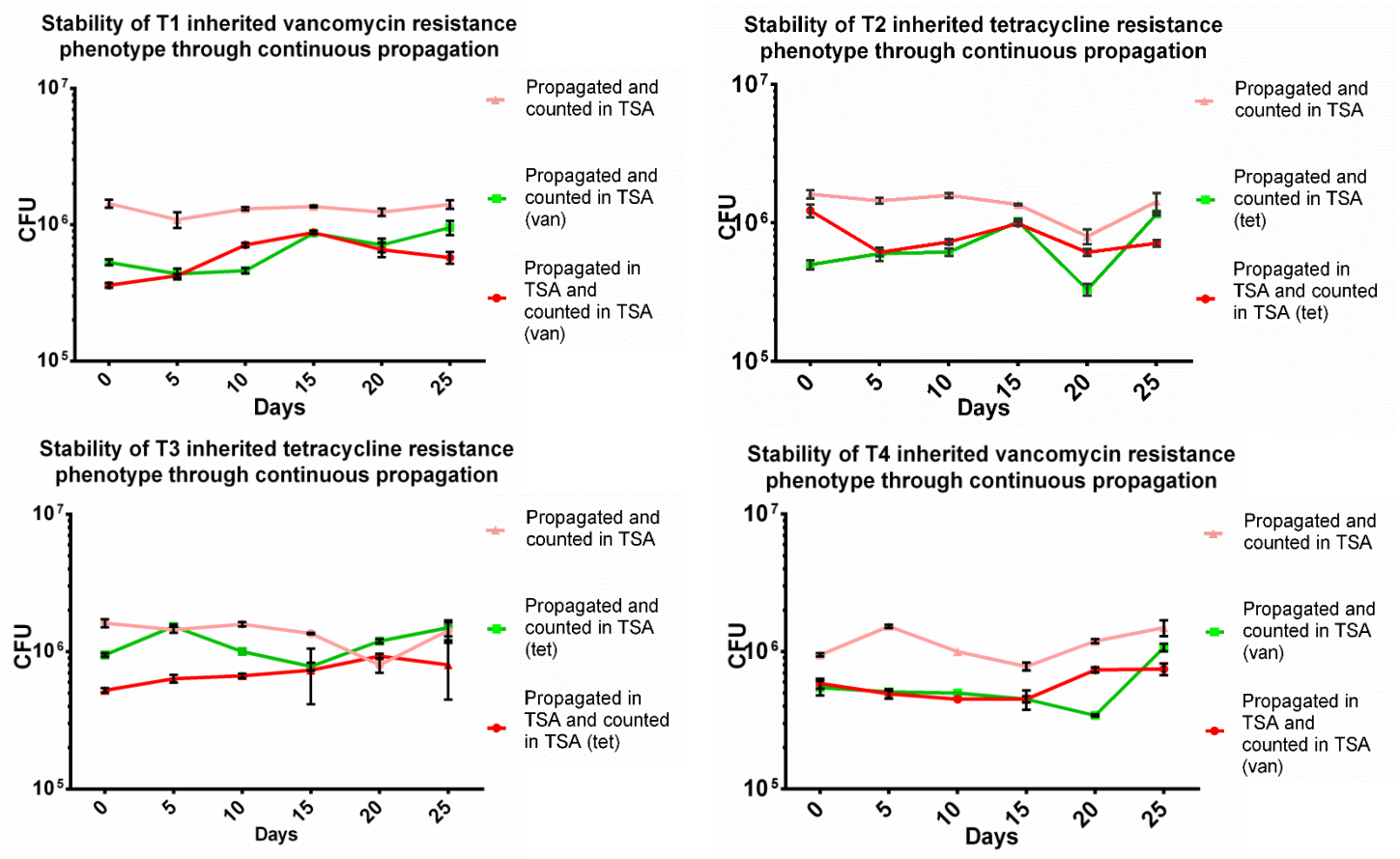


Figure 4.4.11. Stability of transferred antibiotic resistance phenotype. The transconjugants T1-T4 were continuously propagated in and out of selection and tested in selective conditions daily for 25 days to assess if transferred resistance phenotypes would be selected for regardless of external antibiotic stress on the transconjugants. There was no drop-off, of the numbers of progeny which can produce AMR even when placed out of selection for 25 days (red lines) when compared to conditions that ensure upkeep of AMR progeny (green lines) Propagation and counting in TSA (pink line) acted as a negative selection control and propagation in TSA and counted in TSA (*tet/van*) (red line) acted as a positive selection control. No significant change in CFU's as the experiment went on. T1 and T4 CFUs were significantly higher in TSA (p 0.0002 and P 0.0031 respectively) compared to counting in selection (van) across the experimental time course.

4.4.4. The effects of environmental pressures (temperature and nutrient availability) on the conjugation process

In an attempt to understand how conjugation may occur in the natural environment (from which the enterococci were isolated): the conjugation reaction that produced T1 was carried out under sub-optimal temperature conditions. MF06036 and MW01105^{Rif} were conjugated under the same protocol as in figure 4.4.4 with incubation temperatures changed, as shown in table 4.4.4. As expected the total number of transconjugants isolated from the selection plates decreased non-linearly, with reductions in temperature and therefore, so too did conjugation efficiency (37^{o}C - 7.8±0.8x10^{-3}, 20^{o}C - 3.6±0.3x10^{-5}, and 4^{o}C - 3.6±0.9x10^{-9}). When repeating the experiment with nutrient deprivation, efficiencies were further reduced (37^{o}C – 1.0±0.2x10^{-5}, 20^{o}C – 7.4±1.5x10^{-7}, and 4^{o}C - 3.3±1x10^{-12}).

The reduction in conjugation efficiency after removal of nutrients did not produce similar sized decreases (37^{o}C with nutrients compared to nutrient deprivation at 37^{o}C was 780 fold less efficient, 20^{o}C with nutrients compared to nutrient deprivation at 20^{o}C was 48 fold less efficient, 4^{o}C with nutrients compared to nutrient deprivation at 4^{o}C was 1090 fold less efficient) as compared to the temperature reductions in the presence of nutrients. An additional conjugation reaction was performed whereby the normal conjugation protocol was employed with continuous antibiotic stress, reducing normal efficiency tenfold from 7.8±0.8x10^{-3} down to 7.8±0.4x10^{-4} at 37^{o}C.

Environmental conditions such as lower temperature and lack of nutrients as expected reduced enterococcal conjugation efficiency, but did not abolish the ability to produce transconjugants. The “naked” conjugation observed in the laboratory is unlikely to happen in the natural environment and is an inherent bias of laboratory experimentation. It was evident that substrate conjugation is the preferred method of conjugation amongst the collection of isolates in this laboratory.

Table 4.4.4. Conjugation frequency of T1 under temperature and nutrient deprivation

	Conjugation temperature		
Nutrient conditions	37ºC	20ºC	4ºC
Standard nutrients	$7.8\pm0.8x10^{-3}$	$3.6\pm0.3x10^{-5}$	$3.6\pm0.9x10^{-9}$
Nutrient deprivation	$1.0\pm0.2x10^{-5}$	$7.4\pm1.5x10^{-7}$	$3.3\pm1x10^{-12}$

Conjugation reactions creating T1 under nutrient deprivation and sub-optimal temperature. The conjugation reaction depicted in figure 4.4.5 was repeated with reduced temperature and again with reduced temperature and nutrient deprivation.

4.4.5. Atypical conjugal interactions between enterococci using sponge as a substrate

The environmentally isolated, conjugal *Enterococcus* partners have been shown to conjugate under starvation, and at low temperatures. There was interest in establishing if they could find another host when out of their natural GI environment. *Spongilla lacustris* is a sponge that exists in fresh water streams within similar environments from which the enterococci were isolated. A simple assay was tested on *S. lacustris* to see if enterococci interact in any way with the sponge. After 24-hour incubation in water with a sponge hatchling at 20^{o}C, with multiple washing steps, it was evident that enterococci attach to *S. lacustris* spongin (figure 4.4.12).

As enterococci demonstrated an ability to attach to the spongin of *Spongilla lacustris*, a protocol was devised and optimised (figure 4.4.13) to detect if enterococcal attachment to spongin could provide a host environment whereby conjugation could take place. The sponge conjugation assay was performed on *S. lacustris* and *Ephydatia fluviatilis* with a water only control. As seen in figure 4.4.14, both reactions where sponge is present produce significant numbers of T1 as compared to the control (p values - unpaired t test with welches correction). Sponge controls with no bacteria added did not show any growth on double selection plates. Treatments with both sponge species and enterococci had significantly higher ($p<0.0001$) numbers of transconjugants compared to controls with enterococci and no sponge. No conjugation was observed in 52.4% of the "no sponge" control tubes, compared to 2.5% and 0% in *E. fluviatilis* and *S. lacustris* respectively. *Enterococcus c*onjugation frequencies in water only, with no sponge were $7.00x10^{-8}$. *Enterococcus c*onjugation frequencies in the presence of *E. fluviatilis* was $1.26x10^{-6}$ per sponge at 48hrs on selection. *Enterococcus c*onjugation frequencies in the presence of *S. lacustris* was $1.05x10^{-6}$ per sponge at 48hrs on selection. *Enterococcus*

conjugation in the presence of sponge was over 500 times more efficient than in water alone. There was no significant difference in the number of transconjugants between the sponge species (p=0.5796).

Due to the significant increase in conjugation frequency of enterococci in the presence of sponge, attempts to visualise enterococci within sponge tissue was carried out. Specialised fluorescent *in situ* hybridisation microscopic assays were developed in a rapid assessment format for analysis of bacterial presence within sponge. Whole sponges were treated in this protocol without dissection as illustrated in figure 4.4.15(a). The white spongin tissue in (a) is where the majority of enterococci were identified. Figure 4.4.15(b) and (c) show co-localization between Hoechst nuclear stain and the FISH probe specific for *E. faecalis* (ENF). Further modification of the protocol (chapter 2) permitted capture of pinpoint fluorescence, providing clear imagery of individual enterococcal cells within the spongin tissue of the whole mounted sponge (figure 4.4.15(d) and (e)).

Detailed microscopic investigation of enterococcal presence inside sponge required cryosectioning frozen sponge tissue, post enterococcal conjugation (figure 4.4.16). Fluorescence signal of the ENF probe was significantly brighter as compared to whole mounted sponge (figure 4.4.15.) permitting examination of the spatial location of enterococci in the tissue. Enterococci appear to exist heterogeneously within the sponge tissue, with frequent features that appear to show enterococci in contact with each other (arrows). Features such as DNA content of the sponge (figure 4.4.16.(b)) and anchoring spicules (figure 4.4.16.(d)) identify the sections as sponge tissue.

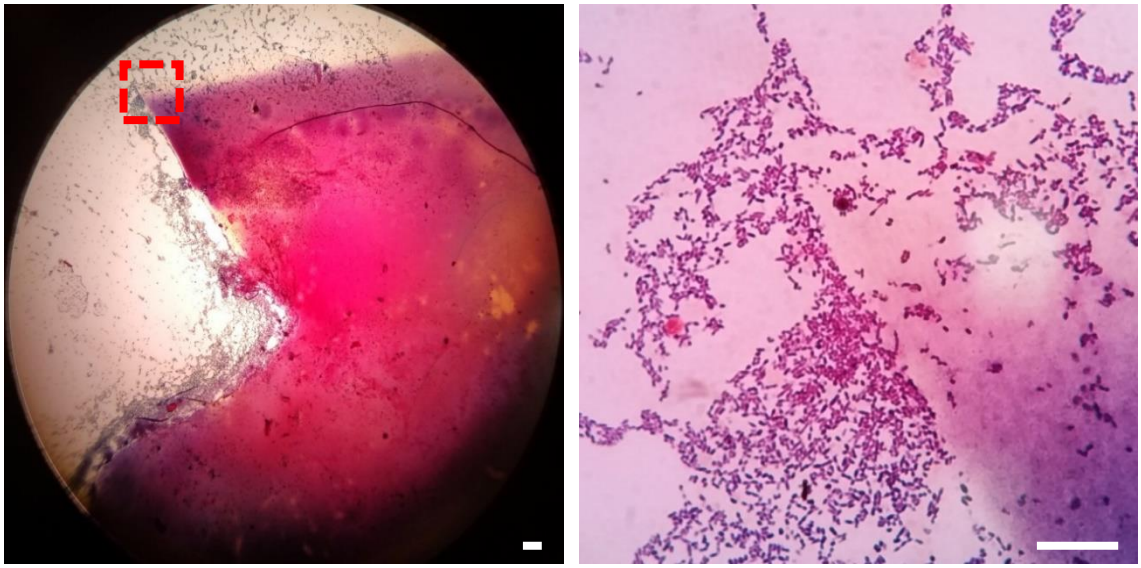


Figure 4.4.12. Interactions between enterococci and sponges from similar geographical isolation sites.

Micrographs depicting colocalization of *E. faecalis* MF06036 with the sponge *Spongilla lacustris*. Sponges were incubated in universal tubes at 20^{o}C with *E. faecalis* (1x10^{5} CFU/ml) for 24 hours. Sponge was removed, washed in sterile PBS, cut in half and stained (1% crystal violet) for 15 minutes. The sponge was dry mounted and imaged on an OLYMPUS CX21 bright field microscope with 40x/0.65 and 100x/1.25 oil, plan objectives. Image on the left is a low power micrograph depicting the brightly stained gemmule (pink) which has a large agglomeration of *E. faecalis* attached to the periphery of the gemmule. The body of the sponge (spongin) exists in the white plane of the micrograph which did not stain brightly. The image on the right is a high-powered magnification, region of interest from the low powered micrograph on the left (zone depicted by a dashed red square). The enterococci are clearly depicted on and adjacent to the gemmule and on top of the spongin. Scale bar represents ten microns.

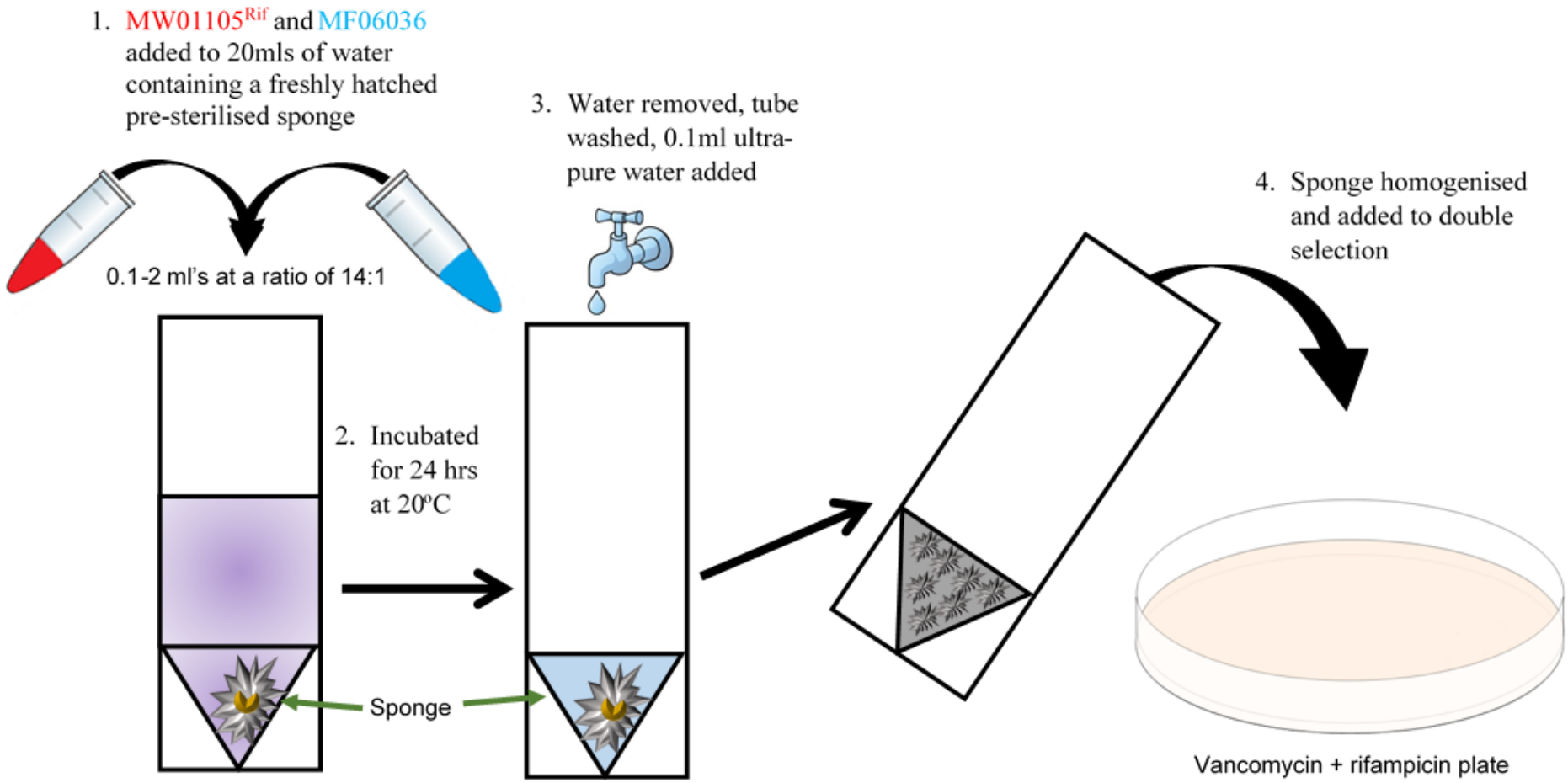


Figure 4.4.13. Protocol created to isolate fresh transconjugants from conjugation reactions in the presence of sponge. The standardised conjugation reaction developed in this study was applied to a universal tube containing a sponge. The ratios were optimised to 14:1 recipient to donor. Reactions took place in water at 20°C for 24 hours. Water was removed and sponge was washed with sterile water. Sponges were transferred to new tubes, homogenised in sterile PBS and added to double selection plates. Controls with no sponge were also performed.

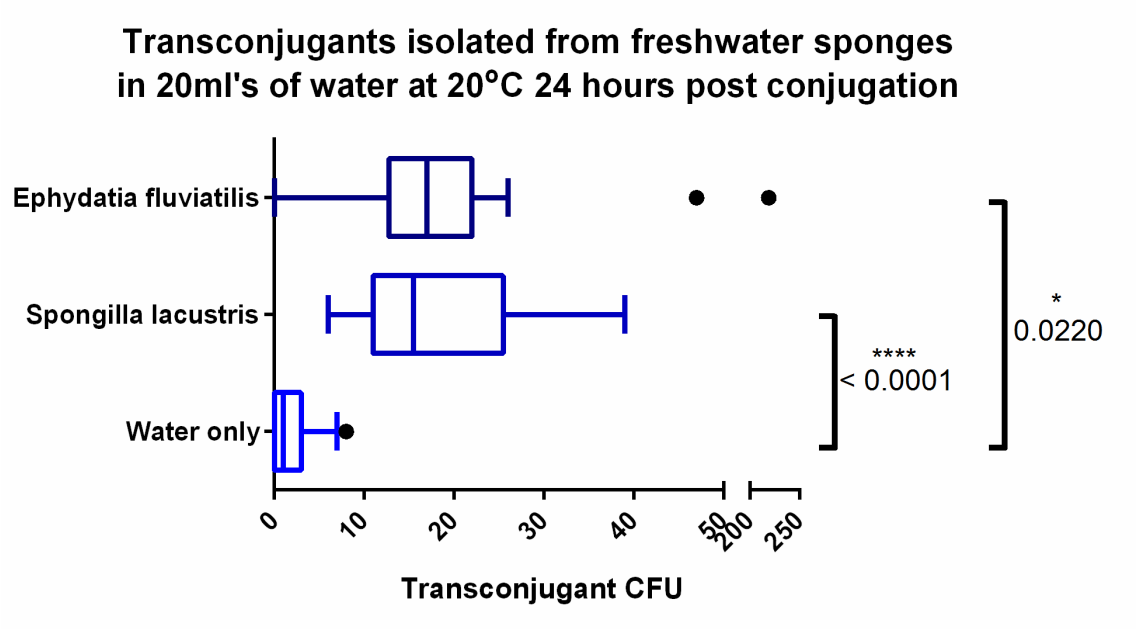


Figure 4.4.14. Enterococcal conjugation in sponge represented with Tukey's box plot.

A Tukey's box plot illustrating the results from the conjugation in the presence of sponge experimentation. Conjugation reactions between MF06036 and MW01105^{Rif} to create T1 were performed in tubes containing either *E. fluviatilis*, *S. lacustris* or water only. All experiments contained seven replicates and were independently repeated three times under the optimised protocol described in figure 4.4.13. *E. fluviatilis* had two outliers which were significantly higher than the median values of both sets of sponge data. Factoring the outliers *E. fluviatilis* had significantly higher numbers of transconjugants located in and on its tissue as compared to the water only control. Recovery of transconjugants from *S. lacustris* experiments yielded positive numbers in every instance as compared to *E. fluviatilis* and water only and numbers recovered were significantly higher than the water only control. Significance was calculated using the unpaired t test with welches correction. Sponge controls with no bacteria added did not show any growth on double selection plates. Zero rate conjugation was observed in 52.4% of the "no sponge" water control tubes, compared to 2.5% and 0% in *E. fluviatilis* and *S. lacustris* respectively. Conjugation efficiencies as transconjugant per donor per sponge are: *E. fluviatilis* – 1.26×10^{-6}, *S. lacustris* -1.05×10^{-6}, water - 7.00×10^{-8}.

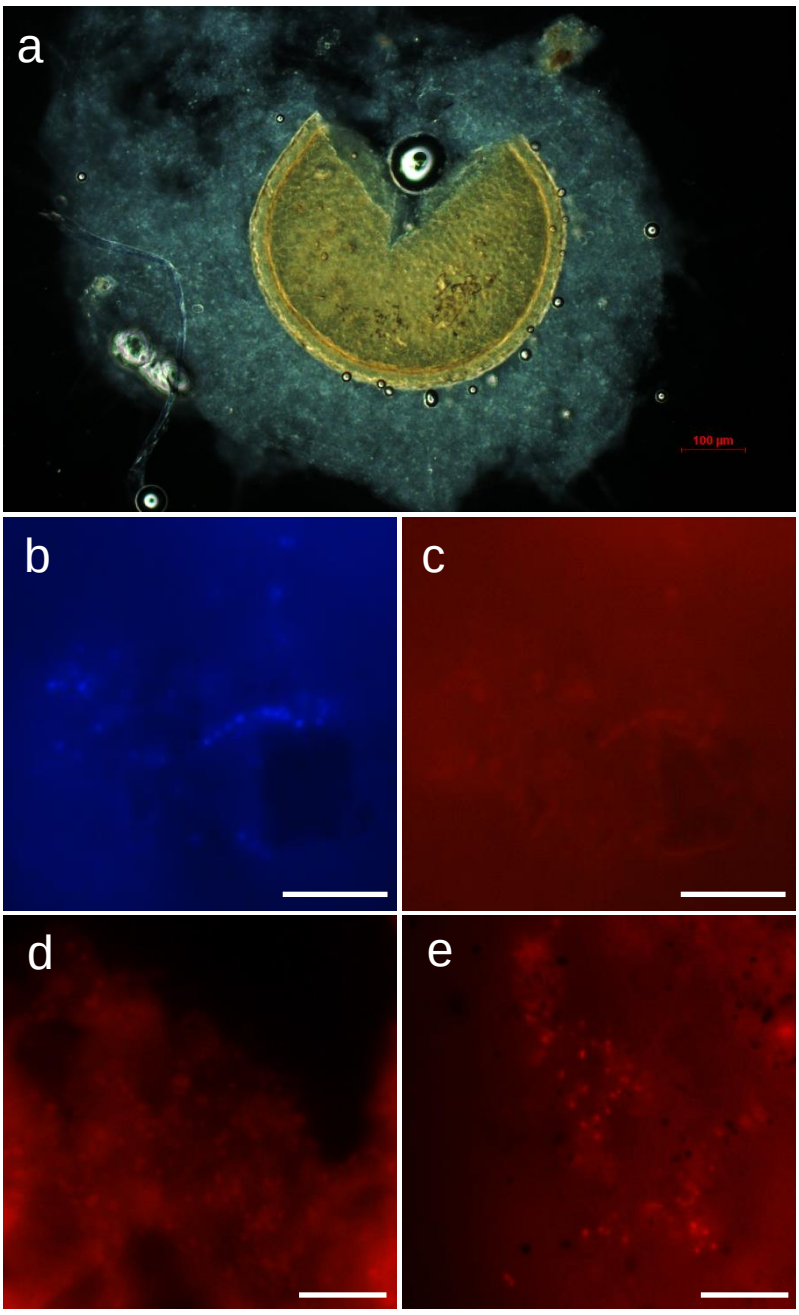


Figure 4.4.15. Optimisation of fluorescent *in situ* hybridisation to detect enterococci in whole mount sponge after conjugation. Fluorescent micrographs using *in situ* hybridisation to visualise MF06036 on and inside sponge whole mounted tissue (a) is a low magnification dark filed micrograph of a whole mounted *S. lacustris*: gemmule – central yellow shell, spongin – fibrous matrix of white collagen based tissue. (b+c) Unprocessed sponge whole mount imaged at 100x magnification with Hoechst (b) and ENF *Enterococcus faecalis probe* (c). In an unprocessed state, it is possible to visualise enterococci using FISH, with significant background signal. Hoechst staining gives good contrast for bacteria. (d) Red channel fluorescent micrograph of ENF FISH staining on a flattened sponge between a glass coverslip and slide incubated for additional time with probes (24 hours). ENF probe signal is much improved over (c). (e) The same processing as in (d) with the addition of Sudan black B, which helps reduce background noise. Enterococci are clearly visible in (e), to the extent that individual cells can easily be observed. White scale bars represent ten microns.

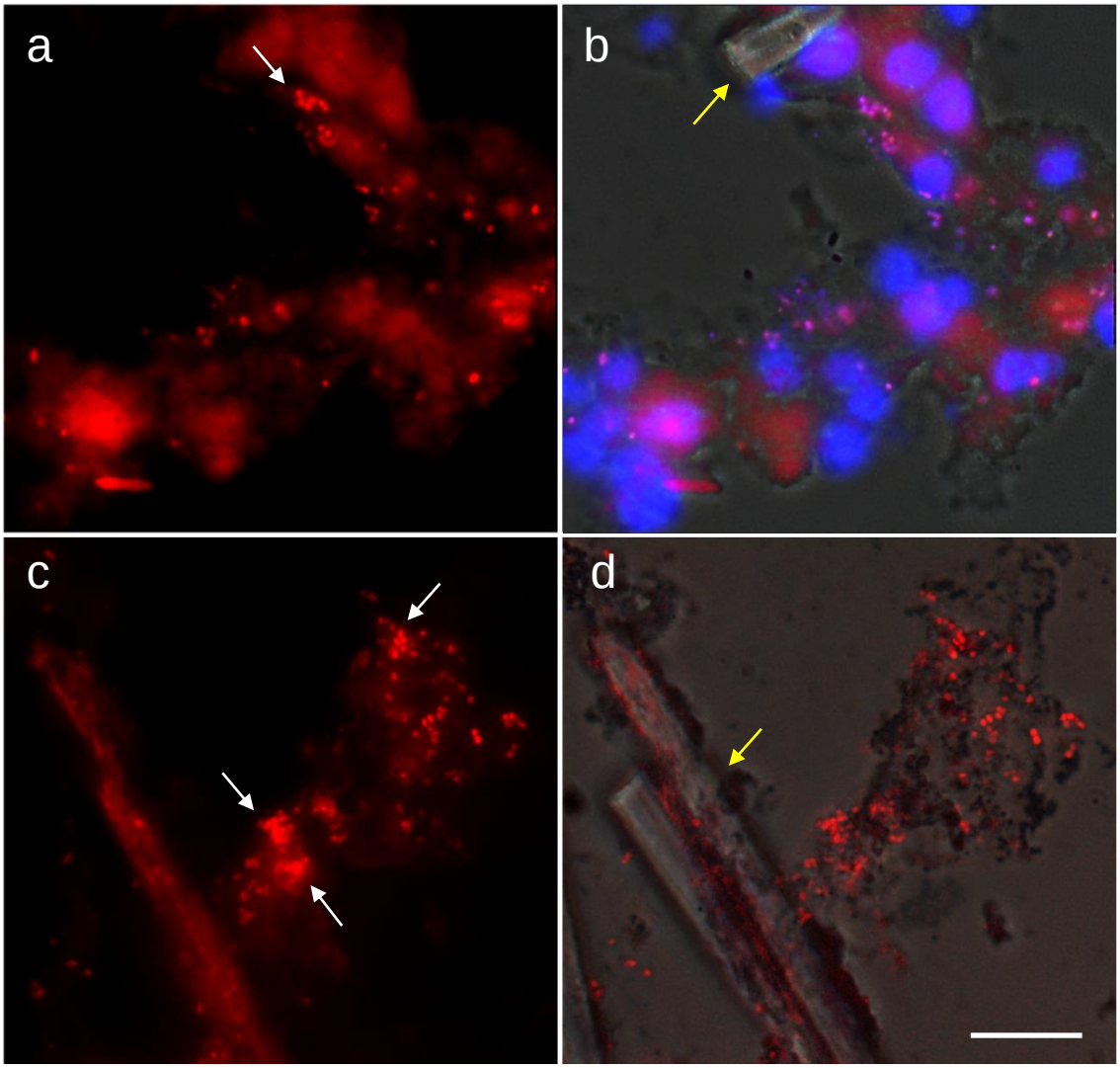


Figure 4.4.16. Fluorescent *in situ* hybridisation on *S. lacustris* cryosections to identify *E. faecalis* MF06036 after a conjugation reaction within the sponge. Fluorescent *in situ* hybridisation on ten-micron sponge sections using the ENF *E. faecalis* probe (Texas red fluorophore) after a conjugation reaction in the presence of sponge. (a) Section of *S. lacustris* examined with red fluorescence. (b) Overlay of the same section as (a) examined with blue, red and bright field imaging channels. (c) Section of *E. fluviatilis* examined under red fluorescence. (d) Overlay of the same section as (c) examined with red and bright field imaging channels. ENF positive (red cells) *E. faecalis* can be observed throughout the entire sponge tissue on all sections. Bacteria often appear in clusters of several individuals in close proximity to each other (white arrows). Enterococci also stain with the Hoechst DNA stain with no photo-conversion (b). Characteristic sponge spicules were present (yellow arrows). Scale bar represents 10 microns.

4.5. DISCUSSION

Enterococci were partnered, and conjugation protocols were developed to generate transconjugants, of which four were analysed (T1-T4). Enterococci were able to conjugate under sub-optimal conditions as well as on sponges'.

4.5.1. Partnering of compatible enterococci for HGT of AMR

As there was a large collection of isolates to work with, partnering involving well known AMR phenotypes as described in the literature and annual clinical surveys (vancomycin, tetracycline) were selected for first (Gilmore *et al.*, 2014; Rossolini *et al.*, 2014). Vancomycin resistance in enterococci is the most well known in clinical settings and tetracycline resistance determinants are present in one of the most studied plasmids (pCF10).

4.5.2. Liquid phase conjugation

Laboratory based conjugation amongst enterococci was first described by Jacob & Hobbs, (1974); and Dunny and Clewell, (1975). The original protocol employed the use of Oxoid nutrient broth number 2 media, and used isolated sex pheromone, which resulted in successful isolation of the transconjugants. Over the years, the protocol has been refined into several varied methods. The method described in figure 4.3.2 is an extension of the original method updated by Tremblay and Archambault, (2013) and optimised for this study. Several groups of conjugation partners were selected for conjugation through this protocol.

The majority of attempts at conjugation through this method were unsuccessful. There was one partnering involving MF06036 transferring vancomycin resistance into the vancomycin susceptible, cephalothin resistant MF06030, MW01043 and MW03061. Initial success was achieved with a conjugation time of 20 minutes and incubation on

selection for 96 hours. The entire conjugation liquid phase for each of the three reactions was plated and transfer efficiency was extremely low, with a maximum of $1.3x10^{-10}$ as compared to the typical $x10^{-3\ to\ -7}$ for plasmids, (Andrup and Anderson, 1999; Cook *et al.,* 2011). The experiment was repeated with longer conjugation times, up to 24 hours, which improved efficiency by 66% to $3.4x10^{-10}$ (figure 4.4.2 (e)).

The reaction involving MW03061 went from a negative reaction in the 20-minute exposure to a positive reaction with the same efficiency as the reaction with MW01043. It has been reported, and is generally accepted, that successful, repeatable conjugation between two enterococci should produce transconjugants on selection growth plates in the first 24 hours with maximum efficiency obtained at 48 hours (Lampkowska *et al.,* 2008; Gilmore *et al.,* 2014). The successful conjugation attempts observed in figure 4.4.2 display conjugation efficiencies similar to those observed for conjugal transposon transfer ($x10^{-9}$) by Marcinek *et al.* (1998). However, the issues with repeatability of the data collected here combined with final protocols that do not represent what is reported in the literature rendered this experiment too unreliable, and an alternative methodology was needed.

4.5.3. Isolation of a transconjugant (T1) using solid phase conjugation

The first change made to the conjugation protocol after the results from the initial experiments was to use recipients with a single unique antibiotic resistance phenotype for all partnering attempts. In this case it was rifampicin resistance. These isolates were $MW01105^{Rif}$, $MW02043^{Rif}$ and $ST01109^{Rif}$, demonstrating aptness as recipients in pheromone based compatibility for conjugation as well as having broad susceptibility profiles to antibiotics (excluding their rifampicin resistance). Changes in AMR profiles could only be from successful conjugation. The second change made to the process was the inclusion of a solid phase conjugation surface (Figure 4.4.4) to replace the liquid phase

that was used in the previous protocols. This solid phase allowed successful transconjugants to grow in tandem with both parent isolates, before being inhibited by double selection. Certain plasmids can transfer more efficiently under specific circumstances. Plasmid pCF10 transfers efficiently during filter mating, where cells are concentrated in close proximity allowing efficient exchange between donors and recipients (Gomez *et al*., 2014). Efficient mating in liquid cultures would typically occur with bacteria that possess conjugation pili, such as the F pilus attributed to E. *coli* (Lawley *et al*., 2003). Enterococci are more likely to efficiently conjugate on solid surfaces, where cell contacts are readily available.

The revised protocol using a solid phase for conjugation ensured only transconjugants could grow. When comparing this method to the previous one, solid phase conjugation was the superior method, repeatedly producing transconjugants with efficiencies over $1x10^8$ million times higher than the initial liquid phase method.

The nature of these transfers indicated the potential for a specific class of plasmid being transferred, (Inc18), as discussed in the introduction (Zhu *et al.,* 2010). To confirm that this reaction was indeed conjugation and not a spontaneous MF06036 rifampicin mutant, post-conjugation selection was performed on the parents and the transconjugants (Figure 4.4.5 (e-f). The control plate (e) shows growth of all three enterococci and (f) shows growth of transconjugants only.

4.5.4. Isolation and phenotypic characterisation of transconjugants T1-4.

Four transconjugants were selected for further testing using the protocol as observed in table 4.4.1. T1 (figure 4.4.5) and three more (T2-T4). These transconjugants had their AMR phenotypes analysed using MIC's and compared to their parental isolates (table 4.4.2) The phenomenon of multiple AMR transfers is reported but occurrences are much lower than reports of single AMR transfer. Vignaroli *et al*. (2011) identified multiple gene

transfer (*ermB* and *vanA*) between enterococci isolated from farm animal sources. Reported cases of antibiotic resistance transfer from enterococci of this origin usually report co-transfer with other determinants, such as *vanA* and aggregation substance reported by Paoletti *et al.* (2007).

Transconjugants T1 and T4 received four AMR phenotypes (vancomycin, erythromycin, trimethoprim and teicoplanin) from MF06036 and MF06035 respectively. Moubareck *et al*. (2003) demonstrated the potential for up to four antibiotic resistance genes to be conjugally transferred (*vanA, ermB, tetL ant(6)*) in a large 180kb plasmid, amongst enterococci of animal origin. Those that received four genes had low transfer efficiency (10^{-9}) as compared to single or double gene (*vanA* and *ermB*) transfer ($10^{-2 \text{ to } -5}$). Silveira *et al.* (2013) reported multiple gene conjugal transfer of the same genotype/phenotypes (vancomycin, erythromycin and tetracycline) from similar sources to Moubareck *et al*. (2003) and the enterococci used in this study. They alluded to one or several MGEs. Enterococcal conjugation on the scale of up to four transferred phenotypes is rarely reported, those that do are from animal origin. Regarding this study, this is the first report of four phenotype transfers to include trimethoprim and teicoplanin determinants with high conjugation efficiency ($10^{-1 \text{ to } -3}$), demonstrating the prolific abilities of enterococcal conjugation beyond straightforward single gene transfer.

As concerns of *Enterococcus* as a nosocomial pathogen continue to escalate, combined with observations of increased virulence, questions on the source origins of AMR phenotypes and real examples of natural horizontal transfer of AMR genes have emerged (Arias and Murray, 2012; Di Cesare *et al*., 2014). Jutkina *et* al., 2016 describes how metagenomic analysis of HGT in the environment is not enough to understand the origins and spread of AMR. There is a global desire for direct detection of such events. This body of work highlights the natural HGT of AMR phenotypes and genes at high frequency in

enterococcal isolates from the natural environment, an ecological niche never tested for propagation of AMR in enterococci previously.

The vancomycin donor MF06036 was responsible for AMR HGT events which resulted in the isolation of two different types of transconjugants (T1 and T3). T3 was created through an interspecies conjugation reaction from *E. faecalis* into *E. faecium* demonstrating the broad host range of these mobile AMR phenotypes. When tested, these two transconjugants have differing AMR profiles that match the donor in parts but not each other. This is the first observation, of this kind in enterococci isolated from an agrarian environment and suggests a two-component conjugation system that can independently transfer a single component during conjugation. Christie *et al.* (1987) observed two separate conjugation systems for the tetracycline pCF10 plasmid. A 25kb *tra* region and a 16kb region (Tn925) different from *tra* that also transferred tetracycline resistance. Interestingly, there were several increases to the MIC of the tested antibiotics in the transconjugants, raising concerns on the peripheral effect of horizontal gene transfer to the hardiness of transconjugants.

T1 had a quadrupling of tetracycline MIC, T2 had a doubling of chloramphenicol MIC and T4 had a doubling of vancomycin resistance. These increases in MIC to non-transferred AMR phenotypes indicates an underlying danger in the propagation of AMR, in that through the action of additional mechanisms involved in bacterial survival, the transfer of mobile elements can increase non-specific responses to antimicrobials. Alterations to the putative enterococcal multidrug efflux pumps could cause general increases to groups of antimicrobials (Molale and Bezuidenhout, 2016). Overexpression of the *E. faecalis* EfrAB (ABC transporter), a putative multidrug efflux pump in *E. coli* caused efflux of norfloxacin (Lee *et al*., 2003). Hürlimann *et al*. (2016) examined EfrAB for its ability to function as an effective efflux pump in *E. faecalis*. They discovered that

deletion of the efflux genes had minimal effect on MIC for drugs such as acriflavine. However, when they overexpressed EfrAB, they discovered that the efflux pump could transport a large consortium of drugs including norfloxacin. They concluded that the standard state of this efflux pump functions at a low level in *E. faecalis* and they postulated that plasmids could cause its overexpression. This information is pertinent in the future directions of enterococcal AMR HGT research.

T3 was the only *Enterococcus* to demonstrate a reduction in general MIC as compared to the parental strains. MIC to kanamycin and erythromycin was halved. Similarly, Gevers *et al*. (2003) demonstrated that when *E. faecalis* JH2-2 transconjugants were created from *tetM* resistant *Lactobacillus,* MIC's to tetracycline were lower. No explanation was provided in Gevers *et al.* (2003) however an explanation for this reduction could be the introduction of altered machinery components encoded on the plasmid which could cause a reduction in efflux function. An inverse reaction to what was observed by Hürlimann *et al*. (2016).

Lee *et al.* (2007) determined that when CMY-2 and DHA-1 beta lactamases were conjugally transferred between *Klebsiella pneumoniae* clinical isolates, OmpK35/36 porins greatly diminished (loss). This caused uptake limited reductions to the MIC of several antibiotics including imipenem. Doménech-Sánchez *et al.* (2003) determined that modification of porins can reduce MICs to aminoglycosides, such as chloramphenicol. Porins do not feature in enterococci, however antibiotic uptake rates can influence the MIC of aminoglycosides. Aslangul *et al.* (2006) determined that alterations to gentamycin MIC was directly based on drug uptake impairment caused by variations in the affinity of the *N,N′*-dicyclohexylcarbodiimide receptor responsible for transportation of the

cationic aminoglycosides. Direct evidence for such observations has not yet been determined, however deductions related to drug uptake mutations are entirely plausible. Transfer efficiencies reported in table 4.4.1 coincide with those observed in the literature. Transfer of vancomycin and/or erythromycin resistance was observed with a frequency of 10^{-1}-10^{-3} obtained for donors isolated from poultry litter. In our hands the transfer frequency for tetracycline was lower (10^{-4}-10^{-5}). However, Vignaroli *et al*. (2011) showed frequencies of 10^{-6}-10^{-9}, where transfer occurred from donor strains isolated from animal faeces to a human *E. faecium* strain.

4.5.5. Transfer of AMR genes in enterococcal transconjugants

As there were strong observed phenotypic transfers of AMR amongst the transconjugants, PCR investigations into gene transfer events were carried out with primers listed in table 2.2. Figure 4.4.7 shows the transfer of the *vanA* gene into T1 and T4 from MF06036 and MF06035. MW01105^{Rif} tested negative for *vanA*. The same reaction occurred for the transfer of *ermB* the erythromycin determinant gene as seen in Figure 4.4.8. Co-transfer of *vanA* and *ermB* has been shown before from pig isolates to human isolates (Vignaroli *et al.*, 2011). Tetracycline gene PCR testing was time consuming and required significant optimisation (as described in the general materials and methods), however *tetL* was present in T2 and its donor MF04010, and *tetM* was present in T2, T3 and their respective donors. All other tetracycline primers did not amplify tetracycline genes in the tested enterococci. However, the identification of *tetL* and *tetM* was indicative of the action of mobile transposons and has frequently been observed in the literature. Moubareck *et al*. (2003) described the movement of vancomycin, erythromycin and tetracycline genes from food animal origins to human gut commensals. Silveira *et al.* (2013) determined cross transfer of similar genes between enterococci from animal, human, the natural environment and food origins. Examples such as these highlights how *ex vivo* reservoirs

of enterococci can potentially cross propagate with isolates with the ability to cross the GI barrier into the bloodstream causing peripheral infection. Table 4.4.3 is a summary of the gene transfer events identified in the four transconjugants.

4.5.6. Stability of acquired AMR phenotype in the transconjugants

To assess if the transferred AMR phenotypes demonstrated in the transconjugants were stable, a continuous propagation and selection assay was carried out for 25 days (figure 4.4.11). If AMR phenotype was not stable, there would be a gradual reduction in CFU of transconjugant propagated in TSA and counted in selection (red lines). T1 and T4 were tested in vancomycin, T2 and T3 were tested in tetracycline with no significant difference amongst any growth condition. All transconjugants retained their acquired AMR, suggesting that the conjugal MGE's studied here also code for maintenance and highly conserved replication systems (Werner *et al.*, 2013) which supports their stability in transformed cells and prevents benign progeny from succeeding. In chapter 3 Alkaline lysis experimentation yielded inconsistent results, and although phenotypic data was acquired that strongly indicated plasmid/MGE transfer, another approach can be considered for future investigations. Whole genome sequencing of target isolates and transconjugants would provide quantifiable information on the genotype of any MGE's within. This is a chap and affordable solution to the issues of the methodology reported here.

4.5.7. Variations in temperature and nutrient deprivation reduce conjugal efficiency

In this study, conjugation of enterococcal AMR genes has been demonstrated in several isolates; as well as inter-species transfer. This novel interaction was accomplished using environmentally isolated individuals. In keeping with the environmental conditions from which the enterococci were isolated, an experiment was carried out to analyse whether

enterococci can continue to conjugate under sub-optimal temperatures and lack of nutrients. Both nutrient deprivation and reductions in temperature from the optimal 37ºC will lower bacterial metabolic activity (Rivkin and Legendre, 2001). Retention of MGE's and HGT is undoubtedly metabolically intense (Glick, 1995). Conjugation reactions that would create T1 were carried out at temperatures detailed in table 4.4.4. The optimal temperature of 37ºC; the maximum temperature that freshwater tributaries' reaches in the height of summer 20ºC; and 4ºC, which accounts for median lowest water temperature for over 75% of the year (unpublished data).

Conjugation occurred at 20ºC, and at 4ºC. In fact, recovery of transconjugants at 4ºC ($x10^{-5}$) is statistically similar to recovery rates observed in the literature for standard *in vitro* conjugation at 37ºC ($x10^{-3\ to\ -7}$) (Andrup and Anderson, 1999; Cook *et al.,* 2011). The fact that enterococci still retain enough metabolic activity to carry out HGT at temperatures that should significantly reduce their biological functionality, and under nutrient starvation reveals the potency of the mechanism.

4.5.8. Possible interactions of enterococci with other organisms

Environmentally isolated enterococci have demonstrated HGT, even in unfavourable conditions. These characteristics help fulfil the criteria to propagate AMR in the environment. It is well understood that enterococci possess virulence genes coding for the attachment to type IV collagen, as well as genes coding for gelatinase (Daniels, 2011). These virulence genes allow enterococci to bind to and ultimately colonise animal tissue, such as an endocarditis vegetation. When enterococci are introduced to the bloodstream of a human, they can bind to the endothelium of damaged heart valves using collagen binding proteins and form a vegetation through encasement in a matrix of fibrin as part of the damage response of the endothelium (McCormick *et al.,* 2002). These vegetations

significantly increase the risk of mortality through septicaemia and cardiac arrest (Wilson *et al.,* 1984; Maki and Agger, 1988).

Fresh water sponge *Spongilla lacustris*, are composed of collagenous matrix called the mesohyl (Krasko *et al.,* 2000; Exposito *et al.,* 2002). They exist ubiquitously across many waterways in Western Europe, including the same sites that the enterococci in this study were isolated (Manconi and Pronzato, 2008).

Therefore, a simple attachment assay was performed (figure 4.4.12) to establish if enterococci bind *S. lacustris.* The results of this assay revealed that MF06036 easily bound *S. lacustris*. Combining this result with evidence from Gevers *et al*. (2003) which suggested sponge-like membranes (cellulose esters filter) with a 0.45µm pore size improves conjugation efficiency, revealed novel opportunities to try and perform *Enterococcus* conjugation reactions on the sponge. They noted that filter mating using a 0.2µm filter produced zero transconjugants using *E. faecalis* JH2-2 as a recipient and 14 (*tetM*) *Lactobacillus* isolates. Using the sponge like membrane they achieved success (50% success rate at a frequency of $10^{-4 \text{ to } -6}$). In our study, replicating environmental conditions would ensure any results can be extrapolated to occur in the agrarian aquatic ecosystem.

4.5.9. Enterococcal conjugal interactions using sponge as a substrate

It was immediately noticeable that enterococci concentrate on sponge (figure 4.4.12) which explains the higher number of transconjugants recovered from *S. lacustris* and then *Ephydatia fluviatilis* as observed in figure 4.4.14. When applying the protocol to sponge negative tubes, conjugation does occur, but the frequency is significantly (P=0.001) lower than what is observed in positive tests (figure 4.4.14). This can be explained due to the lower concentration of enterococci, when diluted in 20mls of water without nutrients. These findings are similar to what was observed in table 4.4.4.

However, when freshwater sponge was introduced, not only is conjugation frequency increased, the majority of transconjugants were isolated from homogenised sponge tissue. The concentration of transconjugants inside sponge demonstrated here coincide with the success attained by Gevers *et al*. (2003).

In order to examine the entire sponge matrix, a FISH protocol was derived from protocols used to examine sponge conjugation and optimised (figure 4.4.15). All transconjugants were located in the white sponge mesohyl as seen in figure 4.4.15 (a). The FISH protocol was so successful at identifying enterococci, that individual sponges were simply subjected to the FISH protocol and whole mounted on a glass coverslip and imaged with a simple epifluorescent microscope. The signal was significantly improved when Sudan black B was added 4.4.15 (e). The protocol devised in in figure 4.4.15 allowed for rapid speciation of any bacteria of choice in a complex eukaryotic organism, unveiling prospects of extensive research opportunities for bacterial interactions located *in situ* and *in vivo*.

When whole sponge sections were examined with FISH, *E. faecalis* was found throughout the sponge mesohyl as seen in figure 4.4.16. Although enterococcal distribution appears sporadic, distinct groupings of enterococci were observed, indicating possible conjugation sites. This study was not able to identify at this stage whether conjugation occurs by sponge filtration of bacterial water suspension, or by attachment of enterococci directly to the mesohyl. Most likely this is a consequence of the sponges' filter feeding activity. Freshwater sponges pump large water volumes through their canal system while stripping them of particulate matter, which inevitably concentrates bacteria and other waterborne particles in close physical proximity to each other (Ostroumov, 2005). However, a physical concentration effect of the filtration process may not be the sole explanation for the observed distribution of *E. faecalis* in the sponge mesohyl. Many

enterococci can produce gelatinase, which may be a reason for their high pathogenic potential with regard to endocarditis (Thurlow *et al.,* 2010). This enzyme may also facilitate entry into the sponge tissue and may even enable them to feed on collagen, which is an important component of the mesohyl, particularly in new tissue (gemmules) (Alexander *et al.,* 2015).

The tight grouping observed by FISH could indicate a sponge based substrate for conjugation to occur. Irrespective of the method of enterococcal conjugation that occurs here, what is known is that these naturally isolated sponge hatchlings, concentrate *E. faecalis* that have had vancomycin resistance transferred to them within a closed sterilised system. Un-inoculated sponges were also homogenised and plated on TSA as a control of the sponge pre-sterilisation process. These plates were persistently free of bacteria with an occasional fungal growth. Studies in the literature could not be found for bacterial conjugation in sponges, or other filter feeding organisms. Therefore, the work presented here is completely novel.

This novel process demonstrates for the first time how *E. faecalis* can survive in agrarian waterways, conjugate their resistance genes to other bacteria and propagate prolific resistance upwards to bacterial pathogens of humans. The sponge experiments can be seen as an analogy to such comparative studies, in that the sponge represents the filter medium and the tube wall represents the other solid surface. Similarly, to an artificial filter medium in the aforementioned assays, at the end of the experiment the sponge tissue contains a much higher number of transconjugant cells than the tube walls as the other solid surface.

Conjugative horizontal gene transfer could also be enhanced as a stress response (Beaber *et al.,* 2004). The production of agents with inhibitory properties that could exert a sub lethal stress or selection on bacteria has been verified for many sponge species. Therefore, an apparent sponge-microbe association can be concluded to exist (Thomas *et al.,* 2010).

Antibiotics found in cell culture of *B. cereus* isolated from *Halichondria japonica*, a Japanese marine sponge produced potent antibacterial activity specifically to enterococci (Nagai *et al*., 2003). In the natural environment, these interactions between sponge and microbes could create a microenvironment, where conditions could be conducive to conjugation. The observed effect of a high number of transconjugants in the sponges has been very similar for both species in the test. Hence it can be quite safely assumed that it may also apply to other species of freshwater sponges. Whether the potential of aquatic filter feeders to facilitate AMR transfer also extends to other taxonomic groups, requires further investigation. Considering the enormous scale of filter feeding activity in many lakes and rivers and the transformative effect filter feeding can have at ecosystem level (Karatayev *et al.*, 1997), research into the impact of filter feeding organisms is bound to provide valuable insights in the scale of AMR transfers in aquatic systems.

Chapter 5

Enterococci produce biofilm that supports the inter and intraspecies transfer of antibiotic resistance traits

5.1. INTRODUCTION

5.1.1. Biofilm: an introduction

Biofilm is the nomenclature given to the physical orientation and localisation of bacteria from the planktonic phase to solid phase on a substrate (McLean *et al.,* 2010). Once this change in phase is initiated, development both through cellular division and the introduction of new individuals generally occurs, until a multispecies community has formed (Rickard *et al.,* 2003). As bacteria are generally found on solid surfaces it is accepted that the biofilm state is common for all bacteria; especially when analysing human infection (enterococcal endocarditis and UTI's) where binding usually occurs to human tissues (Mah and O'Toole, 2001). It has been theorised that bacterial biofilm communities exist as a complex microcosm capable of higher order processes, compared to observed planktonic prokaryotic processes (Davies *et al.,* 1998). Discovered process such as the *N*-acyl-L-homoserine lactone signalling systems in Gram negative bacteria, are responsible for conjugation and virulence gene expression inside biofilm (Taga and Bassler, 2003). Analogous quorum sensing pathways also exist to control virulence gene expression in enterococcal biofilm (Qin *et al.,* 2000). Biofilm matrices and their cellular components have a multifaceted structure comprising selective organisation of individuals (Dunny *et al.,* 2014). Cells congregate around an inter-branched extracellular milieu in order to facilitate a quorum based coordination of expression characteristics and metabolic processes (O'Toole *et al.,* 2000). Biofilm models of 'development' and biological function were initially based upon research carried out on *Pseudomonas aeruginosa* and *P. fluorescens,* (Monds and O'Toole, 2009). With regards to enterococci, models of development are not fully understood, and an understanding of biological functions is just beginning to emerge (Gilmore *et al.,* 2014).

5.1.2. Enterococcal biofilm: Mechanism of infection

The first reports of enterococcal biofilm associated infection came in 1986, from patients who were suffering with infections sourced from vascular access ports containing mature single and multispecies biofilms, including *E. faecalis* (Reed *et al.,* 1986). Then in 1990, enterococcal high molecular weight, surface exposed protein expression levels were discovered to increase when enterococcal biofilms were grown on silicone substrates (Lambert *et al.,* 1990). This led the way for the identification of *E. faecalis* as a component of catheter biofilm, and as such raised awareness of persistent enterococcal infection. Since then efforts have been made to effectively create substrates non-supportive for biofilm formation, for use with human implants and usage of medical equipment/devices over extended periods of time (Raad *et al.,* 1995). To this day *Enterococcus* infections can still translocate to bacteraemia from biofilm associated catheter infections, even with effective antimicrobial treatments vancomycin, telavancin in combination with heparin (Luther *et al*., 2016).

It was shown that 84% of all tested *E. faecalis* isolates from human origins had the ability to form biofilm, whereas only 15% of human *E. faecium* isolates could produce biofilm (Baldassarri *et al.,* 2001; Duprè *et al.,* 2003; Seno *et al.,* 2005; Prakash *et al.,* 2005; Baldassarri *et al.,* 2006). Opportunistic infections associated with endocarditis disease models and persistent catheter infections (which can translocate and cause septicaemia), had a greater biofilm formation capability than commensal counterparts (Dworniczek *et al.,* 2005). Seno *et al.* (2005) were the first group to conclude that biofilm production in enterococci could be graded into weak, intermediary or strong, based on biomass.

It is well known that bacterial biofilm makes up a significant portion of tooth plaque, and *E. faecalis* makes up the majority of all species of enterococci isolated from dental

infection (Dahlén *et al.*, 2000). Interestingly, studies have produced conflicting results on whether enterococci isolated from different human isolation sites form strong or weak biofilm as a result of biofilm formation assay choice. Duggan and Sedgley, (2007) using the polystyrene 96-well biofilm formation assay concluded, that oral enterococci don't produce biofilm using the threshold set by Mohammed *et al.* (2004), where OD^{570nm} greater than 0.5 are considered biofilm producers. However, when comparing to the Sandoe *et al*. (2003) method (where OD^{570} greater than 0.0 are considered biofilm producers) concluded that all their tested isolates were biofilm producers. Indications that methodology choice is responsible for variation in the literature rather than strain difference between enterococci is apparent when comparing the biofilm producing assessment criteria of Sandoe *et al*. (2003) and Mohammed *et al*. (2004). Additionally, studies that use substrate coated with human components have been shown to form strong biofilms with human associated enterococci. George and Kishen, (2008) demonstrated that substrate modification with human saliva on glass and using single rooted anterior teeth yielded strong biofilm formation for oral *E. faecalis* compared to the results from Duggan and Sedgley, (2007).

5.1.3. Cell communication and conjugation potential

Cook *et al.* (2011) described for the first-time, plasmid transfer inside enterococcal biofilm, where sex pheromone signalling recruited horizontal gene transfer between well characterised human isolates. Additionally, they demonstrated increases in enterococcal plasmid copy numbers from a maximum of five copies per planktonic cell versus a maximum of 15 copies per biofilm cell, something indicative of increased conjugation competency. This form of conjugation relies on signalling peptides in the same fashion as the two component *fsr* quorum response for biofilm formation, development and virulence (Hancock and Perego, 2004; Chen *et al.*, 2017). Herein lies the potential to

conclude novel cell to cell signalling interactions unique to the biofilm environment of enterococci.

5.1.4. Enterococcal biofilm extracellular matrix

The extracellular matrix of enterococcal biofilm is composed of polymeric substances such as polysaccharides, proteins and extracellular DNA (eDNA) (Erlandsen *et al.*, 2004). The importance of eDNA in initial formation of enterococcal biofilm has been tested and shown to be a regulator of early set down, but not as influential on biofilms close to maturity (Teng *et al.,* 2009). Evidence has surfaced likening the function of polysaccharides to eDNA, supporting the structure of the extracellular matrix (Barnes *et al.,* 2012). The polysaccharide gene *epa* has been shown to function to supplement biofilm formation through mediated synthesis of cell surface polysaccharides (Teng *et al.*, 2002). The observed sugar dependant fluctuations in *Enterococcus* biofilm forming ability can be partially explained by experimentation in the *bopA-D* (biofilm on plastic) genes first carried out by Hufnagel *et al*. (2004). Using plasmid pTV1-OK, a chromosomal transposon mutation was created with a single insertion in the *bopB* open reading frame (ORF). This insertion caused an 84% reduction in biofilm production in in a strong biofilm producing *E. faecalis*. A triple deletion mutant was created by deleting the 3' end of *bopA*, the 5' end of *bopC* and all *bopB* ORFs. Leaving only *bopD,* a putative sugar binding transcriptional regulator with high similarity to maltose metabolism proteins. Increased presence of *bopD* mRNA was attributed to increased biofilm formation in the transposon mutant with the triple deletion of *bop* genes. Hufngel *et al*. (2004) concluded that the functional characteristics of the *bopD* protein could be used to explain sugar dependant variation in biofilm formation.

Biochemical characterisation of enterococcal biofilm has been partially accomplished by analysing water and alkaline soluble polysaccharide compositions of biofilm extracellular

matrix, in alkaline and starvation conditions (typically observed in human oral cavities) (Chen *et al.,* 2017). This study revealed reductions in matrix water soluble polysaccharides under these stresses. However, studies on the composition of extracellular matrix are still lacking (Gilmore *et al.,* 2014).

The lectin, concanavalin A, binds to α-mannopyranosyl and α-glucopyranosyl residues of carbohydrates (McSwain *et al.,* 2005). Enterococcal cell wall components feature lipoteichoic acids with kojibiose containing an α-D glucopyranosyl residue (Hogendorf *et al.,* 2010). When concanavalin A is coupled with a fluorescent stain such as Alexa Fluor 594, polysaccharides contained on the cell surface of enterococci as well as extracellular polysaccharide components may be visualised. Therefore, this simple system can be used as an enterococcal biofilm stain.

5.1.5. Contradictions within enterococcal biofilm literature

Whilst biofilm and its formation is a well investigated topic, enterococcal-specific biofilm characteristics and formation are less understood (Barnes *et al.,* 2012). There are less than two dozen reports in the literature relating to the understanding of enterococcal biofilm characteristics. Studies tend to focus on interventions to prevent or destroy enterococcal biofilm. Due to the low numbers of publications interspaced by years, there are no universally accepted standard methods for analysing biofilm in enterococci. Variations in reports of biofilm formation and characteristics for enterococci are reported, even as far as standard biofilm formation media, which varies greatly from publication to publication as discussed in (Dunny *et al.,* 2014).

The consensus of literature reviews appears to be that biofilm formation capability is only a function of the specific characteristics of growth, substrate and biofilm promotors in the system (Fisher and Phillips, 2009; Gilmore *et al.,* 2014). There are also distinctive biofilm formation variations based on static or laminar flow growth conditions (Garrett *et al.,*

2008). Optimisation of biofilm biomass using these characteristics may have a negative impact on the functionality of bacteria in the biofilm state, as a model of *in vivo* persistent antibiotic resistant infection (Nguyen *et al.,* 2011). There is a growing consensus that bacteria modulate their produced biofilm to adapt to changing conditions of stress; rather than producing biofilm in large quantities as a function of their pathogenicity or supplemental growth conditions (Garrett *et al.*, 2008; Stewart *et al.*, 2015).

Biofilm studies are assay dependent. Whether it's a measure of biofilm formation based on cell counting alone, the ratio of cells to biomass, or dry biomass alone (Hufnagel *et al.*, 2004; Kristich *et al.*, 2004; Rosa *et al.*, 2006; Creti *et al.,* 2006). There is a clear need for a consensus regarding standardisation of growth conditions for enterococci prior to testing clinically relevant hypothesises.

There exists a drive for insight on the workings of enterococcal biofilm, as the environment is conducive for exchange of information, especially when coupled to the knowledge of intercellular signalling pathways such as the *fsr* and Acyl-homoserine lactone systems (Parsek and Greenberg, 2000; McDougald *et al.*, 2012). It may be that horizontal gene transfer within a biofilm is inefficient as compared to laboratory methodologies (Cook *et al.,* 2011). However, any level of horizontal gene transfer that spreads antibiotic resistance is significant. Understanding these processes will unlock the opportunity for a calculated approach, dealing with increasingly resistant opportunistic infections through effective treatment and preventative strategies.

It is clear that, in order to make relevant comparisons to clinical pathogenesis, laboratory biofilm assay assays need to reflect the conditions that enterococci are exposed to during infection. Such parameters would include nutrient content, substrate composition and mechanical/chemical stress (Van Wamel *et al.,* 2007; Mohamed *et al.,* 2007). Biofilm

assays that work on Gram negative, flagellated *P. aeruginosa*, which binds to most abiotic surfaces, are likely to be inefficient when used in conjunction with Gram positive, non-flagellated *E. faecalis* which binds to biotic surfaces (O'Toole and Kolter, 1998; O'Toole *et al.*, 2000).

5.2.AIM AND OBJECTIVES

The primary aim of this chapter was to determine that *E. faecalis* and *E. faecium* of environmental origin can produce biofilm that supports the transfer of antibiotic resistant traits among themselves.

Objectives:

- Enterococci were assessed on their ability to form optimal biofilm when subjected to favourable growth conditions and substrate
- Substrate composition was tested against the ability of enterococci to form biofilm
- Components such as DNA and cell lysate were tested during enterococcal biofilm formation assays to assess any modulations biofilm formation
- Stress conditions such as cellular starvation was applied to enterococcal biofilm formation studies.
- Using customised florescence microscopy assays, antibiotic resistance genes were examined inside enterococcal biofilm
- Visualisation of enterococcal conjugation inside biofilm

5.3 MATERIALS AND METHODS

All the materials and methods carried out in this chapter are detailed in chapter two.

5.4 RESULTS

This chapter demonstrates that enterococcal biofilm can be a fickle substance, verified by the inconsistent results obtained with established biofilm assays (figure 5.4.2). Polystyrene materials inversely affect biofilm formation proficiencies of enterococci (figure 5.4.3 and 5.4.4). Traditional staining and optical density assays yielded variation and miscategorising of specific enterococcal biofilm forming capabilities (5.4.2, 5.4.3 and 5.4.4). Utilising inert glass surfaces, substrate improvements and biofilm indices, accurate representation of enterococcal biofilm forming ability was achieved (5.4.4). Even with biofilm formation optimised, established assays heavily damaged macro-structures and often removed significant quantities of material (5.4.2). Therefore, a new assay was created to address issues associated with fragile biofilm (5.4.7 and 5.4.8).

The gene frame biofilm apparatus (GBA) allowed for a less intrusive examination of enterococcal biofilm, revealing novel characteristics (5.4.9). Due to the *in situ* nature of the gene frame biofilm on microscope glass, initial set down and early biofilm formation of enterococcal biofilm was captured for the first time microscopically (5.4.10). The biofilm stain ConA was used to identify initial biofilm producing cells in early biofilm development and highlight the biofilm scaffold during biofilm maturation. ConA was tested as a visual aide to identify biofilm biomass amongst enterococci with success. Nutrient deprivation as well as antimicrobial stress was shown to have dramatic effects on the macrostructure, scaffold and cellular distribution of bacteria within enterococcal biofilm (5.4.11, 5.4.12 and 5.4.13). Fluorescent *in situ* hybridisation assays were created to identify enterococci inside biofilms (5.4.14). This assay was used to examine mobile genetic element bound genes such as *vanA* vancomycin resistance gene for the first time inside intact biofilm (5.4.15). Finally, the gene frame biofilm model was adapted to visualise and capture conjugation within biofilm amongst environmentally isolated enterococci for the first time (5.4.16-5.4.20).

5.4.1. Enterococcal growth characteristics in standard and nutrient depleted growth media

The first steps in developing enterococcal biofilm were to determine the specific growth characteristics of each isolate that produced transconjugants in chapter 2 (MF06036, MF06035 MF04010, ST01109^{Rif} and MW01105^{Rif}). Isolates were grown in various media and their absorbance at 600nm was read in a spectrophotometer at the indicated time points (figure 5.4.1). Growth in Tryptone Soy Broth revealed that all isolates grew in a similar fashion both at 37^{o}C and 20^{o}C. At the higher temperature, log phase was achieved after the first hour and growth reached stationary phase at six hours, decline started at 48 hours. At the lower temperature, the discrepancies between the growth rates of each isolate were more apparent. A slow steady log phase over the first 24 hours was apparent, and as such, stationary phase was much more stable over the time course of the experiment.

Starvation growth conditions in spent TSB revealed an increase in average optical density amongst the isolates from 0.003 at 0 hours to 0.02 at 48 hours at 37^{o}C indicating residual growth. The overall lack of growth for isolates in starvation was expected. Isolates grown at 37^{o}C were all consistently similar whereas isolates grown at 20^{o}C were more varied. By eight hours all isolates at the higher temperature were in stationary phase and the same occurred at 48 hours for isolates at the lower temperature.

With the growth curves calculated for the conjugation partners, biofilm formation capabilities could be tested in systems that could be used for microscopic visualisation of conjugation.

Figure 5.4.1. Enterococcal growth characteristics in broth.

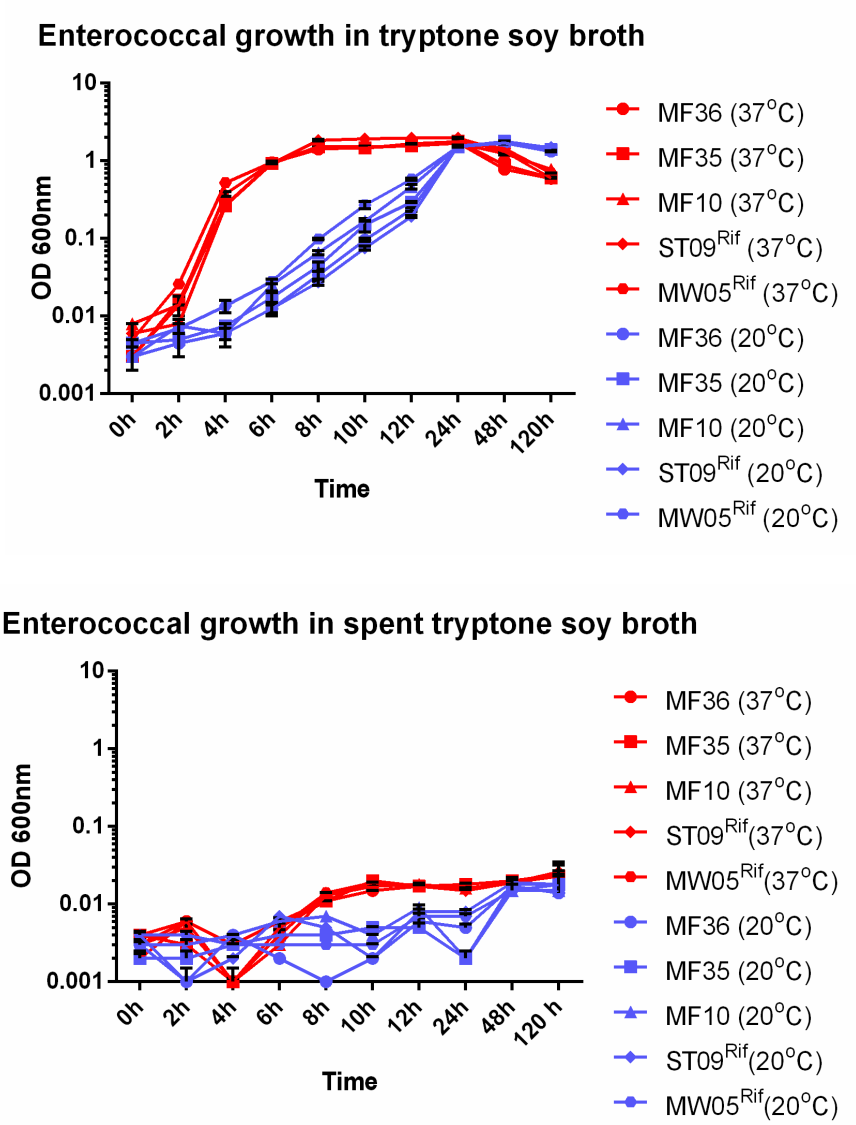


Figure 5.4.1. Enterococcal growth curves represented as logarithmic graphs showing absorbance at 600nm for the indicated time periods, incubated statically up to 120 hours. Optical density correlates to relative turbidity of each well. Isolates were grown in both 37°C (red) and 20°C (blue) in Tryptone Soy Broth (above) and spent Tryptone Broth (below). MF36 - MF06036, MF35 - MF06035, MF10 - MF04010, ST09^{Rif} - ST01109^{Rif} and MW05^{Rif} – MW01105^{Rif}. Error bars represent standard error of the mean. Experiment repeated three times. Using analysis of variance one-way, p values for 37°C versus 20°C were <0.0001 for both graphs.

5.4.2. Traditional (standard) biofilm formation assays applied to enterococci

Once growth characteristics were established, biofilm formation capabilities of the isolates used in the previous conjugation assays (as well as a positive and negative biofilm control) were tested with various established assays.

The crystal violet microplate assay was carried out for 48 hours at 37ºC statically in polystyrene microplates. This technique applied to the isolates confirmed the biofilm strength of the negative and positive control isolates (MF01028 and MW02102) (figure 5.4.2(a). The conjugation recipient MW01105^{Rif} was established as a biofilm non-producer, well below the margin of weak biofilm formation capability. This was in contrast with the biofilm producing result of MW01105^{Rif} in figure 3.4.2. ST01109^{Rif} and MF04010 presented as strong biofilm producers. Additionally, MF06035 and MF06036 presented as weak biofilm producers similarly to what was shown in the initial biofilm formation assay (figure 3.4.2).

The tube formation assay was carried out for 48 hours grown at 37ºC for isolates MW01105^{Rif}, ST01109^{Rif}, MF04010, MF06035, and MF06036 (figure 5.4.2(b)). Tubes 1, 3 and 4 are indistinguishable from each other, tube 2 is marginally more stained with crystal violet and tube 5 displays the greatest crystal violet staining. These results display no correlation to what was observed in the crystal violet assay. MW01105^{Rif} and MF04010 appear with the same staining intensity in the tube formation assay but are described as non-biofilm producing and strong biofilm producers respectively in the microplate assay.

Microscopic visualisation techniques were employed on MF04010, the strongest biofilm producer determined with the crystal violet absorbance assay. The air liquid interface assay investigated the growth of enterococci on a glass coverslip at an interface of two media for 48 hours, and incubated statically at 37ºC (figure 5.4.2(c)). Whilst the isolates grew on the glass coverslip interface, the formation characteristics were weak, (visible

removal of biofilm during washing steps) and when imaging coverslips, a degree of variation was observed between replicates. Variations include identifiable changes to biomass on the coverslip between replicates (observed by eye). Biofilms were imaged both dry and 'wet' in mounting medium. Dry biofilms displayed small quantities micro-aggregates typical of early biofilm formation in the first few hours. Figure 5.4.2(d): little biofilm was detected using this assay. The submerged coverslip in a microplate incubated for 48 hours statically at 37^{o}C produced the clearest results, when stained with crystal violet and imaged using phase contrast. The clear boundary between the internal and external biofilm structure is apparent with the majority of micro colonies contained within the biofilm boundary. Cells appear to concentrate at the leading edge of the biofilm. This assay suffered the same variation in depicting biofilm biomass between replicates (data not shown) as the coverslip assay.

It was clear that the enterococci were not adhering sufficiently to the substrates employed with the standardised assays for biofilm formation. Therefore, steps were taken to improve adhesion and subsequent biofilm formation.

Figure 5.4.2. Traditional biofilm assays applied to enterococci yield inconsistent biofilm.

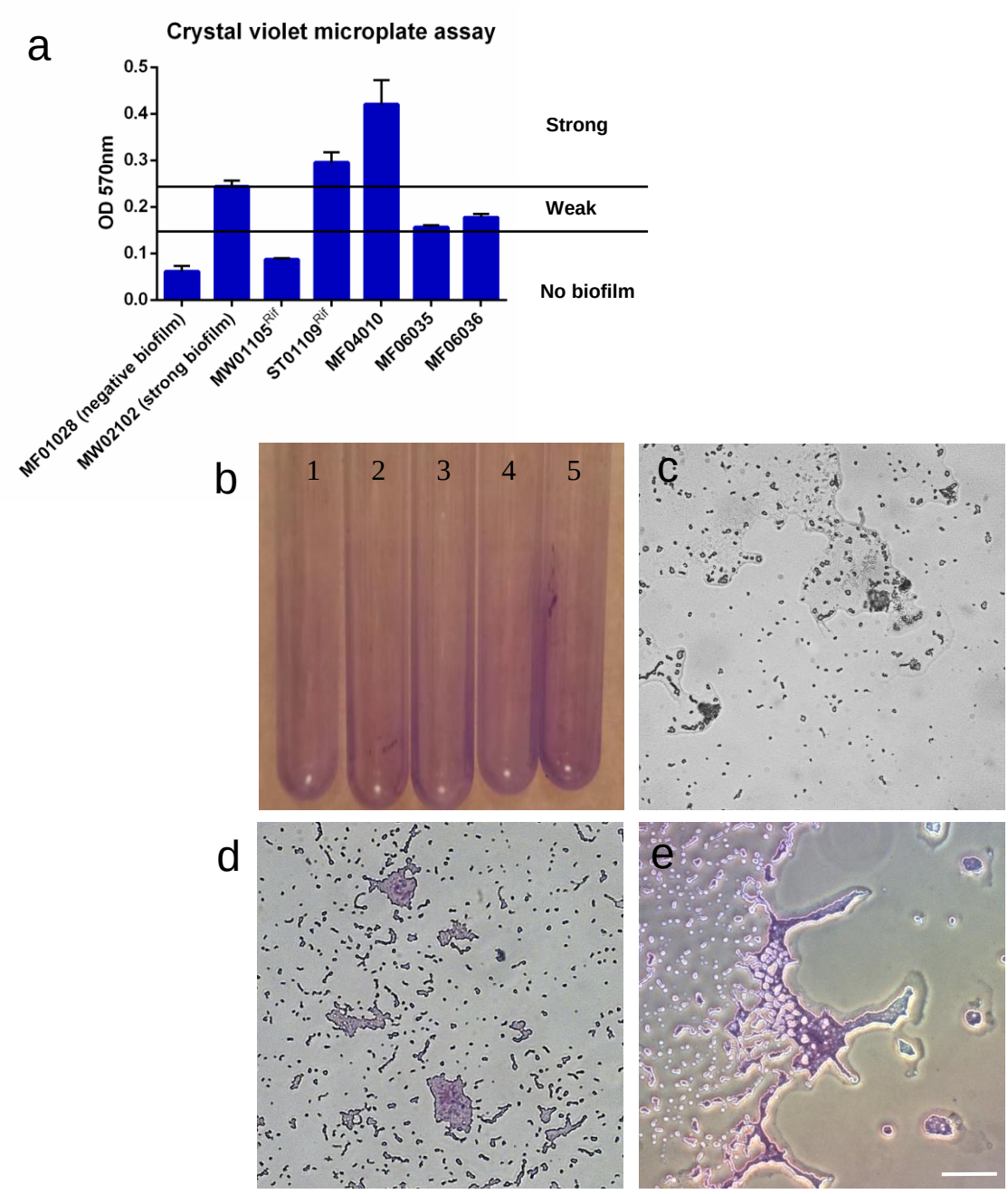


Figure 5.4.2. Data depicting variations in enterococcal biofilm formation based on the assay applied using TSB (1% glucose). (a) A crystal violet microplate assay carried out for 48 hours at 37ºC statically in polystyrene microplates with a known non-biofilm producing (MF01028) and strong producing (MW02102) control. (b) The tube formation assay was carried out for 48 hours at 37ºC and stained with crystal violet (1% for 15 minutes) for isolates MW01105^{Rif} (1), ST01109^{Rif} (2), MF04010 (3), MF06035 (4), MF06036 (5). (c) A 40x magnification micrograph of the air liquid interface assay applied to MF04010 on a glass coverslip at an interface of two mediums for 48 hours, incubated statically at 37ºC. (d) A 40x magnification micrograph of the air liquid interface biofilm assay carried out on MF04010 stained with crystal violet (1% 15 minutes). (e) A 40x magnification micrograph of the submerged coverslip assay in a six-well microplate incubated with MF04010 for 48 hours statically at 37ºC stained with crystal violet (1% 15 minutes) and imaged using phase contrast under wet conditions. Scale bar represents 50 microns across all three micrographs. MF04010 significantly different from other isolates ($p<0.0001$). Experiments have eight biological repeats, independently repeated three times.

5.4.3. Improvement of enterococcal biofilm formation on standard biofilm apparatuses using substrate modifications

The issues with established biofilm retention on substrate (especially glass) were assessed with the addition of collagen and gelatin surface coating. MW01105^{Rif}, MF04010 and MF06036 were selected as representatives of strong, weak and non-biofilm producing isolates. They were grown normally on collagen and gelatin coated microplates for 24 hours, stained with safranin, examined visually and with absorbance readings (figure 5.4.3(a)). Through examining the microplates, MW01105^{Rif} had comparable staining intensity between no coating and collagen coating, with reduced staining intensity for gelatin coating. MF04010 had strong staining across all three conditions, with a large central biomass on the uncoated well. An additional, large biomass was observed at the edge of each well. MF06036 had weak staining across all three conditions with no coating appearing to have stronger staining. Isolates displayed a degree of variation between replicates but were consistently distinguishable.

Absorbance readings of microplate assays (figure 5.4.3(b)) revealed that MW01105^{Rif} grown on a microplate with no coating produced strong biofilm, whilst MF04010 and MF06036 produced strong and weak biofilm respectively. This data displays inconsistencies for MW01105^{Rif} from figure 5.4.2, where the isolate was non-biofilm producing. Additionally, MW01105^{Rif} and MF04010 displayed marked reductions ($p < 0.0001$) in biofilm formation ability on coated substrate.

There were clear issues with the biofilm formation capabilities of the tested enterococcal isolates. Therefore, examination of biofilm production as a function of different substrate compositions was carried out (figure 5.4.4), normalising inter-isolate growth kinetics by using the biofilm index. This would allow comparison of biofilm production factoring in the growth rate of each isolate. This approach would provide a more accurate result of

biofilm formation capability over total biomass staining. Enterococcal isolates were incubated normally for 24 hours in polystyrene and glass bottomed microplate plates with no coating or coated with collagen or gelatin. The total cell count absorbance was measured at 600nm, wells were washed and stained with safranin and measured at 450nm. Biofilm index values were created by dividing the OD450/600 absorbance readings. Biofilm strength was measured as <0.1 weak, 0.1-1 moderate and >1 strong (Leuck *et al.*, 2014).

When examining the differences between biofilm formations on differing material substrates, stronger biofilms were produced on polystyrene for all isolates (figure 5.4.4(a)), excluding MF06036. Paired T-testing between glass and polystyrene indicated significant differences (p 0.0029). The effects of substrate material on biofilm were most significant with isolates MW01105^{Rif} and MF04010 with a 75% and 65% difference observed respectively. Standard deviation was 58% greater among the biofilms developed on polystyrene versus glass substrate biofilms. Biofilms were also grown on glass coated with either collagen or gelatin and compared to no coating controls (figure 5.4.4(b)). MF01028 had significant increases in biofilm formation when grown with substrate coatings of both gelatin and collagen (p <0.0001). It exhibited almost a doubling of biofilm formation projecting its abilities into the moderate biofilm formation category. MW02102 also had significant increases in biofilm formation, with substrate coating. Yielding over a threefold increase in biofilm formation ability on both gelatin and collagen (p <0.0001) projecting it well above the strong category of biofilm formation. MW01105^{Rif} had a significant (p <0.0001) increase in biofilm formation with gelatin coating projecting its formation capabilities into the strong biofilm formation category with a tripling effect.

The only remaining significant change in biofilm formation occurred with MF04010 gelatin coating, whereby a significant (p 0.0004) 23% increase in biofilm was determined. ST01109^{Rif} MF06035 and MF06036 saw no significant change in biofilm formation ability in the presence of different coatings. However, ST01109^{Rif} was the only isolate whereby introduction of coatings caused a decrease in biofilm production (statistically insignificant).

The data collected here identified that substrate composition had a direct effect to the ability of the tested enterococci to form biofilm. With generalised improvements from the inclusion of collagen or gelatin substrate, the effects of total cell lysate and eDNA (known factors in biofilm formation) were tested next.

Figure 5.4.3. Microplate surface coating to improve *Enterococcus* cellular adhesion and subsequent biofilm formation.

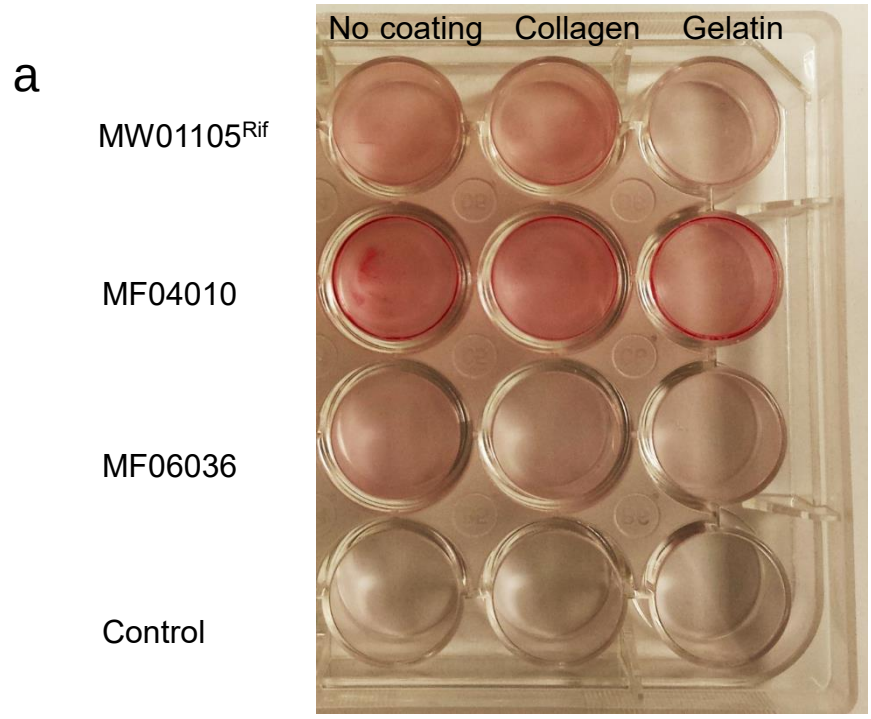


Figure 5.4.3. Addition of collagen and gelatin to microplates to improve biofilm formation. Standard biofilms were grown with a representative non-biofilm producing (MW01105^{Rif}), weak-producing (MF06036) and strong-producing (MF04010) *Enterococcus* on coated wells of a microplate, incubated at 37^{o}C statically under aerobic conditions. Wells were stained with safranin (0.1% 15 minutes) and examined visually (a) and with absorbance readings at an optical density of 450nm (b). Non-biofilm producing isolates had optical density readouts below 0.17, weak biofilm producers were between 0.17 and 0.23 and strong biofilm producers had optical densities higher than 0.23. Scale bars represent standard error of the mean. No coating was significantly different for MW01105^{Rif} ($p < 0.0001$) and MF04010 (p 0.0001) compared to collagen and gelatin. Experiments have eight biological repeats with three independent repeats.

Figure 5.4.4. Substrate composition directly affects enterococcal biofilm formation.

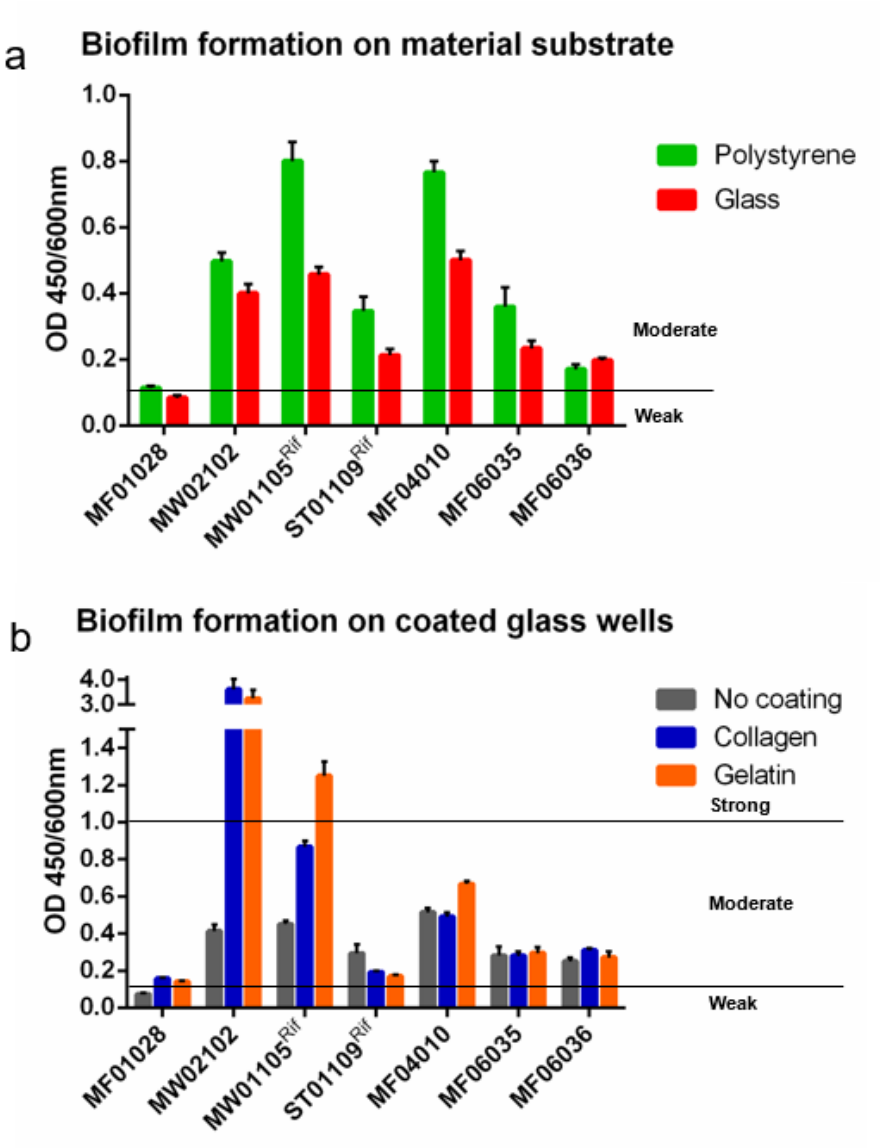


Figure 5.4.4. Effects of substrate composition on biofilm formation. Previously known non-biofilm producing (MF01028) and strong producing biofilm (MW02102) enterococcal isolates were incubated with the five conjugation partners in TSB (1% glucose) at 37ºC for 24 hours in (a) polystyrene and glass bottomed microplate plates as well as (b) no coating, collagen (10µg/cm^2 from 0.1% stock) or gelatin (10µl/cm^2 from 2% w/v stock). After incubation, absorbance was measured at 600nm, wells were washed and stained with safranin (0.1% 15 minutes) and measured at 450nm. Biofilm index values were created by dividing the OD 450/OD600. Biofilm strength was measured as <0.1 weak, 0.1-1 moderate and >1 strong. Error bars represent standard error of the mean. Polystyrene was significantly higher compared to glass across all isolates (p 0.04). Using two-way analysis of variance, no coating was significantly lower (p 0.001) compared to collagen and gelatin. Experiments were carried out with eight biological controls and four independat repeats

5.4.4. Assessing the effects of cell lysate and eDNA on the ability of enterococci to form biofilm

Extracellular DNA makes up part of the enterococcal biofilm matrix and is present in the initial set down of cells during biofilm formation. Additionally, fratricide is common in the biofilm environment. Therefore, total cell lysate (figure 5.4.5) and eDNA (figure 5.4.6) were added to enterococcal biofilms to assess any improvements in formation that would occur.

MW01105^{Rif}, MF06036 and T1 were selected as they were conjugation partners and the subsequent transconjugant from successful transfer of genetic material from chapter 4. From figure 5.4.5, MW01105^{Rif} was neither significantly affected by the lysate of the other isolates or had any affect with its lysate on the other isolates including self-testing. The lysate of MF06036 had a significant effect on both itself (p=0.0003) and T1 (p 0.0002) with increases in biofilm formation by 2.6-fold and 0.5-fold, respectively. The lysate from T1 had the most profound effect observed with almost a tripling of biofilm formation ability when added to MF06036 (p <0.0001). When its own lysate was added back it produced a significant increase (0.63 fold) in biofilm formation ability (p <0.0001).

When testing the effects of extracellular DNA added to biofilm media (figure 5.4.6), MF01028 had no change in its biofilm forming ability. MW02102 had significant decreases in its biofilm formation ability at all concentrations of DNA (p values of 0.0009 for 0.1% DNA, <0.0001 for 1% DNA and 0.0004 for 10% DNA, respectively). MW01105^{Rif} had insignificant increases in biofilm formation proficiency. ST01109^{Rif} had significant increases in biofilm proficiency by 3.2-fold at 0.1% (p 0.00019) and 1.8- fold at 10% eDNA (p <0.0001) but not for 1% eDNA. MF04010 gained the most proficiency in biofilm formation anility with the addition of 0.1% eDNA (p 0.002) producing a 2.3-fold increase and most significantly a 3.8-fold increase with 1% eDNA (p <0.0001) but

saw a slight decrease when 10% eDNA was added. MF06035 and MF06036 saw some insignificant fluctuation in biofilm formation ability.

The improvements offered by total cell lysate and eDNA were too inconsistent for a general adoption to the laboratory methodology for biofilm formation for the conjugation partner enterococci. Additionally, adoption of these parameters along with substrate improvements would make the methodology too complex for rapid, repeatable and reliable biofilm formation assays.

Figure 5.4.5. Modulation of the enterococcal biofilm: Effect of cell lysate on formation.

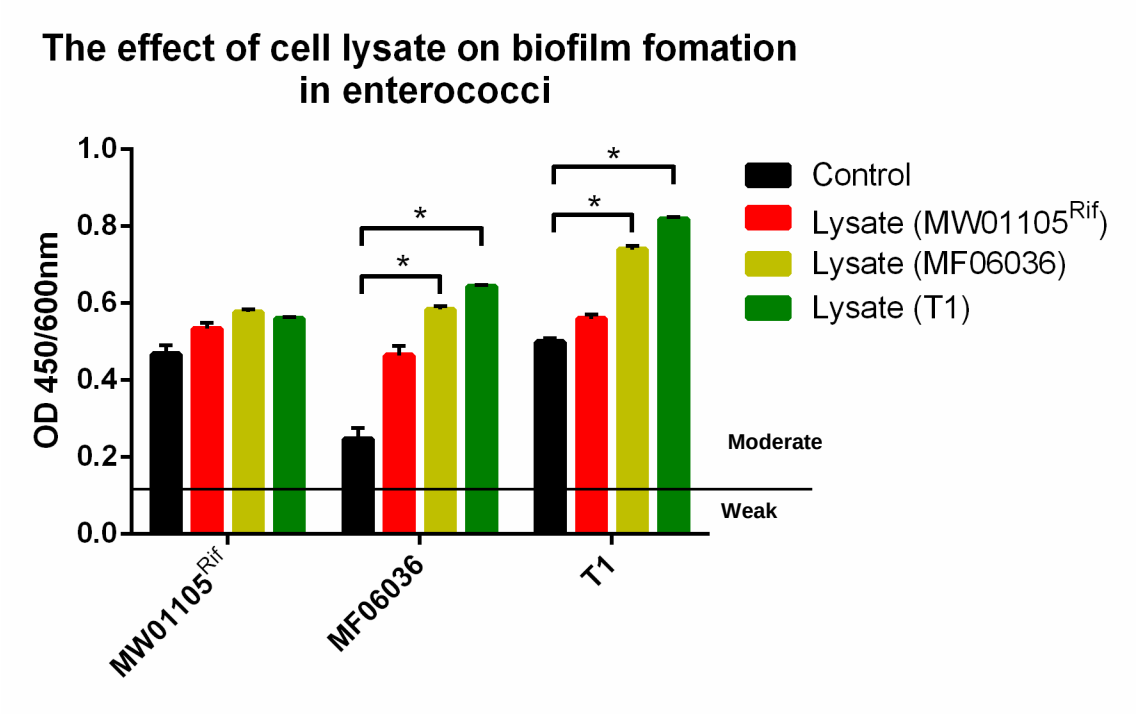


Figure 5.4.5. A graph showing the effect of total cell lysate from conjugal partners MW01105^{Rif}, MF06036 and their T1 on their subsequent biofilms. A one millilitre 16-hour overnight incubation of enterococci were lysed in SDS (1% final concentration for two minutes), washed (PBS) and centrifuged at max RCF for five minutes at room temperature and had the total cell content added back to fresh isolates at the start of a biofilm formation assay to assess the effect, total cell lysate would have on biofilm growth. Error bars represent standard error of the mean. MF06036 biofilm with MF06036 lysate was significantly stronger compared to control (p 0.0003). MF06036 biofilm with T1 lysate was significantly stronger compared to control ($p < 0.0001$). T1 biofilm with MF06036 lysate was significantly stronger compared to control (p 0.0002). T1 biofilm with T1 lysate was significantly stronger compared to control ($p < 0.0001$). Data representative of eight biological repeats, independently repeated three times.

Figure 5.4.6. Modulation of the enterococcal biofilm: Effect of eDNA on formation.

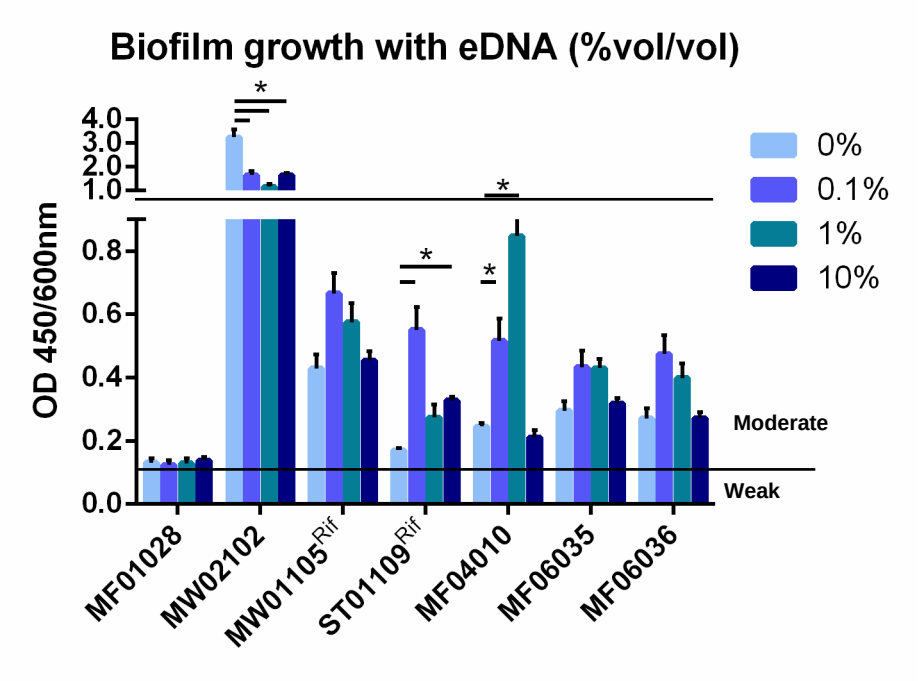


Figure 5.4.6. A graph showing the effect of eDNA on the subsequent biofilms of enterococci. Represented by a non-biofilm producing and a strong producing biofilm formation isolates and the conjugation partners. Extracted enterococcal DNA (stock concentration 20µg/ml) was applied to fresh enterococcal biofilm formation assays as a 0%, 0.1%, 1% or 10% by volume DNA diluted in TSB (1% glucose) media for 24 hours incubated statically at 37°C. Error bars represent standard error of the mean. MW02102 - 0.1% eDNA (p 0.0009), 1% eDNA (p <0.0001) and 10% DNA (p 0.0004). ST01109^{Rif} - 0.1% eDNA (p 0.00019) and 10% eDNA (p <0.0001). MF04010 - 0.1% eDNA (p 0.002) 1% eDNA (p <0.0001). Data representative of eight biological repeats, independently repeated three times.

With the biofilm formation inconsistencies rectified and the true nature of biofilm produced by environmental isolates of enterococci established, it was imperative to address the issue of unwanted biofilm destruction during microscopic visualisation processing discovered in figure 5.4.2. Therefore, the gene frame biofilm apparatus (GBA) was created (figure 5.4.7).

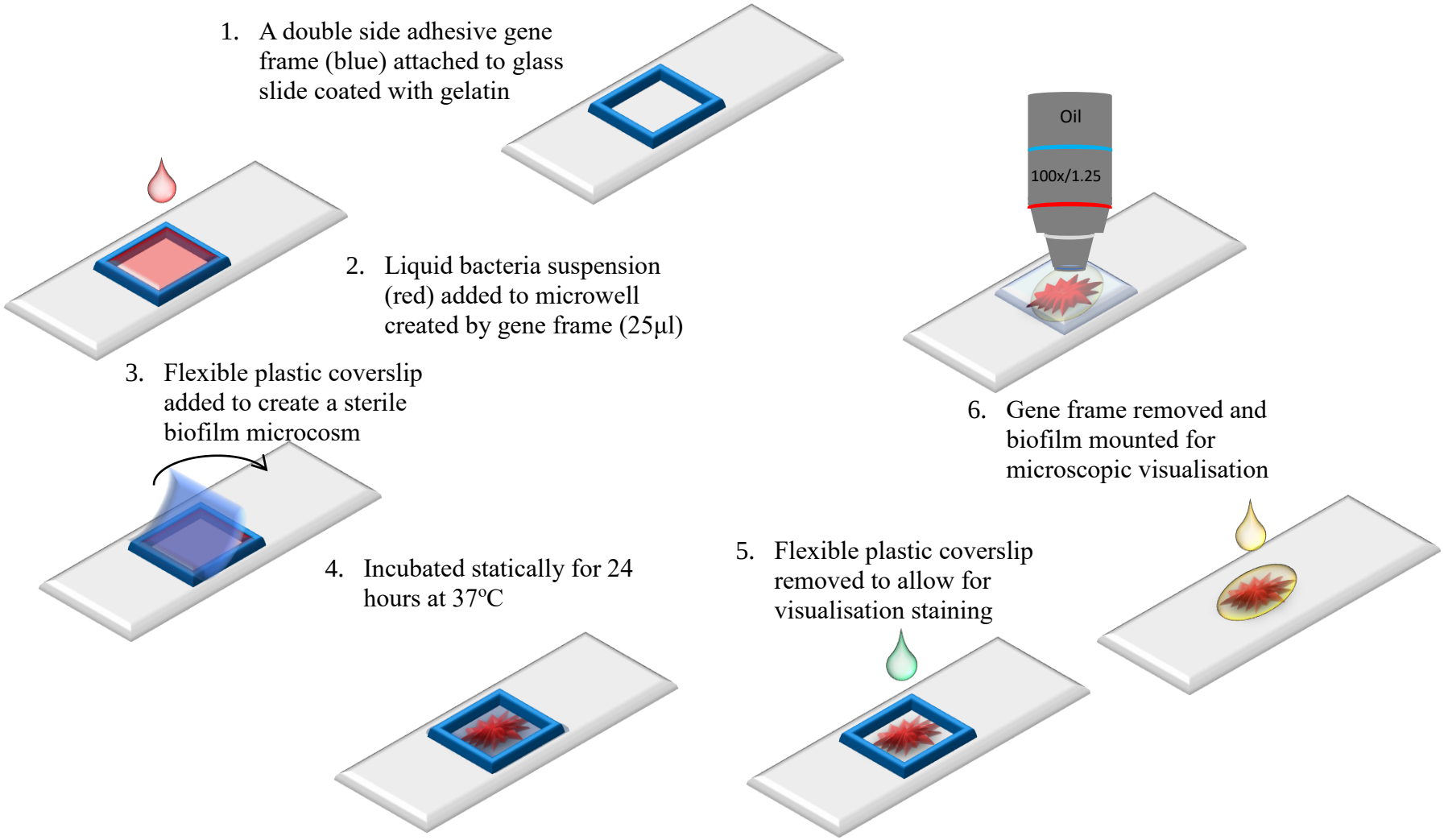


Figure 5.4.7. The gene frame biofilm apparatus (GBA): A novel biofilm development tool for microscopic visualisation of fragile enterococcal biofilm.

5.4.5. The gene frame biofilm apparatus (GBA): A novel biofilm development tool for microscopic visualisation of fragile enterococcal biofilm.

The gene frame biofilm apparatus (GBA) allowed for *in situ* microscopic analysis of enterococcal biofilm without intrusive processing. Due to this novel attribute of the model, comparisons of the traditional visualisation methods and the gene frame method were made. From figure 5.4.8, the coverslip method (i) was employed against the gene frame (ii). All biofilms were grown for 24 hours under standard growth conditions. The coverslip method retained a small quantity of the biofilm pre-processing, evident by the lack of extracellular content within the biofilm boundary (dashed line). The gene frame method produced biofilm with high density that retained the vast majority of extracellular content within the biofilm boundary.

Figure 5.4.8(b(i)) highlights free floating, detached biofilm masses that occur when processed for traditional microscopic visualisation techniques; whereas in figure 5.4.8(b(ii)) the gene frame method generated minimal biofilm surface detachment. There were zero instances of free floating biofilm mass in the gene frame experiments.

Figure (5.4.8 (c)) highlights the differences in cell retention between traditional (i) and gene frame (ii) methods. The traditional methods rend large quantities of cells into the planktonic phase; whereas the gene frame sufficiently minimised this phenomenon, but not without total elimination.

As the gene frame preserved the majority of enterococcal cellular and extracellular content, novel macrostructures were identified as well as unique cellular compositions (figure 5.4.9).

Both figures 5.4.9((a) and (b)) highlight the interconnected branched structure of MF04010 biofilm. This structure was not observed in every isolate.

The high cellular density seen in figure 5.4.9(c) was visualised throughout the entire macrostructure of all observed biofilms and cellular morphology was coccus, compared to the chain-form and diplococcus morphology observed in figure 5.4.9(d). Biofilm visualised in early development contained an abundance of diplococci. All cells in biofilm stain with conA, however what was observed in figure 5.4.9(c) was heterogeneous overstaining on individuals that were interspaced throughout the internal structure of the biofilm. This phenomenon was ubiquitous for all mature biofilms observed. Initial attachment to the substrate can be observed in figure 5.4.9(d), the long chain of 17 clones reveals the nature of production of EPS in abundance for biofilm formation. Cells in the centre of the chain are the first to develop strong localisation of conA staining. The surrounding milieu of younger 'set down' diplococcal cells demonstrated no such staining localisation.

Analysis of cellular composition in early biofilm formation allowed for the screening of biofilm 'development' amongst the enterococcal isolates using the GBA to capture the formation *in situ* figure 5.4.10.

Figure 5.4.10(i) was captured two hours after the biofilm growth experiment started. The cells on the upper left staining red with conA has the appearance of an initial set down diplococcus which has recruited a single *Enterococcus*. On the right, there is a consortium of pairs of diplococci, demonstrating early congealment and the formation of a micro-

colony of biofilm. Instances of these were noted through the surface area of the gene frame.

Figure 5.4.10(ii) was captured four hours after biofilm growth had started. The centre chain of diplococcus clones was again noted in similar fashion to what was observed in figure 5.4.9. This is the first instance of cellular variation in conA staining with strong staining on the transverse of the polar regions of the majority of cells.

Figure 5.4.10(iii) was captured at the six-hour time point. The distinctive feature of this micrograph are the moderately sized aggregates of cells. The upper aggregate displays recruitment of several diplococcus chains into a consortium. This micrograph was a cross section through the two aggregates. The lower aggregate is much larger than the upper aggregate as it projects into the z axis to a much greater extent than the upper aggregate. The lower aggregate was distinctly hollow and devoid of individuals, replaced with EPS, stained red with conA. The upper aggregate was a stack of enterococci two cells thick and the conA staining is not highlighted in this micrograph.

Figure 5.4.10(iv) was captured at the eight-hour time point. At this time, large quantities of extracellular material were identified by the conA staining. This micrograph highlights five small colonising groups of enterococci that accumulated together to form a micro-community of biofilm state cells.

Figure 5.4.10(v) was captured 12 hours after commencement of the experiment. At this stage, there were extensive communities of biofilm states with up to 50 individual members, whereby the surrounding extracellular content was noticeably larger than the cell population. Branching structures can be seen stained with conA at the lower end of the micro-community. ConA staining is heterogeneous at this stage with some cell pole staining observed.

Figure 5.4.10(vi) was taken at the end of the 24-hour experiment. The majority of the surface of the gene frame was coated with biofilm communities varying in size with

macro-colonies containing hundreds of individuals. This micrograph taken at 40x magnification shows the large consortium with DNA overstaining at the upper right of the image. The general appearance of the communities indicates that around 50% were interconnected, whilst the remainder were isolated in small communities.

As the GBA was a successful tool to assay enterococcal biofilm formation under laboratory conditions, assays were carried out to compare standard biofilm versus biofilm formation under stress.

Figure 5.4.8. Comparing ‘traditional’ biofilm assays against the gene frame biofilm apparatus (GBA).

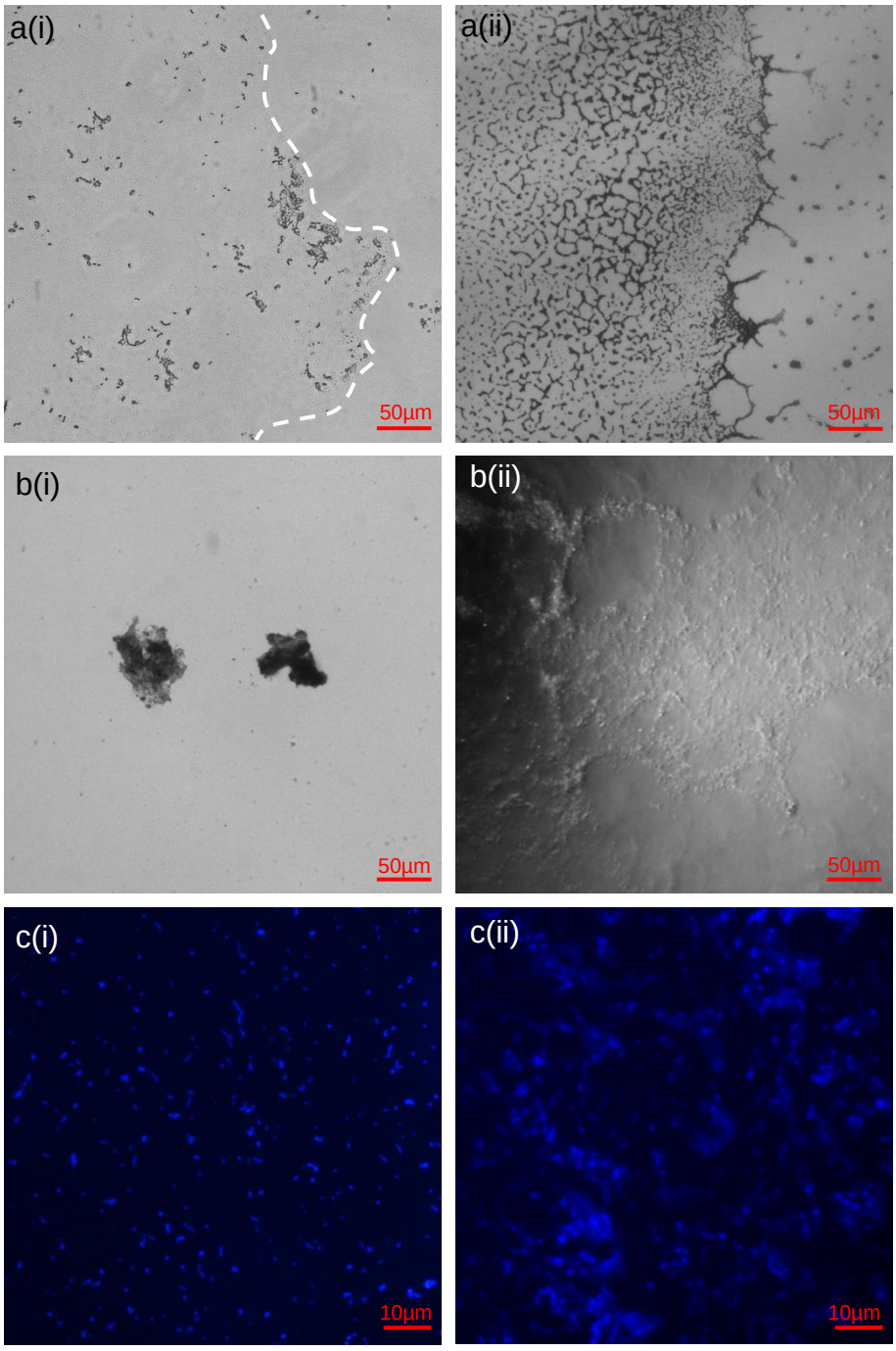


Figure 5.4.8. Micrographs depicting ‘traditional’ biofilm assays versus the gene frame biofilm apparatus (GBA) applied to MF04010. All biofilms were grown for 24 hours at 37^{o}C under static growth conditions with PBS (1% glucose), processed and mounted for microscopic visualisation. (a) 40x micrographs of the coverslip method (i) employed against the gene frame (ii). (b) 40x phase contrast micrographs. (b (i)) highlighting free floating, detached biofilm masses that occurred when processed for microscopic visualisation. (b (ii)) the gene frame method generated minimal biofilm surface detachment. (c) 100x micrographs imaged with fluorescence and Hoechst DNA staining. The traditional method (i) dislodged large quantities of cells into the planktonic phase through processing. The gene frame (ii) minimised this phenomenon.

Figure 5.4.9. Identification of macro-scaffolding and cellular distribution of enterococcal biofilm using the gene frame biofilm apparatus (GBA).

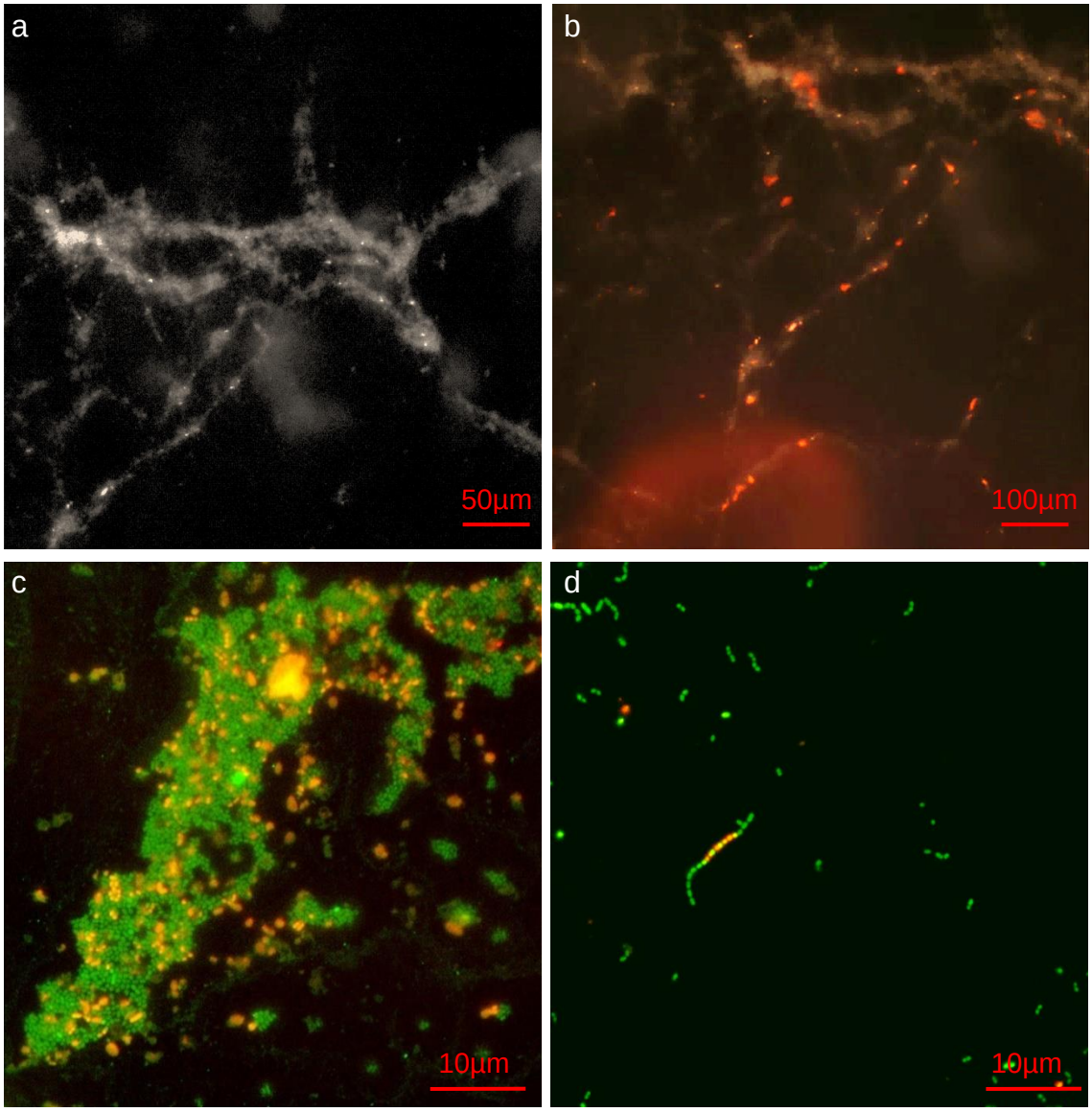


Figure 5.4.9. Micrographs depicting novel macrostructures from enterococcal biofilm growth in the GBA imaged with fluorescence microscopy. Biofilms were grown for 24 hours at 37°C statically. (a) A 20x micrograph of a 24-hour biofilm produced by MF04010 imaged under dark field lighting. (b) A 10x composite micrograph of another 24-hour biofilm produced by MF04010 using dark field and the G2-A filter. The G2-A filter captured the red fluorescence staining of conA bound to the EPS produced by MF04010. Both (a) and (b) highlight the interconnected branched structure of MF04010 biofilm. This structure was not observed in every isolate. (c-d) high powered 100x fluorescent micrographs of MF06036 24-hour (c) and two-hour (d) biofilm stained with SYTO9 (green) and conA (red). The high cellular density seen in (c) was visualised throughout the entire macrostructure of all observed biofilms.

Figure 5.4.10. Enterococcal biofilm development mediated by the gene frame biofilm apparatus (GBA).

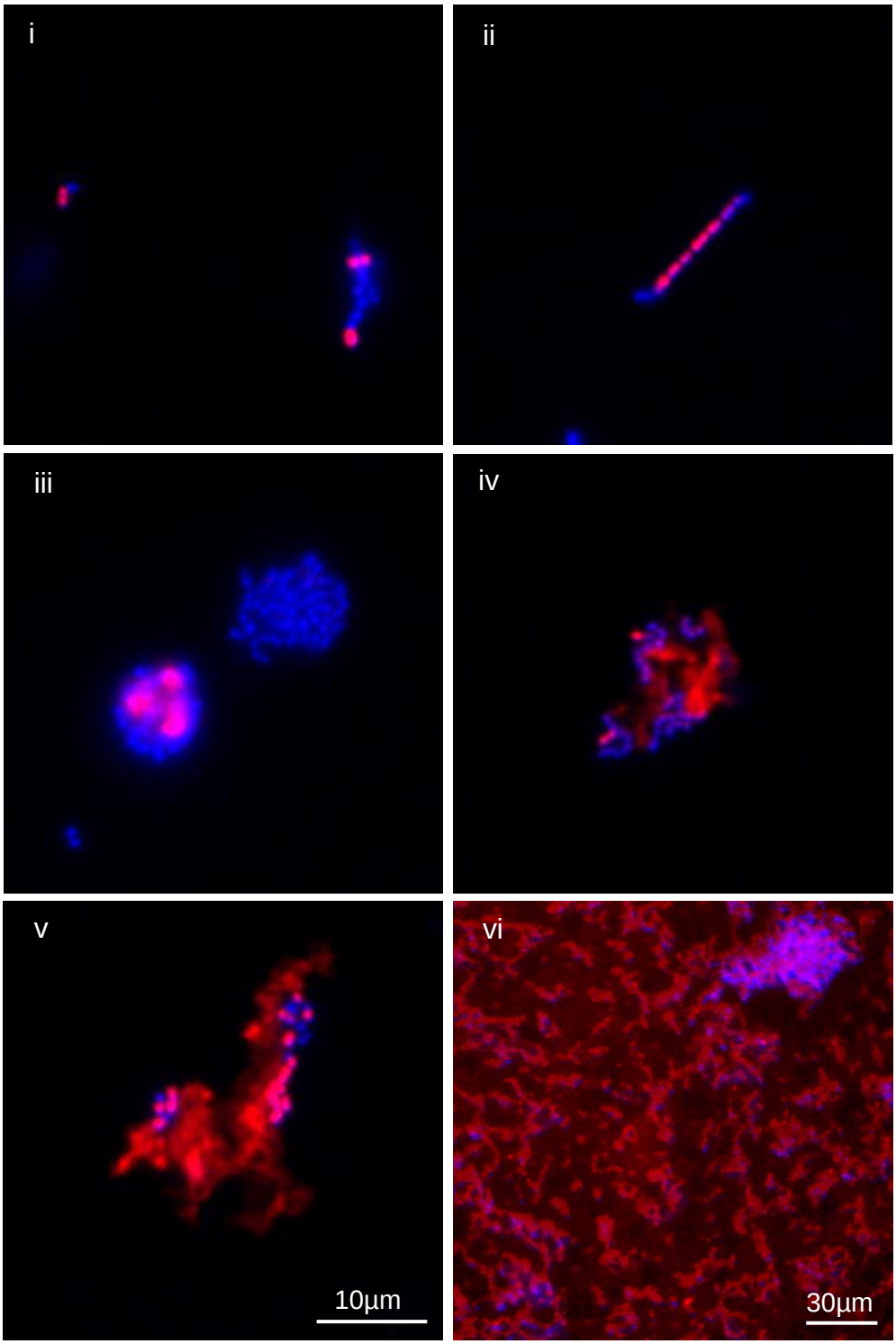


Figure 5.4.10. Region of interest Micrographs depicting the biofilm development of MF04010 during biofilm growth in the GBA. Biofilms were stained with conA (red) and Hoechst (blue) and imaged with florescence microscopy. (i) A 100x micrograph of a two-hour biofilm growth. (ii) A 100x micrograph of a four-hour biofilm growth. (iii) A 100x micrograph of a six-hour biofilm growth. (iv) A 100x micrograph of an eight-hour biofilm growth. (v) A 100x micrograph of a 12-hour biofilm growth. (vi) A 40x micrograph of a 24-hour biofilm growth. Scale bar for 100x micrographs (i-v) represents 10 microns. Scale bar for the 40x micrograph (vi) represents 50 microns.

5.4.6. The effects of nutrient deprivation on enterococcal biofilm formation

As enterococcal biofilms are postulated to exist in the natural environment, and nutrient deprivation is a commonality of extra enteric life of a bacterium, biofilm formation experiments were tested with starved enterococci.

The strong biofilm producer MW02102 was grown for 24 hours in gene frames with nutrients (figure 5.4.11(i)) or incubated at stationary growth phase under nutrient deprivation (figure 5.4.11(ii)), stained and imaged with Hoechst and conA. Under both conditions biofilm formation was clearly evident. Figure 5.4.11(a) highlights the clear biofilm community from the planktonic milieu. The white dashed line indicates the boundary of the biofilm. This was the most profound incidence of a defined biofilm boundary under liquid phase microscopy (free floating planktonic cells can be seen to the left of the boundary. This isolate produced long chains containing tens of individuals (5.4.11(a(i)) that formed interconnected biofilm (5.4.11(a(ii)). Under nutrient deprivation the observed cellular content of biofilm was composed primarily of diplococci. However, there was still evidence of aggregation of micro biofilm communities containing 10 to 20 individuals. Cells concentrated at the upper edge of the biofilm environment built upwards in the z-axis, creating a gradient whose boundary is indicated with the yellow dashed line. Figure 5.4.11(c) was a 24-hour biofilm created with nutrient deprivation on isolate MF06035. During set down and formation of biofilm the bacteria created channels embedded into the substrate, from which the majority of biofilm micro-communities are situated.

It was clear that nutrient deprivation altered biofilm formation and did not eliminate the functional characteristics, only microscopic morphology. As in figure 5.4.11, nutrient deprivation still permitted biofilm formation. Therefore, investigations into the effects of nutrient deprivation were carried out on MF01028 (figure 5.4.12): an isolate previously characterised as a biofilm non-producer and confirmed in this study as a weak biofilm

producer (biofilm index on uncoated glass). MF01028 was grown under standard biofilm formation conditions for 24 hours (5.4.12(a)) and under nutrient deprivation conditions (5.4.12(b)) stained and imaged with Hoechst and conA. From figure (5.4.12(a (ii))) there is no appreciable conA staining. Cells do aggregate in the typical way observed up to this point (5.4.12(a(i))). Figure 5.4.12(b(ii)) contrasts with 5.4.12(a(ii)) highlighting strong conA staining typical of good microscopic biofilm adhesion and production of extracellular components. Cells form smaller aggregates and the composition of single diplococcal cells is significantly increased, as is also the case in figure 5.4.11.

MF01028 biofilm images were processed in Image J and pixel intensity was measured and compared (figure 5.4.12(c)) to MW02102 the strong biofilm producer. There was no significant difference between conA staining in standard or starved growth conditions for MW02102. This data corroborates with what was discovered in figure 5.4.11. There was a significant difference ($p < 0.0001$) of conA pixel intensity between standard and starved biofilm growth. Pixel intensity for conA staining with nutrient deprivation for MF01028 a classified non- producing biofilm isolate was the same as MW02102, a classified strong biofilm producer.

Figure 5.4.11. Effects of nutrient deprivation on biofilm formation characteristics and conA staining in proficient biofilm producing enterococci.

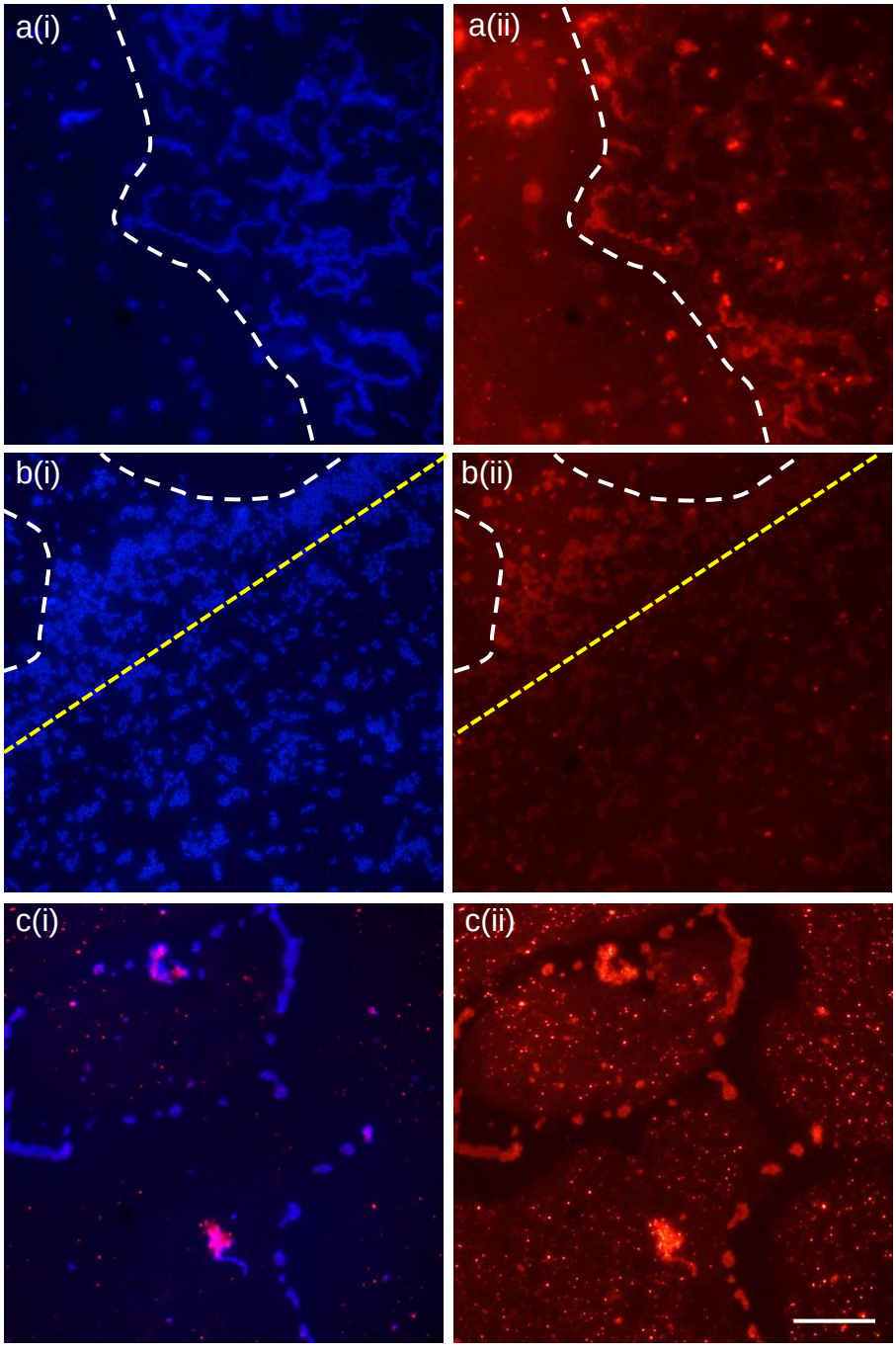


Figure 5.4.11. Micrographs depicting the effects of standard nutrient supplementation (a) and nutrient deprivation (b and c) on biofilm formation in biofilm producing enterococci in the GBA. Biofilms with standard nutrient supplementation (TSB (1% glucose) were grown for 24 hours at 37^{o}C statically. Starved biofilms were grown with nutrient depleted TSB (1% glucose) and a stationary phase culture of enterococci for 24 hours at 37^{o}C statically. (a) 40x micrographs of standard biofilm growth using MW02102, stained with Hoechst (blue (a)) and conA (red (b)). (b) 40x micrographs of starved biofilm formation using MW02102, stained with Hoechst (blue (a)) and conA (red (b)). (c) 40x micrographs of starved biofilm formation using isolate MF06035. Scale bar represents 30 microns. The white dashed lines indicate the boundary of biofilms. The yellow dashed line represents a change in biofilm height in the z-axis.

Figure 5.4.12. Effects of nutrient deprivation on biofilm formation characteristics and conA staining in non-biofilm producing enterococci.

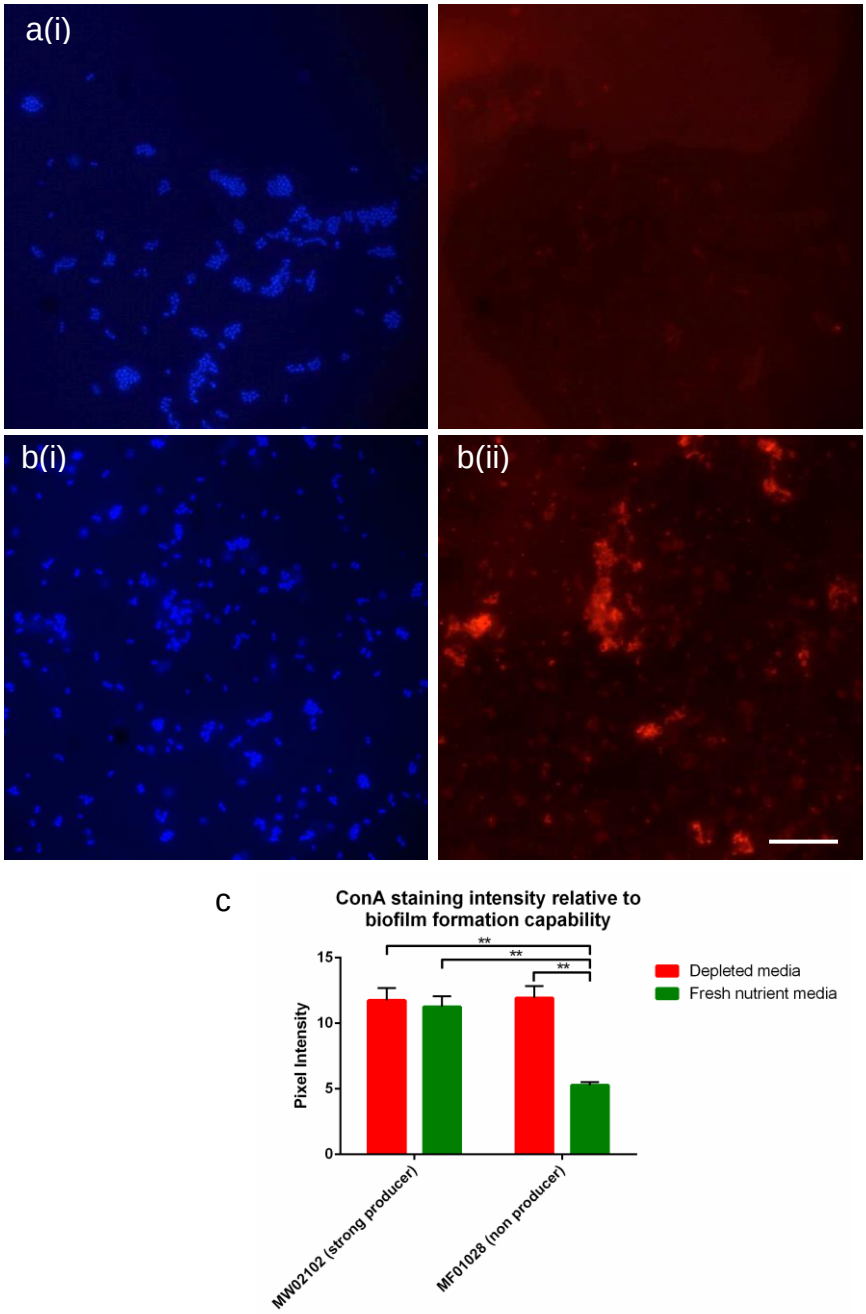


Figure 5.4.12 Micrographs and graph depicting the effects of standard nutrient supplementation (a) and nutrient deprivation (b) on biofilm formation in non-biofilm producing biofilm isolate MF01028 in the GBA. Biofilms with standard nutrient supplementation (TSB (1% glucose) were grown for 24 hours at 37ºC statically. Starved biofilms were grown with nutrient depleted TSB (1% glucose) and a stationary phase culture of enterococci for 24 hours at 37ºC statically. (a) 40x micrographs of MF01028 subjected to the standard biofilm growth conditions, stained with Hoechst (blue (a)) and conA (red (b)). (b) 40x micrographs of starved biofilm formation using MW02102, stained with Hoechst (blue (a)) and conA (red (b)). Scale bar represents 30 microns. (c) A graph comparing conA pixel intensity of both standard and starved biofilm EPS production between the strong biofilm producer MW02102 and the non-producer MW01028. Significance (**) equates to a p value of 0.0001 Ten regions from each gene frame were imaged for statistics, independently repeated three times.

5.4.7. The effects of antibiotic stress on enterococcal biofilm formation and cellular characteristics

As with the effects of starvation, there was interest in seeing if antibiotic selective pressure would affect any characteristics of enterococcal biofilm formation at the microscopic level. Therefore, experiments were carried out involving growing biofilm with MF06036 subjected to antimicrobial stress and compared to starvation and standard growth conditions (figure 5.4.13). The most immediate observed effect is the spatial characterisation of MF06036 as it laid down biofilm.

Under standard (5.4.13(a)) nutrient growth conditions bacteria formed biofilm with a high cellular density, and the cells were primarily arranged as diplococci. Cellular granularity in phase contrast is low, with cells appearing as black spheres. Apparent changes are due to cells not being in the same z-axis. Under starvation (5.4.13(b)), bacteria form biofilm communities with low cell density and have a similar diplococcus morphology. Granularity remains low during cellular starvation. Adding sub inhibitory concentrations of vancomycin (10µg/ml) to pre-established biofilm (5.4.13(c)) had a marked effect on cell density, where cells are almost completely touching. Cells still retained a diplococcus morphology; however cellular granularity slightly increased. When subjecting a biofilm to sub inhibitory concentrations of vancomycin (10µg/ml) throughout the growth phase as well as post growth (5.4.13(d)) there were dramatic changes compared to post growth addition of vancomycin and even more so from standard growth conditions. Cells were visibly larger, had taken on chain form variations. These cells had apparent ‘grains’ of phase contrast compared to standard nutrient and even nutrient deprivation. Enterococci exist in high cell density, but are much less organised compared to the other conditions. Cellular size changes were quantified in image J (5.4.13(e)). Nutrient biofilm average cell size was 0.51µm, starved biofilm was 0.52µm, and vancomycin post-biofilm formation stress was 0.64µm, which was significantly larger ($P<0.0001$). Vancomycin stress

conditions throughout produced an average cell size of 0.92μm, significantly increases cell length along the transverse of the cell ($p < 0.0001$) compared to the other three growth conditions.

Figure 5.4.13. Selective pressure and nutrient deprivation affects spatial density, chain forming characteristics, granularity, and size of MF06036 early biofilm cells.

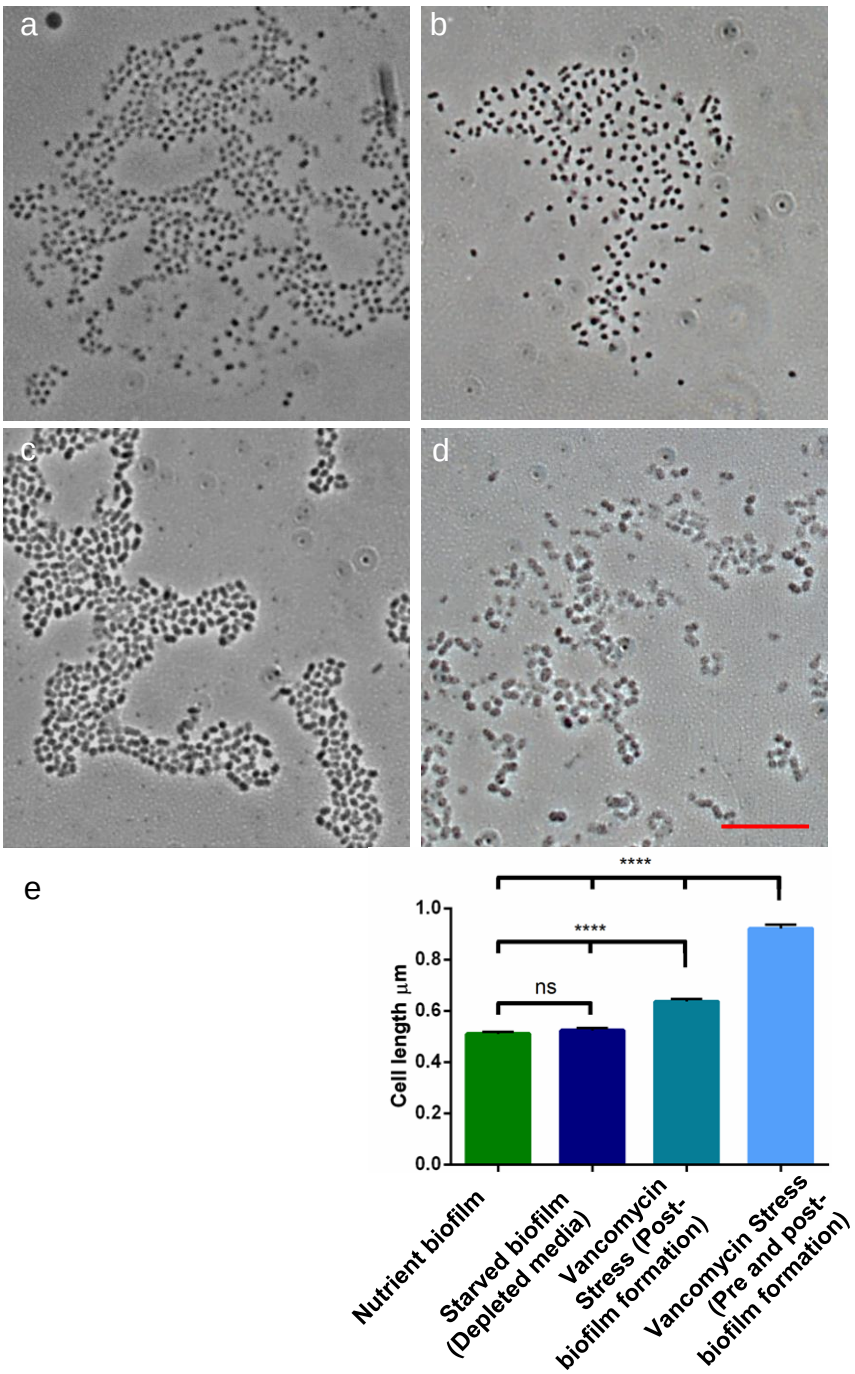


Figure 5.4.13. Phase contrast micrographs and a graph depicting the effects of starvation and vancomycin stress on MF06036's cellular and biofilm morphology. Experiments were carried out at 37°C incubated statically for 24 hours. (a) A 100x micrograph depicting biofilm growth using standard TSB (1% glucose). (b) A 100x micrograph depicting biofilm growth using nutrient depleted TSB (1% glucose) with a stationary phase culture of MF06036. (c) A 100x micrograph depicting the effects of sub inhibitory concentrations of vancomycin (10µg/ml) to pre-established biofilm growth with TSB (1% glucose). (d) A 100x micrograph depicting the effects of sub inhibitory concentrations of vancomycin (10µg/ml) throughout the biofilm growth phase as well as post growth. Scale bar represents ten microns. (e) A graph quantifying the change in cellular size observed under the stress of starvation and antibiotic selective pressure. Cellular size changes were quantified in Image J by measuring the diameter of the transverse of cells. Significance (***) equates to a p value of 0.0001. Ten regions from each gene frame were imaged for statistics, independently repeated three times.

5.4.8. Utilisation of fluorescent *in situ* hybridisation to visualise enterococci inside biofilm and minimise artefacts with standard fluorescence techniques

At this stage, there were issues with microscopic visualization that prevented visualisation of enterococcal conjugation inside biofilm. Issues with photo-conversion from Hoechst nuclear stains (data not shown) across the G2-A filter on the microscope, and the lack of specificity of Hoechst warranted a staining protocol that could image enterococci specifically. The solution to these problems was the development of an in-house FISH assay for rapid identification of enterococci in the biofilm state. Figure 5.4.14(a(i)) is *E. faecium* FISH probe imaging of ST01109^{RIf} with Alexa fluor 594 probes targeted to 23s rRNA in a standard 24-hour biofilm. Figure 5.4.14 (a(ii)) is an *E. faecalis* Alexa fluor 594 probe targeted to 16s rRNA on an MF06036 standard 24-hour biofilm. The two *Enterococcus* probes effectively stained *E. faecalis* and *E. faecium* where individual cells were clearly visible within biofilm. Figure 5.4.14 (b) is an EUB FISH probe labelled with fluorescein that targets the majority of non-marine bacteria, shown here binding to MF06036 in a standard 24-hour biofilm. Figure 5.4.14 (c) is a collection of negative binding controls to highlight any natural autofluorescence in the enterococcal isolates. Figure 5.4.14(c(i)) is a phase contrast image of MF06036 biofilm cells. Figure 5.4.14(c(ii)) is an overlay image of the same region using the G2-A red filter showing no autofluorescence with FISH probes. Figure 5.4.14(c(iii)) is an overlay image of the same region using the B2-A green filter showing no autofluorescence with FISH probes. This FISH technique eliminated the photo-conversion caused by Hoechst and allowed methods of investigating the presence of specific isolates of enterococci and even some mobile genes (*vanA*) as in the next section below.

Figure 5.4.14. Creating a fluorescent *in situ* hybridisation (FISH) assay for rapid examination of enterococci in biofilm states.

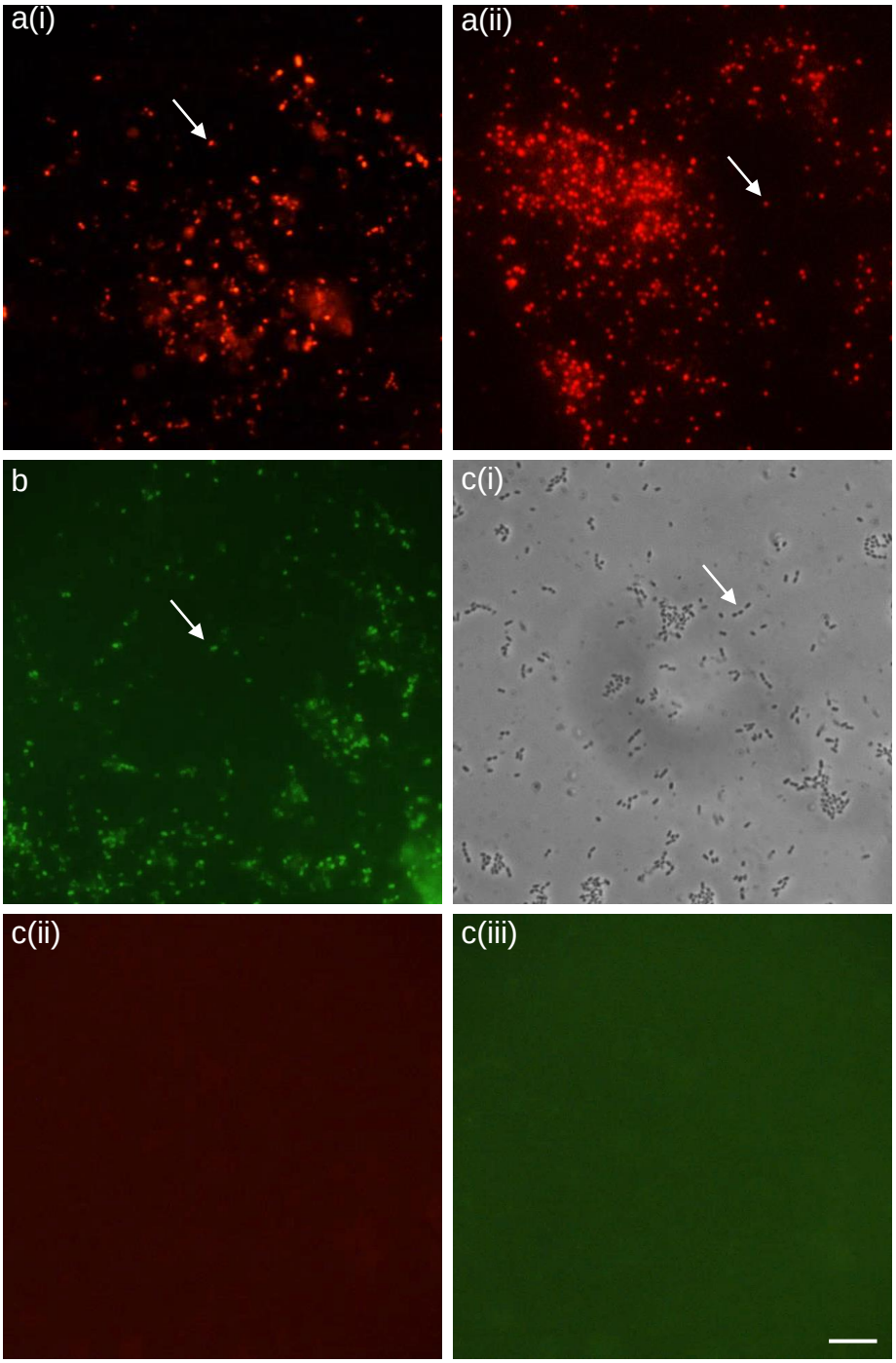


Figure 5.4.14 Micrographs of a fluorescent *in situ* hybridisation (FISH) assay developed to analyse enterococcal cellular presence in biofilm states. (a(i)) A 100x micrograph depicting *E. faecium* FISH probe imaging of *E. faecium* ST01109^{RIf} with Alexa fluor 594 (red) probe targeted to 23s rRNA in a standard 24-hour biofilm. (a(ii)) A 100x micrograph depicting *E. faecalis* FISH probe imaging of *E. faecalis* MF06036 with Alexa fluor 594 (red) probe targeted to 16s rRNA in a standard 24-hour biofilm. (b) A 100x micrograph of the EUB FISH probe labelled with fluorescein (green) that targets the majority of non-marine bacteria, shown here binding to MF06036 in a standard 24-hour biofilm. (c) A collection of 100x magnification, negative binding controls to highlight any natural autofluorescence in the enterococcal isolates. (c(i)) A phase contrast image of MF06036 biofilm cells. (c(ii)) An overlay image of the same region using the G2-A red filter showing no autofluorescence with FISH probes. (c(iii)) An overlay image of the same region using the B2-A green filter showing no autofluorescence with FISH probes. Scale bar represents 10 microns. Arrows identify bacteria.

5.4.9. Modification of fluorescent *in situ* hybridisation to target the mobile *vanA* vancomycin resistance gene conjugally transferred in the creation of T1

The standard FISH assay described in 2.23.1 was modified to target several binding sites across the *vanA* vancomycin gene, as described in 2.23.3. This allowed for the identification *vanA* inside cells inside MF06036 biofilm. Figure 5.4.15 (a) is a negative biological control using MF04010 for fluorescein probes targeted to the *vanA* vancomycin resistance gene. Figure 5.4.15(a(i)) uses phase contrast to highlight MF04010 biofilm, whereby the leading edge is clearly visible. Towards the tail end of the central mass occasional diplococci and short chains of no more than four cells are visible. (5.4.15(a(ii))) The green fluorescence channel showing no localised intercellular staining, with minimal green autofluorescence displayed (high saturation due to the gain intensity utilised to capture high intensity images on the camera).

Figure 5.4.15(b) depicts the addition of sub inhibitory (10µg/ml) vancomycin to MF06036 biofilm milieu for 24 hours after initial biofilm formation. Figure 5.4.15(b(i)) is a phase contrast image of treated biofilm: of note is the large array of interconnected biofilm structures and apparent cellular stacking in the z-axis. Variation in light intensity highlights extracellular components. Figure 5.4.15(b(ii)) is a green channel overlay of the same region as in figure 5.4.15(b(i)): there is an increased diffuse signal when *vanA* probes are added to MF06036 biofilm. Additionally, there are instances of heterogeneous single cell fluorescence not observed in the control.

Figure 5.4.15(c) depicts the addition of sub inhibitory (10µg/ml) vancomycin to MF06036 during biofilm formation (24 hours) and another post biofilm formation addition for 24 hours. Figure 5.4.15(c(i)) is a phase contrast image of treated biofilm. Cell size variations are apparent with phase contrast imagery, with the presence of apparent fratricidal cells. Figure 5.4.15(c(ii) is a green channel overlay of the same region is in figure 5.4.15(c(i)). There is total cellular staining in both single and diplococcal cells.

Cells that had higher granularity with phase contrast emit a stronger FISH signal. Intercellular staining varies from heterogeneous intercellular staining to apparent membranous staining. There were several instances of all cells within a chain-form having staining; and others where only the lead cell presents with positive staining.

Figure 5.4.15. Utilisation of FISH to detect the vancomycin resistance gene in *E. faecalis* using the gene frame biofilm apparatus (GBA).

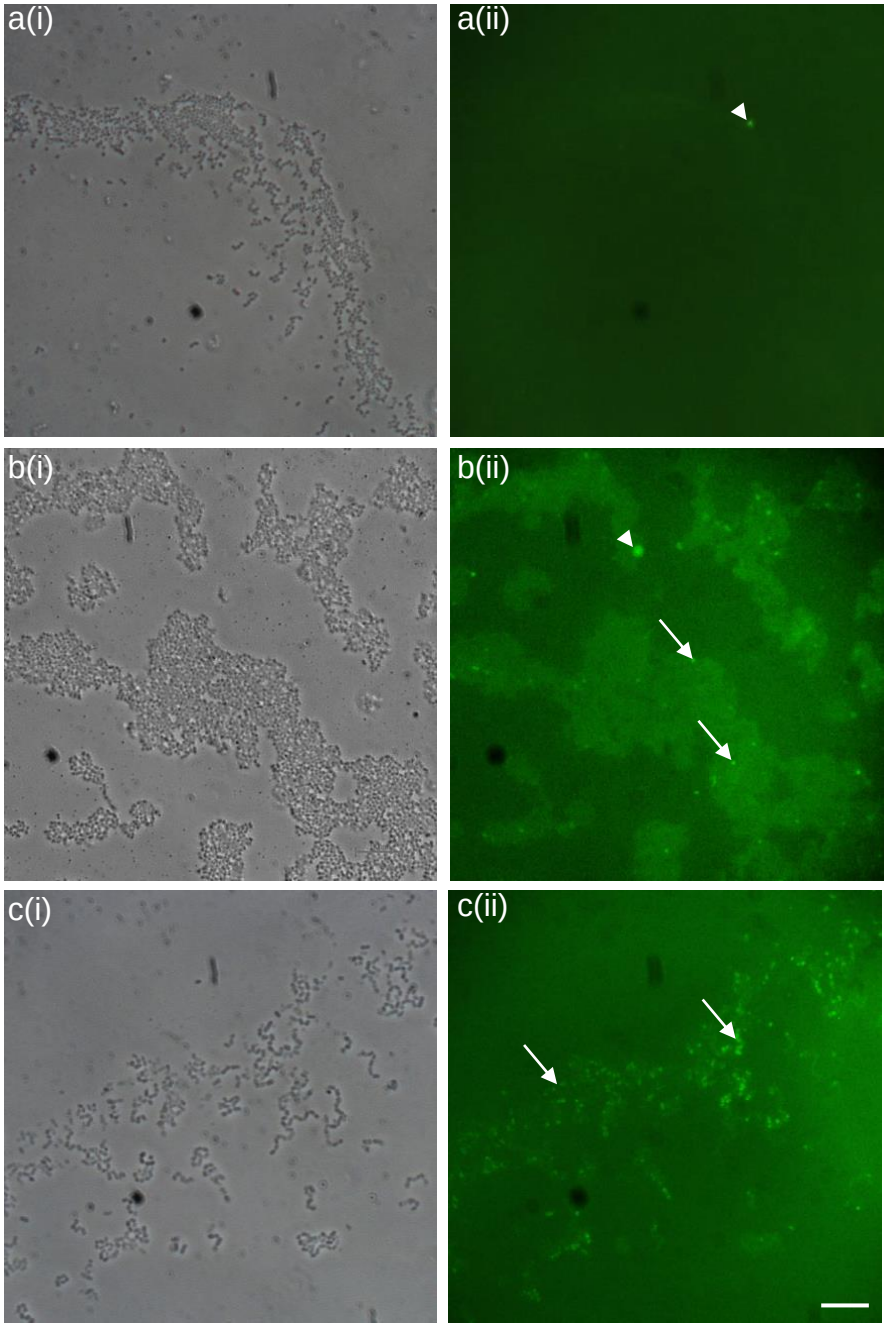


Figure 5.4.15. Micrographs of FISH detection of plasmid bound *vanA* fluorescein (green) resistance genes in enterococcal biofilm. (a) 100x micrograph overlays of a negative biological control using MF04010 and fluorescein probes targeted to the *vanA* vancomycin resistance gene. (a(i)) Phase contrast imaging to highlight MF04010, 48-hour biofilm cells grown with TSB (1% glucose) at 37ºC incubated statically. Media was replaced at the 24-hour time point. (a(ii)) Green fluorescent imaging of the same region. (b) 100x micrograph overlays of the addition of sub inhibitory (10µg/ml) vancomycin for 34 hours, to the *vanA* positive MF06036 in a 24-hour pre-established biofilm grown with TSB (1% glucose) at 37ºC. (b(i)) Phase contrast imaging MF06036 vancomycin exposed biofilm. (b(ii)) Green fluorescent imaging of the same region. (c) 100x micrograph overlays of the addition of sub inhibitory (10µg/ml) vancomycin to the biofilm formation media TSB (1% glucose) during the formation of isolate MF06036's biofilm (24 hours). Media was replaced at the 24-hour time point with vancomycin TSB (1% glucose) for an additional 24 hours. (c(i)) Phase contrast imaging MF06036 vancomycin exposed biofilm. (c(ii) Green fluorescent imaging of the same region. Scale bar represents ten microns. Arrowhead – fluorescence artefact, Arrow – cell positive for vanA staining.

Sufficient data had been acquired demonstrating novel conjugation of environmental isolates of enterococci, and that enterococci can form biofilm: The final step in this thesis was to test if environmentally isolated enterococci can conjugate inside biofilm. The in-house protocol devised (Figure 4.4.15) in this study was capable of efficiently establishing biofilms of similar surface size which could be grown in a sealed environment that could then have a fresh planktonic conjugation partner added to established biofilm cells. This reaction could then be incubated without the risk of contamination and be assessed by traditional selection plates or by novel microscopic approach.

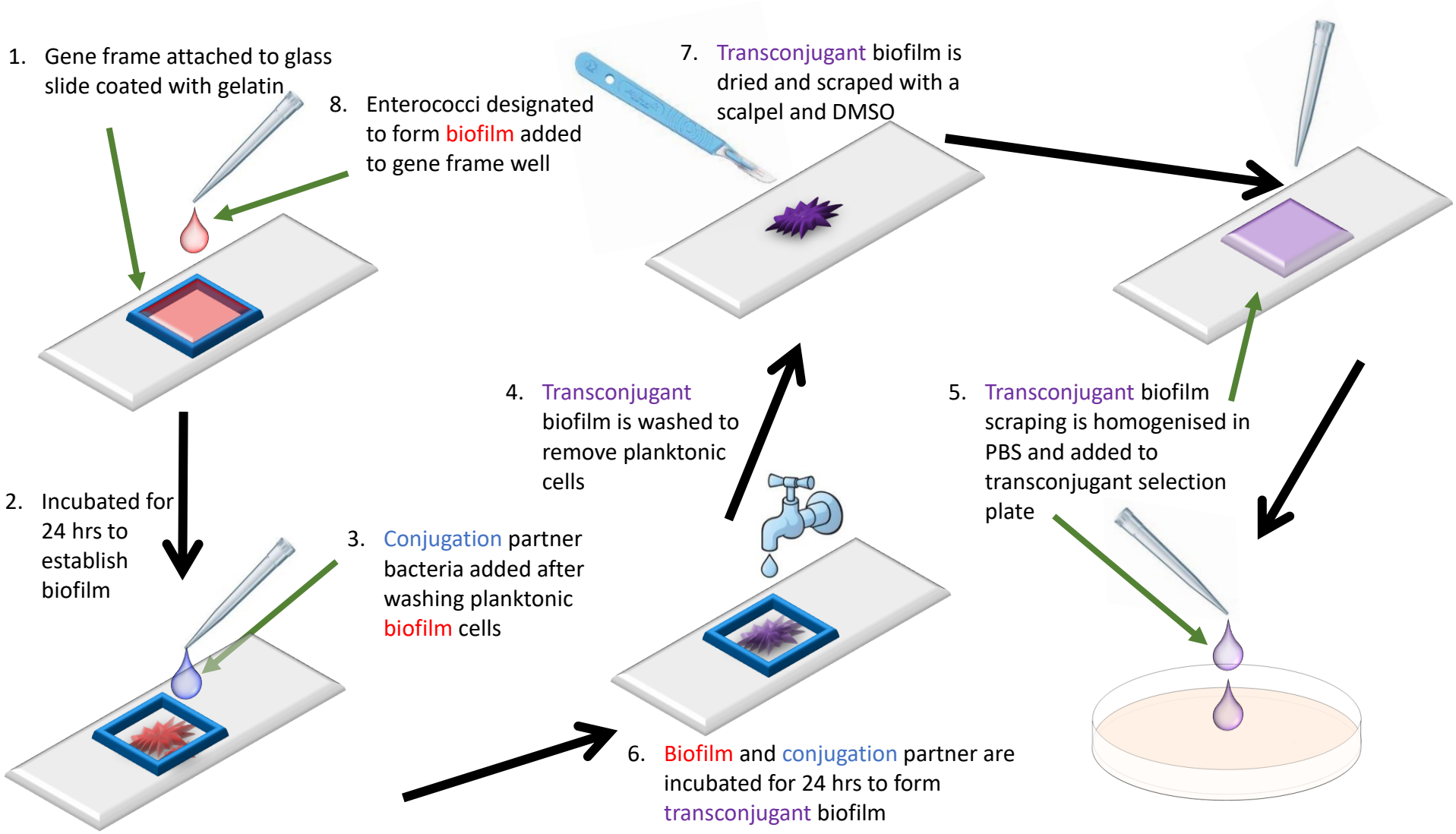


Figure 5.4.16. The gene frame biofilm model utilised to detect conjugation between enterococci in a biofilm substrate.

5.4.10. Using the gene frame biofilm apparatus, 'triple selection' and fluorescent *in situ* hybridisation to isolate transconjugants from conjugation reactions within enterococcal biofilm

Using the gene frame biofilm model isolation of T1 from true biofilm was possible.

From three bars shown in figure 5.4.17, compatible enterococci can and do conjugate within a biofilm. Interestingly, conjugation in a biofilm created primarily by the recipient (biofilm created with MW01105^{Rif}) had an efficiency of $2.01x10^{-3}$; double that of the biofilm primarily created by the donor (biofilm created with MF06036) at $1.01x10^{-3}$. Conjugation in the mixed enterococcal biofilm was 3 times as efficient as the donor biofilm with an efficiency of $3.04x10^{-3}$. Statistically there is no difference between these three types of biofilm conjugation tests excluding a comparison of the donor only biofilm against the mixed biofilm (p value of 0.0048 ** using welches correction). However, it is clear how a mixed biofilm (without accounting for planktonic enterococci) yields an improvement in the generation of transconjugants.

Applying nutrient deprivation conditions to the same biofilm conjugation reaction produced transconjugants. The efficiency of conjugation was reduced ($7.18x10^{-4}$, $1.03x10^{-3}$, and $1.54x10^{-3}$) and the ratio of transconjugants between the MF06036, MW01105^{Rif} and the mixed biofilm remained the same.

After proving that the tested enterococci can conjugate inside biofilm of their own creation as an end-point assay, protocols were devised to visually observe the result of enterococcal conjugation inside biofilm. The simplest way to carry this out would be to eliminate the parental isolates post conjugation inside biofilm. This would allow for the visualisation of transconjugants using a simple live/dead stain. Figure 5.4.18 was the first protocol tested using double antibiotic selection to eliminate the parental isolates and leave the transconjugant alive. Figure 5.4.18 shows that the action of double selection

alone inhibits MW01105^{Rif} and MF06036. However, this assay clearly produces cells that stain green with the live stain (SYTO9). As expected T1 has a much higher ratio of Live: dead as compared to its parents. This assay demonstrates a flaw in the process of using double selection alone. It was simply not enough to visualise live transconjugants alone. Some parental cells tested positive with the live stain, at concentrations known to cause no growth.

As a response to the data gathered and shown in figure 5.4.18. Lysozyme was added to the reaction to degrade compromised cells that may stain as live using the live/dead staining protocol. Figure 5.4.19(a) demonstrates the action of lysozyme on the parent isolates and T1 when employed at the MIC. Lysozyme alone is effective at inhibiting planktonic MW01105^{Rif}, MF06036 and T1 (MIC's = 10.4 mg/ml, 20.8 mg/ml, and 10.4mg/ml respectively), and is even more effective when compounded with double selection (MIC's = 0.8, 0.162, and 2.6 mg/ml respectively).

The live dead assay with lysozyme (2 mg/ml) was performed on the isolates again with dramatic effects (figure 5.4.19(b)). The results yielded a total kill response for MW01105^{Rif}, a 3% live staining response for MF06036; whereas T1 had a 55% live staining response. The grey compromised bars in this graph considers cells stained with SYTO9 but exhibited gross morphological changes associated with compromising of cellular integrity. Cells were recoded as live if SYTO9 staining was present on a cell with the absence of propidium iodide staining; however, staining alone was not enough to rule a cell as 'live'. Many cells that were stained with SYTO9 only were counted as 'live', but when examining cell morphology, it was clear that the cells had varying degrees of compromised envelopes.

Figure 5.4.19(c-e) highlight the variations in singular SYTO9 staining. The red arrow indicates healthy cells, noting the characteristic morphology of enterococci: diplococcus,

with smooth cellular envelopes and clear bounded green fluorescence. The yellow arrows highlight compromised cells which are dead but overpoweringly stain green. The cells highlighted with the white arrow appear normal, but when examined closely cell elongation is present as well as dysmorphic furrowing in the lower cell. These cells vary in morphology, however there are clear differences between those shown in yellow arrows versus those shown with red arrows. The blue arrow is an example of total destruction of enterococcal chain form.

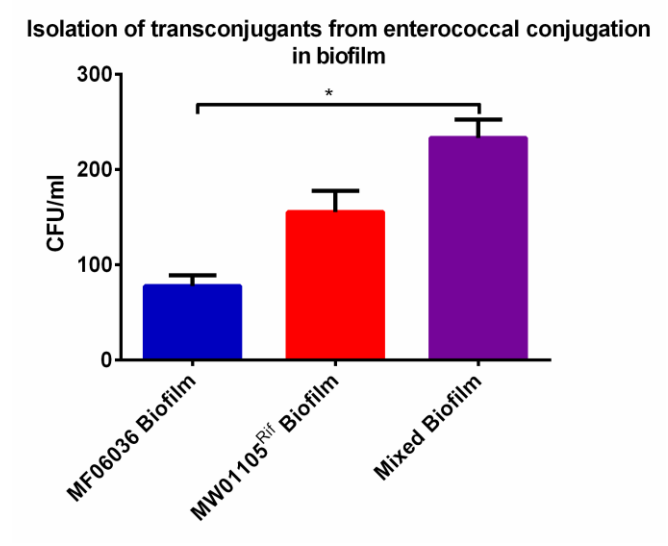
With the data gathered in figure 5.4.19 a biofilm conjugation reaction was established with MW01105^{Rif} and MF06036 (Figure 5.4.20). Figure 5.4.20(a-c) clearly demonstrates microscopically the synergistic killing of MW01105^{Rif} (a), MF06036 (b) and what can only be the successful conjugation of MW01105^{Rif} and MF06036 (c) highlighted with live SYTO9 green imaging.

Figure 5.4.20(d) is a total cell count of the three biofilms visualised in 5.4.20(a-c). Compared to figure 5.4.19(b) the number of cells stained live was higher (MW01105^{Rif} 3.6%, MF06036 1.75%). However, when comparing compromised cells in the same fashion the results are the same. The total cell count of the conjugation biofilm yielded higher numbers of live cells, as well as dead cells (including compromised cells). As this was a biofilm conjugation experiment with MW01105^{Rif}, MF06036 and freshly created T1, these increased numbers of dead and compromised cells were expected when compared to figure 5.4.19 (b) T1 only biofilm.

Figures 5.4.20(e-f) are fluorescent micrographs of a biofilm conjugation reaction between *E. faecalis* MF06036 and *E. faecium* ST01109^{Rif}. Due to the selection conditions, the only remaining enterococci in this biofilm are MF06036 (all blue stained cells that do not co-localise with red staining) and the resultant transconjugant from the conjugation reaction, which is stained with the FISH probe. This assay provided visual evidence for

enterococcal conjugation within biofilm. Washing steps during the methodology ensured planktonic cells were washed away and binding controls ensured that non-specific signal did not interfere with data collection. The Hoechst photo-conversion does not enter the B2-A blue filter on the fluorescent microscope.

Figure 5.4.17 Selection plate isolation of T1 from biofilm under both starvation and standard growth conditions.



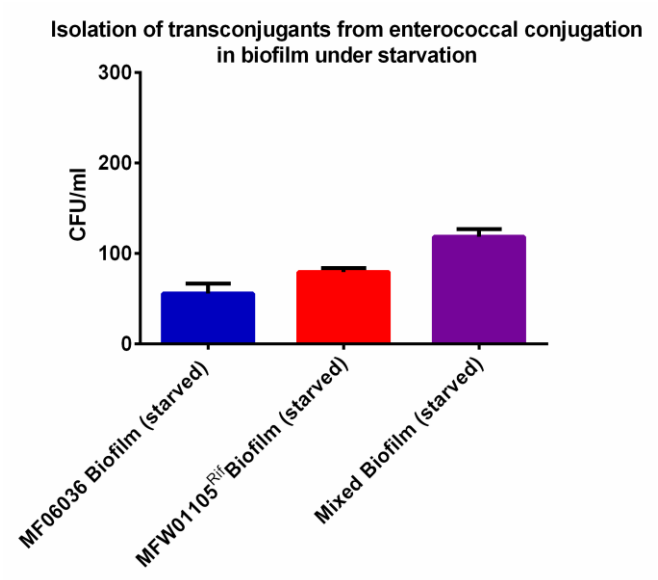


Figure 5.4.17. Graphs depicting the isolation frequencies of T1 from standard and starved biofilm where MF06036 and MW01105^{Rif} successfully conjugated inside biofilm. Biofilms were grown with one isolate for 24 hours. Planktonic cells were removed with PBS and the corresponding conjugation partner was added (at the same cell concentration as the initial isolate at the beginning of biofilm formation), and incubated for a further 24 hours. Planktonic cells were removed with PBS and biofilms were scraped, re-suspended in PBS and added to selection plates (vancomycin 10µg/ml and rifampicin 100µg/ml) for a further 24 hours. Transconjugant growth was recorded and displayed in the graphs above. Starved biofilms used nutrient depleted TSB (1% glucose) and stationary phase isolates. MF06036 biofilm represents biofilm created by MF06036 which had MW01105^{Rif} added after 24 hours. MW01105^{Rif} biofilm represents biofilm created by MW01105^{Rif} which had MF06036 added after 24 hours. Mixed biofilm represents biofilm created with the addition of MW01105^{Rif} and MF06036 and grown for 48 hours with media replacement at the 24-hour time point. Error bars represent standard error of the mean. Mixed biofilms had statistically higher CFU/ml compared to MF06036 biofilms under standard conditions (p 0.048) and starvation (p 0.013). Experiments were carried out with 6 biological repeats and five independent repeats.

Figure 5.4.18. Using double selection with rifampicin and vancomycin to eliminate *Enterococcus* conjugative partners but not their transconjugant in a biofilm state.

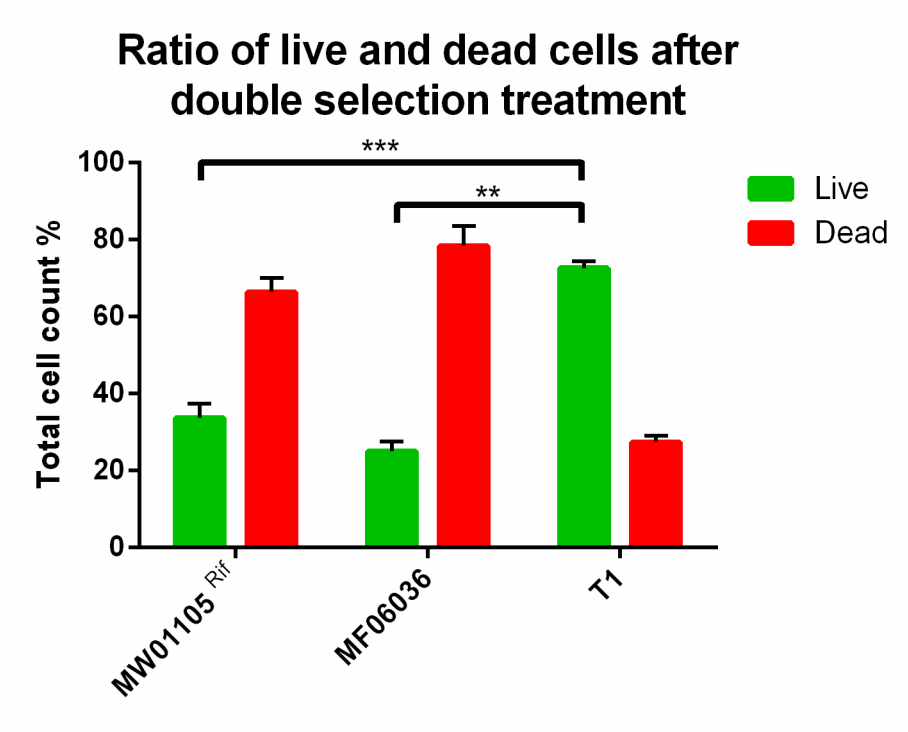


Figure 5.4.18. A bar graph depicting the total cell count of live and dead bacteria stained with SYTO9 (green) and propidium iodide (red). Individual biofilms were grown for 24 hours (TSB 1% glucose, 37ºC). Biofilms were washed to remove planktonic cells and exposed to double selection (vancomycin 10µg/ml and rifampicin 100µg/ml) for 24 hours. Error bars represent standard error of the mean. Significance *** p 0.0002, significance ** p 0.0032. Experiments were carried out with 6 biological repeats and five independent repeats

Figure 5.4.19. Using rifampicin, vancomycin a nd lysozyme to eliminate the conjugal partners MW01105^{Rif} and MF06036 but not their transconjugant, T1.

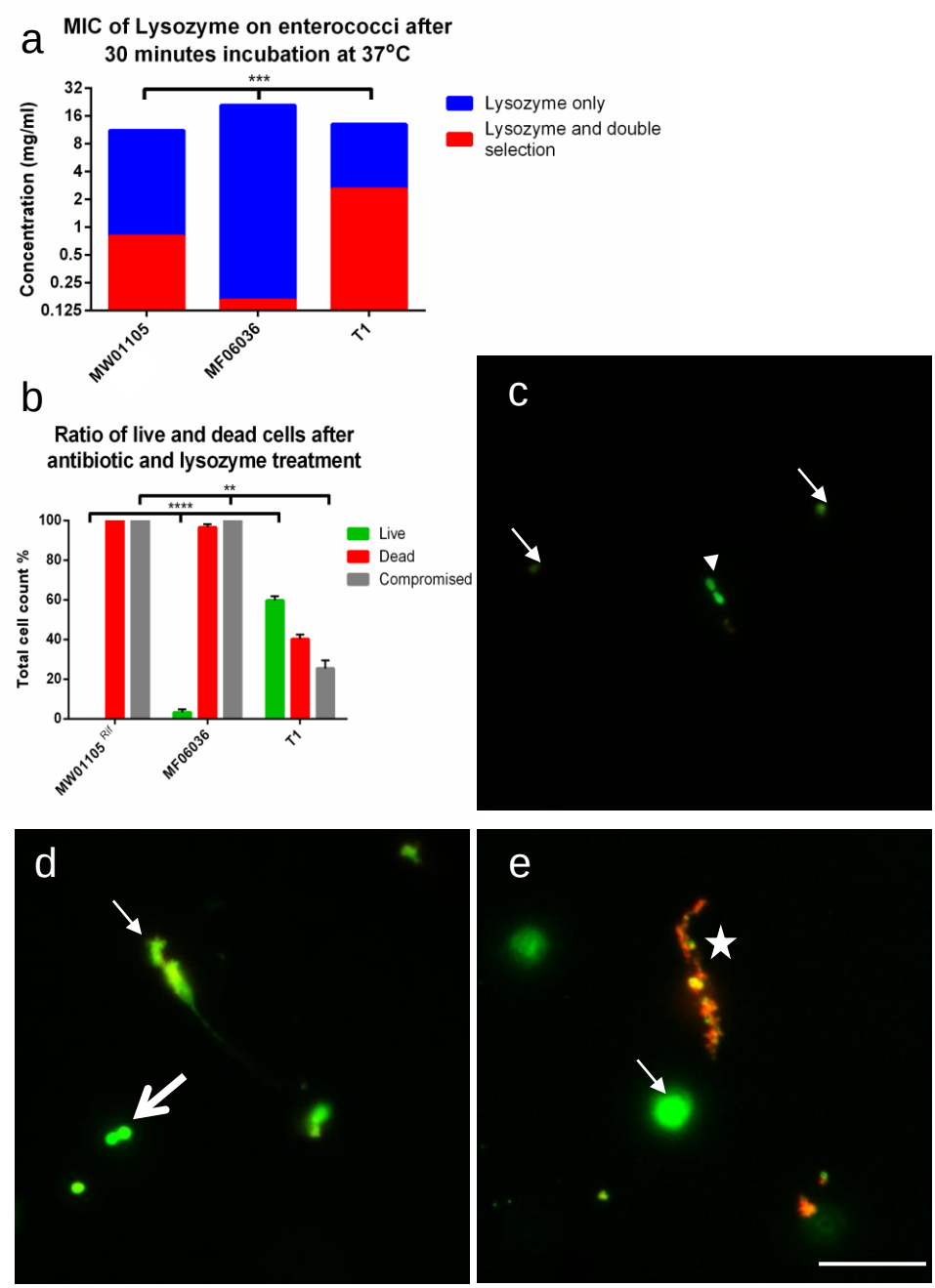


Figure 5.4.19. Graphs and micrographs depicting the action of adding lysozyme to double selection elimination conditions of the T1 conjugation reaction to eliminate MF06036 and MW01105^{Rif} in biofilm. (a) A minimum inhibitory concentration graph demonstrating the killing action of lysozyme on the parent isolates and T1 with and without double antibiotic selection (vancomycin 10µg/ml and rifampicin 100µg/ml). (b) A repeat experiment of (a) using triple selection - vancomycin (10µg/ml) and rifampicin (100µg/ml) and lysozyme (2 mg/ml). Error bars represent standard error of the mean. (c - e) 100x region of interest micrographs highlighting the variations in using the live (green)/dead (red) staining kit for assessment of total killing of MF06036, MW01105^{Rif} and survival of T1 under 'triple selection'. The outlined arrow indicates healthy cells. Filled arrows highlight compromised cells which are dead but overpoweringly stain green. The cells highlighted with the arrowhead appear normal, but when examined closely cell elongation is present as well as dysmorphic furrowing in the lower cell. The star is an example of total destruction of enterococcal chain form. Scale bar represents ten microns. Significance *** p 0.0001, significance ** p 0.002. Lysozyme and double selection group MIC was significantly (p 0.02) lower than lysozyme alone. Experiments were carried out with 6 biological repeats and five independent repeats.

Figure 5.4.20. Microscopic visualisation of transconjugants in biofilm under standard growth conditions by killing parents with double selection and lysozyme.

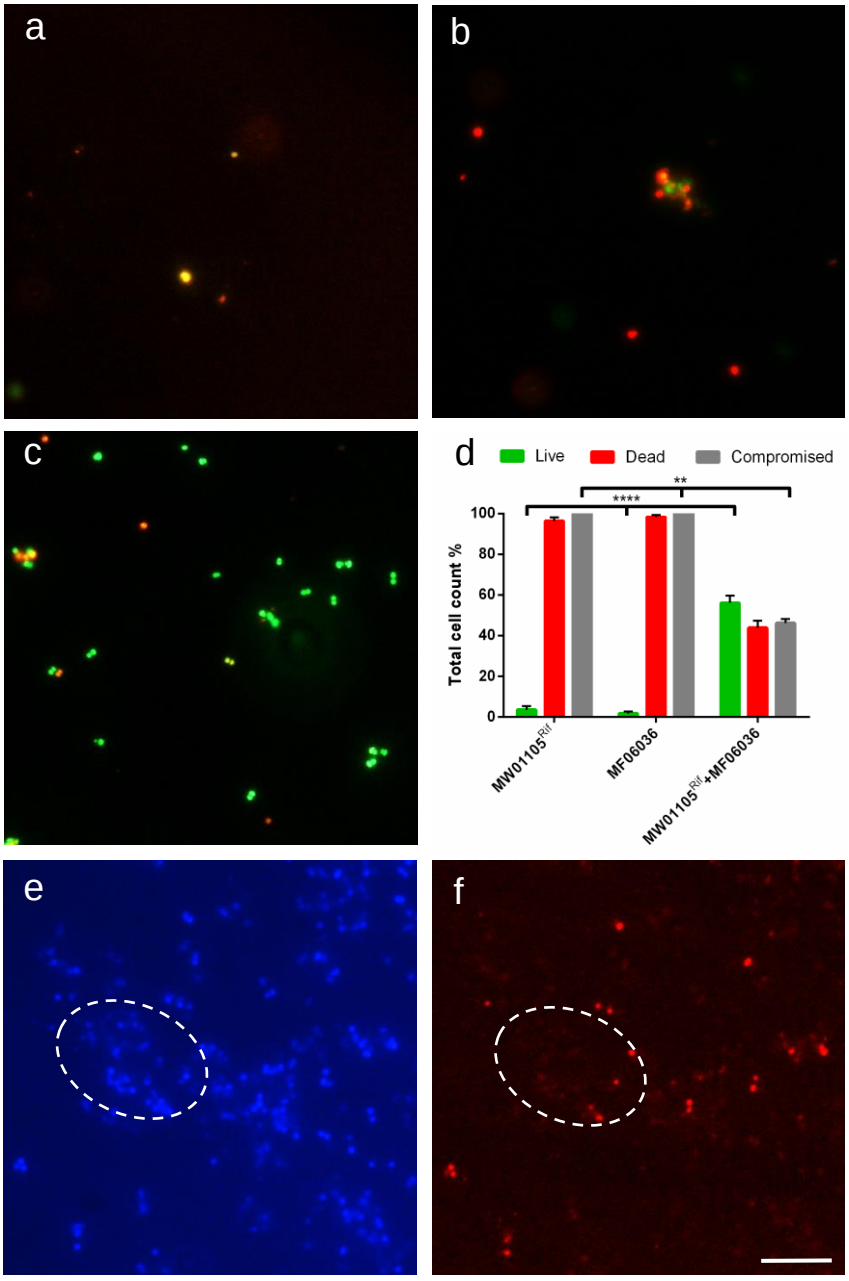


Figure 5.4.20. Micrographs and a graph visualising the successful selection of T1 from MF06036 and MW01105^{Rif} conjugation inside biofilms. (a) 100x fluorescent micrograph of a standard 24-hour biofilm made with MW01105^{Rif}. (b) 100x fluorescent micrograph of a standard 24-hour biofilm made with MF06036. (c) 100x fluorescent micrograph of a standard 24-hour conjugation reaction with MW01105^{Rif} inside biofilm. Subfigures (a-c) were visualised with Live/dead (green/red) after treatment with double selection (10µg/ml vancomycin, 100µg/ml rifampicin) to inhibit/kill cells. The biofilm was then treated with lysozyme (2 mg/ml) for a further 24 hours to eradicate the compromised cells. (d) A graph showing the total cell count of the three biofilms visualised in (a-c). Error bars represent the standard error of the mean. Sub-figures (e-f) are fluorescent micrographs of a conjugation reaction between *E. faecalis* MF06036 and *E. faecium* ST01109^{Rif} inside biofilm. Biofilm was created, and enterococci allowed to conjugate for 24 hours. Post conjugation, biofilm was treated with tetracycline (30 µg/ml) for 24 hours, and then treated with lysozyme (2 mg/ml) for an additional 24 hours. (e) 100x fluorescent micrograph showing Hoechst staining of tetracycline and lysozyme treated biofilm post enterococcal conjugation. (f) FISH staining of same region as (e) with probes specific for *E. faecium* only. White dashed ovoid depicts region of interest with total cell count stained blue (e) and stained red (f) with *E. faecium* cells only. Scale bar represents ten microns. Significance *** p 0.0001, significance ** p 0.002. Experiments were carried out with 6 biological repeats and five independent repeats.

5.5 DISCUSSION

5.5.1. Optimisation of biofilm formation: Enterococcal growth characteristics.

The conjugation partners MW01105^{Rif}, ST01109^{Rif}, MF04010, MF06035 and MF06036 were responsible for the creation of T1, T2, T3 and T4. They formed visible biofilm when tested with the polystyrene biofilm formation assay (figure 3.4.2). These experiments were designed to analyse and characterise biofilm formation in enterococci. Tryptone soy broth (TSB) was chosen as the standard growth medium as previous studies determined that biofilm growth phase was longer (6-8 hours versus 4 hours) as compared to brain heart infusion (BHI) (Kristich *et al.*, 2004). Spent TSB was used to induce cell starvation amongst the *Enterococcus* isolates. Conjugation was successful on *S. lacustris* and *E. fluviatilis* at 20^{o}C, near the maximum recorded temperature for waterways in the United Kingdom (Hanna and Garner, 2015). Therefore, growth characteristics at 20^{o}C were also determined to account for the feasibility of the phenomenon occurring in the natural environment.

As expected, the *Enterococcus* isolates had reduced growth rates at lower temperatures, and therefore took longer to reach stationary phase, as also observed by Morandi, (2005) when 21 enterococci isolated from dairy products displayed reduced growth rates at 25^{o}C as compared to 37^{o}C in non-fat dry milk reconstitutions. *Enterococcus* growth rates in standard TSB medium at 20^{o}C were comparable to those described by Čermák *et al*. (2009) where bacteria reached stationary phase at the 24-hour time point. Initiation of growth, duration of the log phase and establishment of stationary phase at 37^{o}C were comparable to what was observed by Pessione *et al*. (2012). Measurements of optical density and recording of values were carried out according to the same guidelines demonstrated previously (Čermák *et al.,* 2009). Mothey, (2013) used minimal growth

medium to effectively limit the growth characteristics of *Streptococcus mutans*, In this study the start and endpoint optical densities were around 0.07 (675nm). When spent TSB media was applied to *E. faecalis* and *E. faecium*, a similar growth limiting effect was observed. Spent TSB was created by filter sterilising and re-using the TSB used to grow the isolates. The average starting optical density at 570nm for each isolate was started as low as possible for the spectrophotometer and at end point, OD trended upwards consistently. This indicated that although the media was used to grow the isolates to stationary phase, growth continued either as a function of metabolic activity of the bacteria (Figdor *et al.*, 2003) or limited presence of nutrients available in the spent medium when added back to isolates for growth analysis (Ahmed *et al.*, 2014). The *E. faecalis* and *E. faecium* used in this study grew in a similar way to those grown in M1 semi defined medium, with the slight increase in optical density equal to what was observed by Zhang *et al.* (2013a). Zhang *et* al. (2013a) reported that when wild type *E. faecium* was grown in M1 medium as a negative control for other supplements, OD^{600nm} increased from 0.0025 to higher than 0.1 by the end of the experiment (15 hours). These growth curves enabled biofilm formation experiments to be created accounting for the differences in formation at lower temperatures; as well as how long it takes for the enterococci to reach log, stationary and decline phase in both standard and spent growth media.

5.5.2. Traditional biofilm assays applied to enterococci yield inconsistent biofilm.

To better understand the biofilm formation characteristics of the *E. faecalis* and *E. faecium* isolates used for conjugation, a selection of the most popular standard assays was tested. Toledo-Arana *et al.* (2001) demonstrated the ability of enterococcal strains to adhere to polystyrene and form biofilm, creating the microplate biofilm assay: reading the optical density of solubilised crystal violet as a direct measurement of biomass.

Therefore, the crystal violet assay was tested on the conjugation isolates; as well as two isolates known for their biofilm capabilities, tested previously (Daniels 2011). *E. faecium* MF01028 and *E. faecalis* MW02102 were reconfirmed as non-biofilm and strong biofilm producers, respectively. A selection criterion for biofilm formation intensity from Rosa *et al.* (2006) was applied to the other isolates.

First described by Christensen *et al.* (1982): the tube biofilm formation assay assesses identification of biofilm by visualisation of distinctive crystal violet staining on the inside surface of the cell culture tubes. Stain generally concentrates at the air liquid interface and at the bottom of the tube itself. However, results were difficult to compare between isolates and the assay itself is entirely subjective. Results from the tube formation assay were different from the more widely used crystal violet microplate assay. Hassan, (2011) assessed the capabilities of the tube formation assay against the 'gold standard' microplate assay and determined that three isolates produced false positive results and 19 were false negative. Explanations such as these determine that the tube formation assay is an unreliable assay for characterisation of biofilm formation.

The air liquid interface has been demonstrated as a relevant assay for the analysis of bacterial biofilm adherence and formation on a glass substratum, especially when applied to motile bacteria (Koza *et al.*, 2009). As *Enterococcus* are non-motile, expectations of the validity of this assay were low; barring the fact that they are facultative anaerobes, and would have relatively uniform planktonic density throughout the media for the duration of active growth. The *E. faecalis* and *E. faecium* isolates could form biofilm on glass coverslips at the air liquid interface. However, these biofilms had the appearance of an immature biofilm and had weak attachment to the glass surface. Cellular density was low considering the isolates were grown in nutrient rich biofilm media. The air liquid

interface method has not been widely used to evaluate *Enterococcus* biofilm formation. Opperman *et al.* (2009) conducted a study on biofilm formation inhibition of Gram positive (including *Enterococcus*), but not Gram-negative biofilms. This study utilised the air liquid interface biofilm model for *P. aeruginosa* and *E. coli* but not for *Enterococcus*. This is most likely due to relative motility of bacteria determining where biofilm will be deposited. O'Toole, (2011) showed motile bacteria such as *P. aeruginosa* form strong biofilm at the air-liquid interface, whereas the non-motile *S. aureus* forms strong biofilm at the bottom of the same microtiter plates. *E. faecalis* and *E. faecium* are relatively non-motile bacteria and as such would be expected to behave similarly to *S. aureus*.

The submerged coverslip biofilm formation assay is essentially the microplate assay with a glass insert at the bottom of the well to capture biofilms for imaging. This protocol was first utilised on OG1RF: a laboratory modified human oral isolate of *E. faecalis* (Guiton *et al.,* 2009) to quantify biofilm produced on polyvinyl chloride (PVC) coverslips using the same approach as the microplate assay, with an additional application of confocal microscopic visualisation. This experimental approach was used on the *Enterococcus* isolates with varying degree of success. Results obtained in figure 5.4.2(d) are comparable to what was observed by Guiton *et al.* (2009) and their wild type OG1RF. The observed appearance of *Enterococcus* biofilm shown here is a typical feature of enterococcal organisation (Lyer and Hancock, 2012). The typical *E. faecalis* biofilm appears as a static monolayer of well-spaced cells with various quantities of heterogeneous extracellular polymeric substances as observed in Barnes *et al.* (2012). Figure 5.4.2(e) highlights cell concentrating at the boundary of the biofilm. Microscopic visualisations of *Enterococcus* biofilm boundaries are rarely reported and identification of structures whereby cells accumulate at the boundary is novel. To our knowledge these findings have never been

reported before with environmentally isolated, *E. faecalis* especially one that can conjugate its tetracycline resistance genes.

The main limitation of the biofilm visualisation assays was that the processing removed large quantities of cells and extracellular material. *Enterococcus* can exist in chain form and has been suggested by Guiton *et al.* (2009) that if cells exist in this form prior to initial 'set down' in early biofilm formation that the individual cell surface is reduced, lowering available binding sites to the abiotic substrate. The microscopic processing would easily create enough mechanical sheer force to remove chains that may only be bound to the substrate by a small number of individuals. Whilst biofilm formation on plastic is initially profound, the electrostatic charges at play with polystyrene material could influence adherence once a threshold number of cells had set down (van Merode *et al.*, 2006). Therefore, a direct approach to improving cellular adhesion to substratum was employed.

5.5.3. Microplate surface coating to improve cellular adhesion and subsequent biofilm formation.

Reporting on variance and discrepancies between biofilm assays is rarely reported for enterococcal biofilm research. Leuck *et al.* (2014) described an inherent limitation of the polystyrene microplate biofilm assay which results in increased variation of biofilm formation capabilities of clinically relevant *Enterococcus* strains. Biofilm formation assay results described above highlight the variations first mentioned by Leuck *et al.* (2014) Their proposed amendments for analysis of clinically relevant biofilm formation included: measurements of biofilm formation as a fraction of direct biomass and cell density (biofilm index), as cell density may affect biofilm biomass. They suggested that a polystyrene substrate may not allow for the expression of all adhesins that would be

used in the attachment to a complex substrate such as tissue. Whilst the 'gold standard' polystyrene dish assay remains a useful tool with capabilities of correlating biofilm formation on abiotic surfaces to human infection (Sandoe *et al.,* 2003), suggestions of improvements to the assay for reduced standard deviation and relevance to infection models such as endocarditis have been made (Leuck *et al.,* 2014).

Improving biofilm formation to allow for increased substrate adherence would manage the limitations of the microscopic visualisation and characterisation of enterococcal biofilm. MF04010 and MF06035 tested positive for the collagen adhesion protein *ace* by PCR, and MF06035 tested positive for the ability to hydrolyse collagen and gelatin previously (Daniels, 2011). Therefore, investigation of improved binding assays was undertaken (figures 5.4.3 and 5.4.4). The application of type IV collagen or gelatin to the bottom of the microplate surface significantly reduced the biomass of MW01105^{Rif} and MF04010, but not MF06036 as expected based on the absence of specific collagen and gelatin interaction pathways. It was clear from figure 5.4.3 that either polystyrene itself or coating the wells with material specific to cell adherence during *in vivo* infection played a role in the biomass production of enterococcal biofilm. Re-evaluation of substrate composition and measurement of biofilm relative to bacterial cell number was measured next.

5.5.4. Substrate composition directly affects enterococcal biofilm formation.

Microscopic investigations into the *Enterococcus* biofilm are often used to examine biofilm forming ability or to assess the extent of chemically mediated biofilm destruction (Toledo-Arana *et al.,* 2001; Hancock and Perego, 2004; Dunavant *et al.,* 2006). Investigations into improvement of adhesion to aid in visualisation of characteristics have

rarely been carried out. Paganelli *et al.* (2013) used poly-L-lysine coated glass coverslips to improve cell adherence to glass for microscopic biofilm visualisation. Figure 5.4.4 details the undertaking of improving biofilm formation in enterococci. Since it has been postulated that polystyrene material composition can affect the deposition of bacteria onto its surface, glass was tested as a suitable replacement substrate for biofilm formation. The visual effect on enterococcal biofilm created by formation on glass and polystyrene has been shown by Janek *et al.* (2012). Glass is used in the majority of microscopic investigations and is an inert substance that should minimise any potential electrostatic interactions with *E. faecalis* and *E. faecium* to ones of cellular attachment only (van Merode *et al.*, 2006). Glass substrate significantly reduced the biofilm forming ability of all tested enterococci excluding MF06036. The effect of glass substrate was enough to reduce MF01028 to a weak biofilm producer from a moderate one. Clearly polystyrene has a cumulative effect on the *Enterococcus* biofilm unreflective of true enterococcal adhesion for clinically relevant biofilm formation; and glass substrate generally reduces an isolate's biofilm relative to cell number (Paganelli *et al.*, 2013). The biofilm index measurements revealed biofilm formation ability relative to cell growth, reflecting a more precise measure of biofilm (Leuck *et al.*, 2014).

Collagen and gelatin were chosen as testable substrate improvements that could be applied to glass as they best reflected the materials that enterococci attach to during infection. Application of collagen and gelatin to the glass substrate had a profound improvement on biofilm formation ability for MF01028, MW02102, MW01105^{Rif} and gelatin coating improved biofilm formation for MF04010. Bukhari, (2013) showed that collagen IV coated microplates in one instance significantly improved biofilm formation in an *ace* positive *E. faecalis* strain BS12297. Birkenhauer *et al.* (2014) noted that collagen coating of microplates improved cell surface attachments and subsequent

biofilm formation (40% increase for MRSA M05-35 against uncoated wells) in *Staphylococcus* biofilm compared to *P. aeruginosa*. They described *Staphylococcus* as inefficient biofilm producers as compared to *P. aeruginosa*. The improvement noted in staphylococcal biofilms could be associated to their mechanism (*eap* -extracellular adherence protein) of attaching to biotic substrates in a similar fashion to enterococcal attachment mechanisms (*esp, gelE*). From the literature and the results, it was clear that substrate coating was a viable method of improving enterococcal biofilm formation. Overall gelatin coating improved biofilm formation the greatest (gelatin - 62% average increase; collagen – 60% increase) amongst all tested isolates. Gelatin glass coating was also easier and faster compared to collagen coating. Therefore, gelatin coated glass was selected as the substrate of choice for all further examinations.

5.5.5. Modulation of the enterococcal biofilm: Effect of cell lysate and eNDA on formation.

Enterococcal cell lysis during early biofilm development is a well-documented phenomenon (Thomas *et al.,* 2008; Barnes *et al.,* 2012; Paganelli *et al.,* 2013 and Dunny *et al.,* 2014). Fratricidal lysis and subsequent DNA release can contribute towards enterococcal biofilm formation and eDNA is an integral component of early biofilm formation. Therefore, two experiments were devised to test the presence of total cell lysate or total DNA content on the *Enterococcus* isolates. Dunny *et al.* (2014) discussed enterococcal fratricide and biofilm development: cells sensitive to the peptide lactone GBAP express the fratricidal inducing *GelE* and the immunity from fratricide *SprE* genes. Cells insensitive to GBAP, or immunity mutants would not be protected from fratricidal lysis. Therefore, the conjugation partners MW01105^{Rif}, MF06036 and T1 had their total cell lysate tested on each other as well as self-tested. The lysate from MW01105^{Rif} had

no effect to biofilm formation; however, MF06036 and T1 lysate had a significant effect on biofilm formation when tested against each other and self-tested.

Barnes *et al.* (2012) demonstrated potential non-fratricidal sources of eDNA for the accumulation of enterococcal biofilm during early development, as DNA was present in abundance with cellular lysis. Therefore, MF06036 DNA content was applied to biofilm formation investigations as shown in figure 5.4.6. In all cases, excluding MF01028 and MW02102, biofilm formation increased with the application of total DNA content. Increases with ST01109^{Rif} and MF04010 were significant. The data in figures 5.4.5 and 5.4.6 show general increases in biofilm formation capabilities in the presence of total cell lysate or eDNA. These data support the experiments conducted by Paganelli *et al*. (2013): whereby an autolysin deficient *Enterococcus* was resistant to lysis, released less eDNA and reduced biofilm formation. Rostami *et al.* (2017) discussed stabilisation roles for eDNA in multispecies biofilm as well as showing that DNA degradation reduced the diversity of multi-species biofilms: suggesting that DNA is more than scaffolding but rather anchoring points for other species of bacteria. Conclusions can be made that the importance of eDNA may lie beyond stabilisation and improvement of biofilm formation. Experimental levels of SDS were diluted at a final concentration (0.01%) determined to be essentially SDS-free by Post *et al.* (1998) and therefore would not adversely affect results.

Surface adhesion was generally improved with the addition of gelatin to the inert glass substratum. Additions of cell lysate and eDNA improved biofilm formation for select enterococcal single species biofilm. Compounding effects of substrate enhancements, cell lysate and eDNA were not equal to the effects observed on an individual basis. It appeared at least in this case that once maximum biofilm formation had been achieved strength

could not be improved. However, these conditions improved retention when processed for microscopic visualisation of biofilm on glass coverslips; but when manipulation of coverslips was required; it still created damage to biofilm.

5.5.6. The gene frame biofilm apparatus (GBA): A novel biofilm development tool for microscopic visualisation of fragile enterococcal biofilm.

It was easy to damage biofilm when mounting the coverslip to a glass slide. An alternative approach/apparatus was required to preserve samples as much as possible during the processing. There are numerous pieces of equipment for modelling biofilms, minimising intrusions caused by the aforementioned processing: such as the drip-flow biofilm reactor (DBR), Calgary biofilm device (CBD) and the tubular biofilm reactor (TBR) (Xu *et al.*, 1998; Ceri *et al.*, 1999; Winn *et al.*, 2014). Equipment such as these are highly specialised, can be expensive and time consuming to set up. There existed no such model for examination of static biofilm. Many reactors involve insertion of coupons, chips or other inclusions and require physical removal for processing in the same fashion as the coverslip biofilm method.

The gene frame biofilm apparatus was born from the need to analyse biofilm created by bacteria *in situ*, without exposing 'naked' biofilms to mechanical stresses associated with visualisation assays. It needed to be able to allow for attachment and growth of mature static biofilms fixed in position on a useful substrate such as a glass microscope slide. The gene frame was created by ABgene primarily for *in situ* PCR investigations of mammalian cells. It was repurposed here to allow for creation of biofilms that could be imaged without hindrance. The system could be sealed and made sterile. Low and medium power magnification was possible without mounting and could be carried out at

any time during the biofilm growth phase. Figure 5.4.7 outlines the process of using the gene frame biofilm model. One of the most valuable uses of this method is that the gene frame creates a micro-well (25µl) whereby the biofilm can be grown to a specific size: nutrient conditions can be altered without damaging the biofilm and full staining protocols can be carried out in the well itself. The GBA allowed for detailed investigations of enterococcal biofilm that previously were difficult to obtain with adequate precision. Comparisons of the GBA against traditional assays were made before adopting the assay fully.

Instances of biofilm retention, detached biofilm when mounting and cellular retention were measured and compared between 'traditional' assays and the GBA using *E. faecalis* MW02102. Improvements of biofilm and cellular retention are clearly evident in figure 5.4.8. The improvements attributed to the GBA were significant therefore; the GBA was employed to assess all future biofilm experiments.

5.5.7. Identification of macro-scaffolding and cellular distribution of enterococcal biofilm using the GBA.

The gene frame allowed for unobtrusive analysis of enterococcal biofilm, revealing novel macro-organisation of adhered biofilm. Figure 5.4.9(a-b) presents low powered images of MF04010 biofilm. MF04010 was the best biofilm producer of all enterococci used in this study that also was responsible for creation of a transconjugant. These biofilms were grown in the GBA, carefully washed and imaged with dark field microscopy. Subfigure (b) had concanavalin A (conA) Alexa fluor 594 staining applied, but was processed in the same fashion as in subfigure (a). The branching macro structure was composed of cells, could only be visualised in unopened GBA and the feature was removed upon opening and staining of the biofilm. This form of novel structure could not be found in the

literature with reference to *E. faecalis* and *E. faecium* using low powered fluorescence microscopy.

Biofilms formed under enclosed laminar flow can create strings of biofilm called ‘streamers’ from mature colonies of biofilm (Wang *et al*. (2016). The slower the flow the thicker these streamers will become. Enterococci isolated from the natural environment have never been shown to form streamers, however *S. aureus* has been shown to form streamers (Kim *et al*., 2014). Streamers are the result of deformation and or detachment from the main body of biofilm (Biswas *et al*., 2016). As the GBA is an enclosed environment, where cells cannot escape form the mature biofilm, it is possible that seeding of cells from biofilm is incomplete and can form a structure with similar appearance to streamers. De-attachment and reattachment has been shown in staphylococci previously and could occur with enterococci and the unique characteristics within the GBA (Fux *et al*., 2003). Streamers have weak attachments and are difficult to image (imaged *in situ*). Likewise, the novel structures identified in figure 5.4.9 had weak attachments and could be moved and dislodged by moving the GBA whilst imaging on the microscope.

Another explanation for the ‘thick streamers’ observed in figure 5.4.9 could be a result of alteration in genetic regulation, due to the sealed environment creating atypical gradients of growth as a result of nutrient availability or chemical composition (Sauer *et al*., 2002; Van Loosdrecht *et al*., 2002). Additionally, Hall-Stoodley *et* al. (2004) illustrated connecting structures between mature colonies of biofilm which have the same appearance as the structures observed in figure 5.4.9.

As with all pure microscopic visualisation, the possibility that this observation could be from some of ‘artefact’ cannot be definitively ruled out without the use of more advanced microscopic techniques. This was not possible during this research investigation and is

definitely a result that could be developed with methods of laminar flow applied to the GBA.

The typical appearance of enterococcal biofilm is exemplified by Janek *et al.* (2012) where cells aggregate around non-branched EPS boundaries with strain specific cellular morphology. The biofilm stain conA can be seen at this low power magnification displaying localisation with the bacterial cells. High power magnification of this phenomenon was not possible as it interfered with the macrostructure observed here. Experiments utilising high power magnification are shown in subfigure (c-d). Subfigure (c) highlights the strong cellular attachment and biofilm under the same conditions as (a-b). Higher resolution of conA staining was observed in (c) from the heterogeneous macro staining from subfigure (b). The GBA was tested for proficiency at detecting early 'set down' of enterococci and exceled at imaging diplococci attached to the gelatin coated substrate without the need for high resolution electron microscopy as observed in Barnes *et al*. (2012).

At the conclusion of these experiments the GBA was deemed a successful method for the analysis of enterococcal biofilm as it had unveiled new structural organisation of *E. faecalis*. These structures were never seen before for enterococci isolated from the environment, and was accomplished using a simple epifluorescent microscope, cheap materials, was easy to assemble, sterilise and used an emerging enterococcal biofilm stain (conA).

5.5.8. Enterococcal biofilm development mediated by the gene frame biofilm apparatus (GBA).

The GBA detected initial cellular adherence to the substratum allowing visualisation of enterococcal biofilm development using fluorescent staining. Numerous experiments

have analysed in detail many aspects of enterococcal biofilm composition, cellular activity and factors at play as reviewed by Fisher and Phillips, (2009), and Gilmore *et al*. (2014). Nonetheless visual investigations of initial cellular attachment and production of extracellular polymeric substances (EPS) are limited (Bales *et al.,* 2013). Previous investigations have focused on EPS in mature biofilm (Jung *et al.,* 2015). The regulation of adhesins and other factors can greatly influence biofilm formation. As a result, enterococcal biofilm structure at any stage of development can vary species to species or even from isolates of the same species (Dunny *et al.,* 2014). Therefore, investigations into biofilm 'development' regarding cellular composition and production of EPS were carried out.

The use of conA as a stain for EPS in biofilm formation has only recently been established (Jung *et al.,* 2015; Ridan and Benxiang, 2014). Production of EPS occurs during enterococcal biofilm formation and since conA binds to polysaccharide residues, it can be used as a selective stain to examine biofilm formation in enterococci. The EPS fromation during biofilm development as observed in figure 5.4.10 is direct evidence of biofilm development, as cell count increases in tandem with biofilm until EPS staining overcomes the co-localisation with DNA staining as seen in mature biofilm (figure 5.4.10(vi)). Instances of conA EPS staining was lower in enterococci with chain forms. Providing further evidence that, enterococci that exist in chain form do not form biofilm at the same level as counterparts in diplococcus form. Guiton *et al*. (2009) demonstrated that *Atn* (autolysin) deletion *E. faecalis* mutants caused adherence defects, subsequently increasing chaining, potentially reducing binding availability. They suggest that chaining phenotype is an inefficient morphology for biofilm formation which agrees with the decreased conA staining and observed here.

5.5.9. Effects of nutrient deprivation and conA staining to demonstrate biofilm formation in a strong biofilm producing enterococcal isolate.

Nutrient deprivation and antimicrobial stress investigations into enterococcal biofilm formation have been studied previously (Liu *et al.,* 2010; Ran *et al.,* 2015). These studies utilised reference strains *E. faecalis* ATCC 29212 (isolated from human urine), and ATCC 33186 (animal isolated quality control strain). Studies into starvation of enterococci isolated from the natural environment are lacking. Therefore, investigations into the effects of nutrient deprivation were carried out on the environmentally isolated *E. faecalis* and *E. faecium* are discussed below.

Enterococcus faecalis MW02102 created a strong biofilm with extensive cell chain form characteristics (5.4.11) in contrast to evidence of weak biofilm attachments to substratum in chain form as discussed by Guiton *et al*. (2009). This isolate was the only one tested that existed in chain form in a mature biofilm on such a large scale. Starved biofilm eliminated the chain from observed under standard growth conditions. This could reflect the reduction of cellular metabolism and as such, reduction of cellular replication (Giard *et al.,* 2000). The cells enter a state whereby they conserve functions to survive longer. ConA staining is markedly reduced in MW02102 indicating a lack of production of EPS. Hoechst overstaining typical of eDNA presence is reduced in starvation and cellular boundaries are clearly visible. Liu *et al*. (2010) demonstrated the persistence of biofilm forming ability of *E. faecalis* (ATCC 29212) during starvation using both PBS and nutrient depleted BHI on human dentin substrate. They identified that the nature of substratum can affect enterococcal biofilm formation, suggesting that substratum comprising human elements such as dentin improved biofilm formation in a similar fashion to collagen and gelation as observed in figure 5.4.4. They also corroborate the findings of Lleo *et al.* (2007) stating that starved enterococci form the weakest biofilm as

compared to standard growth conditions. The starvation biofilm formation data obtained in figure 5.4.11 agrees with the literature findings with identifiable low density biofilms, as expected with limited nutrient availability. Chen *et al.* (2017) using *E. faecalis* (ATCC 33186) also argued that starvation and increased alkalinity observed in the oral cavity reduces water soluble polysaccharides as a defence mechanism from increased pH stress and therefore reduces biofilm biomass. They reported that starvation alone was the biggest contributor to reduced biofilm formation when using nutrient depleted TSB.

Additional instances of cell concentrating at the biofilm boundary seen under subfigure (b) further highlights the validity of this phenomenon described in 5.4.2. The reduction in conA staining observed in figure 5.4.11 agrees with the results reported from Chen *et al*. (2017). The channelling effect observed in the MF06035 biofilm in figure 5.4.11(c) has never been reported for enterococcal biofilm formation on glass substrate. Explanations as to the occurrence of this phenomenon could be that the bacterial biofilm formation lead projection sites occur in this way; initial microfluidics at play placed cells into these channels (Kim *et al.,* 2012); or imperfections of gelatin surface coating beyond what was observed in surface coating imperfection testing (data not shown) gave way to these channels. The apparent ‘zone of clearing’ of gelatin coated microscope glass around aggregates of cells, has the appearance of MF06035 mediated hydrolysis of the gelatin into the surrounding media and cells (Su *et al*., 1991).

5.5.10. Effects of nutrient deprivation and ConA staining to demonstrate biofilm formation in a "no-biofilm" producing enterococcal isolate.

E. faecium MF01028 was classified as a non-biofilm producing isolate based on several biofilm experiments carried out here and previously by Daniels, (2011). However, during starvation testing it was noted that adhesion to glass substrate was extremely high as compared to nutrient conditions. Microscopic investigation into the biofilm morphology during starvation was carried out as seen in figure 5.4.12. The first observation was that this non-biofilm producing *Enterococcus*, when placed in the GBA with gelatin coating, formed attachments to the substrate. The most immediate difference between growth conditions with DNA staining was that the cellular distribution was more uniform under starvation and there were less instances of aggregation of several enterococci with high cellular density. This effect has been noted throughout this research study.

The most striking effect is the increased detection of EPS during cellular starvation. Evidence from the previous paragraph suggests that EPS (or specific elements of its composition) decreases when known biofilm producers are starved. Liu *et al.* (2010) used a direct measure of CFU per biofilm block to conclude that biofilm was "weakest" under starvation. They did not assess EPS composition directly, regardless of cell population at the end of biofilm formation. Chen *et al.* (2017) employed dry weight measurement and examination of polysaccharides to conclude that biomass decreases with starvation in a known biofilm producer. Both groups utilised known biofilm producing strains of *E. faecalis* and did not use a non-biofilm producing control in their investigations. The phenomenon, of increased EPS production through increased staining, only occurred in a known non-biofilm producing isolate in this investigation.

The work carried out for this thesis and the literature in question both have compelling evidence showing that under starvation, cells have limited growth rates which functions

to lessen biofilm development, as there will be a smaller population of biofilm state bacterial cells. In support of these contradictory findings, Gao *et al.* (2016) demonstrated that during starvation in a multi-species biofilm reaction that enterococci form improved biofilms and resist starvation more effectively, even with limited growth. These improved biofilms excluded cell population in biofilm assessment, similarly to the assessment carried out in figure 5.4.11 and 5.4.12. Ran *et al*. (2015) showed increased levels of *gelE* and *ace* expression when enterococci were starved. These elements improve attachment and biofilm formation on collagenous and gelatinous substrates, such as human tissue. Starvation induction in *E. faecalis* MF01028 and in *E. faecalis* described by Gao *et al.* (2016) and Ran *et al*. (2015) could prime cells to form strong attachments and biofilm to defend against prolonged starvation as a survival mechanism. This would not be observed unless direct assessment of EPS formation relative to surrounding cells was assessed, as shown in figures 5.4.11 and 5.4.12.

Quantification of conA staining was required in order to make such comparisons, as outlined in these last two paragraphs. Quantification of conA staining has been accomplished previously for EPS production in biofilm by Mueller *et al.* (2006). They captured confocal images of EPS staining with conA on marine phototropic biofilm and were quantified using image quantification software. Quantification of conA staining in *E. faecalis* biofilm had never been carried out and with the previous report of Mueller *et al*. (2006), was done so as follows. Measurements of pixel intensity of red fluorescence for conA staining in figure 5.4.11 and 5.4.12 was accomplished and is shown in 5.4.12(c). As evidence demonstrates, there was no significant difference in conA intensity for the strong biofilm producing MW02102 during starvation and normal growth. However, there was a significant ($p<0.0001$) difference in conA staining intensity for the non-biofilm producing MF01028 during starvation and normal growth. Combining the

evidence shown here and investigations into starvation, conclusions on conA staining can be made. ConA is a useful stain for investigating enterococcal biofilm forming ability and re-evaluation of adherence for non-biofilm producing isolates should be considered when performing examinations into enterococcal biofilm formation at the microscopic level (Jung *et al*., 2015). ConA staining, direct assessment with microscopy and image quantification revealed for the first time, an increase in EPS production because of cell starvation in *E. faecalis* that tested negative for biofilm formation using standardised biofilm assays.

5.5.11. Antibiotic selective pressure and nutrient deprivation affects spatial density, chain forming characteristics, granularity, and size of MF06036 early biofilm cells.

Investigations into MIC of antimicrobials have previously demonstrated a profound effect on enterococcal cellular morphology (Chau *et al.*, 2011). Using flow cytometry to analyse stress from daptomycin and vancomycin, Chau *et al*. (2011) determined that 0.25 xMIC (0.5µg/ml) therapy increased bacterial cell size (up to 300% for resistant isolates) significantly ($p=0.02$). Additionally, confocal microscopy revealed that bacterial chain dysmorphology was apparent after just 60 minutes of 1 xMIC treatment. Vancomycin sub inhibitory concentrations in the resistant MF06036 were providing some irregularities when imaging for biofilm experiments (data not shown). Combining these phenomena with the observed changes identified under nutrient deprivation, investigations into the effects of sub inhibitory concentrations of vancomycin on MF06036 were investigated, as seen in figure 5.4.13.

MF06036 under planktonic growth conditions existed in chain form, but when established in biofilm, chain form is mostly abolished (figure 5.4.13(a)). The decreased cell density associated with cellular starvation are visible (subfigure (b)). Conditioning biofilm

growth with sub MIC of vancomycin retains cell density but it appears that cell density is altered, and cell size appears increased. Pre-conditioning MF06036 cells during growth prior to, and during biofilm growth yielded the most striking changes to cellular morphology. The formation of chains was observed in the biofilm state. Babic *et al.* (2011) demonstrated that cells can propagate genes by conjugation down through the chain from the lead member. This therefore could be a defence mechanism employed by MF06036 in response to bactericidal stress. Gholia *et al.* (2004) demonstrated reduced adhesion to uroepithelial cells in *Klebsiella pneumoniae* in the presence of ceftazidime and ofloxacin. When treated with amikacin, *K. pneumoniae* demonstrated increased adhesion to uroepithelial cells. Although *K. pneumoniae* and *E. faecalis* are unrelated microorganisms, specific antimicrobial stress can affect bacterial cellular ability to adhere to biotic surfaces. It is therefore possible that antimicrobial stress can affect biofilm formation ability.

Regarding MF06036 under vancomycin stress, there appears to be a reduction in biofilm forming ability with reduced cell numbers. This could be explained by the increase chaining, death rates of subpopulations of cells without *vanA* gene presence, or an effect on adhesion as described by Gholia *et al.* (2004). With visible increases in cell size as observed in figure 5.4.13(d) analysis of cell sizes were carried out by quantifying images in Image J. It was revealed that there was no significant difference ($p>0.05$) in cell size between starvation and normal growth conditions. Hartke *et al.* (1998) demonstrated that *E. faecalis* cells do not decrease in size with starvation stress. Vancomycin additions significantly impacted the cell size of the tested enterococci. Vijaranakul *et al*. (1995) demonstrated increases in *S. aureus* cells under high levels of ionic stress, and Paulander *et al.* (2014) confirmed that bactericidal antibiotics increase bacterial cell size. These reports confirm that it is possible for Gram positive bacteria to increase in cell size under certain stress and corroborates what was observed in this study.

After biofilm formation visual characterisation experimentation, it became apparent that the GBA could be utilised for cytogenetic identification of enterococcal biofilm cells without histological processing. Therefore, a fluorescent *in situ* hybridisation (FISH) assay was devised for cytogenetic investigations.

5.5.12. Creation of a fluorescent *in situ* hybridisation protocol for the examination of *Enterococcus* cells in biofilm states.

FISH studies into enterococci have been accomplished previously (Waar *et al.,* 2005; Al-Ahmad *et al.,* 2009). However, there is no simple way to identify *E. faecalis* and *E. faecium* in biofilms. The GBA was a suitable model to allow for examination of enterococcal presence in biofilm using FISH, therefore a method was devised and optimised for the identification of enterococci in biofilm state. Probes were sourced from a study that used a rapid identification assay to identify *Enterococcus* species (Wellinghausen *et al.,* 2007). Clear cellular localisation of FISH probes for *E. Faecalis, E faecium* and a general bacteria (EUB) probe were all working for enterococcal biofilm: optimised for minimal background signal and zero instances of autofluorescence as observed in figure 5.4.14. This method could be applied to any *in situ* investigations of multispecies biofilms to examine any interactive roles between species.

5.5.13. Utilisation of FISH to detect vancomycin resistant *E. faecalis* in the gene frame biofilm apparatus (GBA).

Visualisation of enterococcal genes inside biofilm was demonstrated in figure 5.4.14, therefore investigations into the examination of mobile genes was attempted in enterococci for the first time. The *vanA* gene is one of the most prolific and clinically relevant genes relating to enterococcal infections and is widely studied, as described in Gilmore *et al*. (2014). Investigations into the presence of this gene in biofilm would be of

value to gain a deeper understanding of the conjugal interactions of transferring *vanA* inside bacterial biofilm

Initial investigations were unsuccessful. Success in the laboratory for this investigation was accomplished through a multi-probe approach targeting the same gene. The assay is specific, as seen with the negative binding control tested with the application of FISH probes to the *vanA* negative *E. faecalis* MF04010. Probes designed to bind to the *vanA* plasmid bound gene, demonstrate positive staining not observable in previous experiments with a single probe approach. The staining patterns observed on MF06036 are entirely novel. Fratricidal sub populations of MF06036 allowing for ease of access of probes to the genes within the cells would be a potential explanation for the observation of heterogeneous instances of bright cells found within established biofilm conditioned with vancomycin. This explanation coincides with explanations from Thomas *et al*. (2008), whereby they described pockets of eDNA staining spread heterogeneously throughout the biofilm. Seneviratne *et al.* (2017) suggested that the phenomenon of consistent enterococcal antibiotic resistance in biofilm state regardless of biomass, could be the result of a population of highly resistant cells spread within the biofilm. These "persisters" were described by Lewis, (2008). Cook *et al*. (2011) described the phenomenon of heterogenous increases in enterococcal plasmid copy number in biofilm state. These cells would then have larger copy number genes with the potential to rapidly propagate plasmids to recipient isolates, as well as being resistant to corresponding antibiotics. It is believed that bacteria may exist in this form to keep most of the metabolically intense conjugation machinery in a small number of individuals until conjugation is required. The novel staining patterns in figure 5.4.15(b) are characteristic of the descriptions of persister cells on a biofilm exposed to antibiotic stress. Hybridisation assays were created with influence from Waar *et al.* (2005); who were early

adopters of examination of enterococcal morphology and spatial localisation *in situ* with embedded biofilm samples. This assay utilised 16S rRNA probes for speciation of enterococci, using the modified the Waar *et al*. (2005) method for rapid identification of pheromone genes (iCF10 compliment for the pCF10 tetracycline plasmid) in planktonic enterococci. This protocol also used probes with high melting temperatures (77^{o}C and 79^{o}C) and selection permeabilised cells using propionium iodide staining for flow cytometry. Whilst the method described in this PhD investigation involved growing 24-hour biofilm and application of a 24-hour probe protocol; improvements in speed of the assay and application to affixed pre-established biofilms are entirely possible.

Zwirglmaier *et al.* (2003) were the first group to develop a FISH method for identification of low copy number genes, with less than 10 copies per cell, inside plasmids. They were able to detect low copy number genes for beta lactamase AMR genes in *E. coli* and coined the assay name 'recognition of individual genes' (RING) FISH. Whilst the assay was innovative, it required *in vitro* transcription of polynucleotide probes and in-house labelling with fluorochromes interspaced every 10-20 nucleotides. This assay yielded probes up to 1200 base pairs long which gave a bright signal when imaged with fluorescence. Due to the maximum size of probes and their degree of size variation, the staining pattern was that of a halo localised around the periphery of cells. This assay has never been applied to enterococci and whilst it is novel, its adoption has not become widespread as a microbiological FISH assay. The multiprobe oligonucleotide FISH developed in this research project improves upon some of the limitations imposed by RING FISH. Probes were created using a commercial supplier, were all the same length and had the same annealing temperatures. These improvements were crucial in the identification of *vanA* within *E. faecalis* MF06036 without the need for extensive molecular approaches.

Using oligonucleotide FISH in this fashion has never been used for identification of mobile element bound AMR genes in planktonic enterococci, let alone in biofilm cells, and reflects the ability of this assay to identify low copy number genes.

The goal of this research study was to visually assess, true, repeatable conjugation events inside the biofilm of enterococci. The GBA was utilised successfully to analyse biofilm *in situ*. The created biofilms were of adequate size to afford sufficient precision between experiments. Therefore, the GBA was further modified to be able to recover scrapings for biofilm conjugation investigations. The GBA had an additional advantage, in that prior to scraping; biofilms could also be processed for microscopic visualisation of conjugation using all the assays developed in this research project thus far.

5.5.14. Recovery of transconjugants from inside enterococcal biofilm.

The GBA allowed for fully contained, sterile enterococcal biofilms to form. These reactions could be opened and resealed under sterile conditions to allow for multi-stage conjugation assays to be performed in biofilm, whilst eliminating planktonic enterococci from any results. As T1 was the first isolated transconjugant and had the most relevant vancomycin resistance phenotype, it was used, along with its parental isolates to assess if the GBA had an ability to capture biofilm conjugation. The first assay followed the protocol illustrated in figure 5.4.14, whereby several biofilms were created with either MW01105^{Rif} or MF06036, washing the biofilms and introducing the conjugation partner. As seen in figure 5.4.17, successful isolation of transconjugants from enterococcal conjugal biofilm was possible. The nature of the protocol (Figure 5.4.16) ensures that planktonic conjugation is eliminated, due to the removal of all non-adherent cells prior to the addition of the conjugation partner. This is the first observation of true enterococcal conjugation on or within biofilm in enterococci.

Biofilm containing both parents introduced at the same time was also created (mixed biofilm). This practice of having biofilms with both conjugation partners has been reported previously using circular aclar membranes in six-well plates to grow biofilm using BHI broth (Cook *et al.,* 2011). However, their data cannot account for the role of planktonic conjugation reactions occurring prior to, and during the early formation of biofilm. The potential role of planktonic conjugation can be observed in figure 5.4.17 where the mixed biofilm produced significantly more transconjugants (p 0.002) than the MF06036 biofilm. As the isolates were selected from agrarian ecosystem where nutrient conditions can be poor (compared to laboratory conditions) and biofilm formation can be altered based on these conditions, repeat biofilm conjugation experiments were performed under nutrient deprivation (cell starvation) using the same depleted media that was used in figure 5.4.1. The result of this experiment (figure 5.4.17) yielded conjugation recoveries roughly 50% lower than the experiment performed under standard experimental nutrient conditions. Marcinek *et al.* (1998) reported that enterococcal conjugation under the natural conditions present within municipal sewage treatment (and animal GI tract) such as low temperature, oxygen saturation and various toxic chemical compositions decreased significantly. Transfer of the sex pheromone plasmids pAD1 and pIP1017 had a 10^5 lower conjugation rate under these "natural conditions" compared to laboratory conditions. Transfer frequency was ten-fold lower for Tn916. Temperature and nutrient availability were the only factors altered in our laboratory experimentation and the observed reduction in conjugation frequency follows the trends observed by Marcinek *et al.* (1998). The reduction in efficiency is secondary to the significance of conjugation in any form in our system. The novelty of this experiment lies in the fact that even under nutrient deprivation, these enterococci form biofilm, and they successfully conjugate AMR phenotypes under some of the stress observed in the agrarian environment. These

findings corroborate the statements from Marcinek *et al.* (1998), where they conclude that conjugation should take place under "natural conditions".

Cook *et al*. (2011) grew a mixed biofilm where mating most likely occurred in the liquid phase, as sex pheromone signalling, especially pCF10 has been shown to occur planktonically. Elimination of the variables had not been carried out therefore conclusions can be made that the majority of the observed effect could have occurred during the first four hours of growth that the authors allowed for mixed conjugal partner biofilm formation during experimentation. The GBA method used in this research study grew single partner biofilms, and then added the conjugation partner after removal of non-adherent biofilm cells. Secondarily Cook *et al.* (2011) grew mixed biofilms in growth media for 24 hours, where transconjugants can replicate freely increasing pre-biofilm transconjugant CFU; whereas in this study conjugation was carried out without addition of growth media after addition of the second partner and even a total starvation experiment; which yielded biofilm transconjugants analogous to what may be conceived to happen in the extra enteric environment.

To date this is only the second report of enterococcal conjugation recovery from a biofilm state after Cook *et al.* (2011) however, it is the first report to demonstrate definitive *in vitro* biofilm conjugation. The experiments were performed on environmentally isolated *E. faecalis* strains with a spontaneous rifampicin mutant recipient. The experimental conditions employed in this study are closer to the conditions present in the environment from which these enterococci were isolated, than the laboratory conditions found in the literature. A continuation of this research would aim to further simulate the environmental conditions at the isolation sites of the enterococci used here.

5.5.15. Using double selection with rifampicin and vancomycin to eliminate conjugative partners but not their transconjugant in a biofilm state.

After confirming biofilm conjugation through the recovery of transconjugants by means of destruction and homogenisation of biofilm, attempts to observe the transconjugants without removing them from solid phase were carried out. After significant optimisation and testing various approaches to experimental design, the final protocol of eliminating parental isolates *in situ* and visualising transconjugants with fluorescent stains was employed. The first step in the process of successfully visualising T1 was applying selection conditions to the parental isolates MF06036 and MW01105^{Rif} and analysing the cellular effects. Therefore, rifampicin and vancomycin were applied to single species reactions for 24 hours, and enumerated with live dead fluorescent staining as shown in figure 5.4.18. The results of this experiment yielded information on the survivability of T1 over its parents under selection conditions. The concentration of double selection used prevents the growth of the parental isolates on TSA plates.

5.5.16. Application of triple selection (lysozyme in tandem with double antibiotic selection) to destroy conjugative partners but not their transconjugant.

The double selection proved effective at killing and inhibiting parental isolates. However, the cells were still present in the system and for this assay to be effective, parental isolates needed to be eliminated. Further investigation into the removal of those isolates whilst leaving the transconjugant wholly intact was initiated. An additional biocidal agent was required to remove all traces of live staining to improve the assay. Lysozyme was chosen as it hydrolyses the *N*-acetyl glucosamine, *N*-acetyl muramic acid bond in peptidoglycan, therefore weakening the bacterial cell wall leading to cell lysis (Pellegrini *et al.*, 1992). Lysozyme is effective against Gram positive bacteria and used in combination with vancomycin, the effect of bactericidal activity and (cellular destruction) should be

multiplied (Nash *et al.,* 2006). As seen in figure 5.4.19 (a) lysozyme alone had MIC values greater than 8 and less than 16 mg/ml; these values fall within the previously observed ranges for enterococci (Varahan *et al.,* 2013). MW01105^{Rif} and T1 had similar MIC's which was expected. Interestingly when lysozyme was added in tandem with antibiotic double selection the MIC's for lysozyme were reduced. The synergistic effect of both double selection and lysozyme was apparent, and revealed the same trend of inhibition observed in figure 5.4.17. Using lysozyme in combination with antibiotic selection has not been reported for enterococci before. T1 had a combined MIC four times higher than MW01105^{Rif} and eight times higher than MF06036. Therefore, validity of the assay was confirmed.

The experiment shown in figure 5.4.19 (a) was repeated for 5.4.19 (b) with an additional data bar type, representing compromised cells. These were bacteria which had cellular dysmorphia or were destroyed. Using fluorescence to measure the effects of selection was adapted from Chau *et al.* (2011) where they microscopically visualised the action of vancomycin and daptomycin sub-MICs on vancomycin resistant (*vanA*, and *vanB*) and susceptible *E. faecalis* and *E. faecium*. Their VRE isolates had similar MIC's to the VRE isolates used in this thesis (256 and 512µg/ml). The examples of the variation in positive (green) staining are shown in figure 5.4.19 (c-e). MW01105^{Rif} had a total kill count with zero positive staining, and MF06036 had 3% positive staining, however when factoring in compromised cells a total kill count was revealed. T1 easily survived these extreme selection conditions. This method has proven to effectively eliminate the parental isolates whilst leaving the majority of transconjugant cells unharmed. The work outlined with this protocol highlights the limitations of Live/dead imaging, and that it is possible to visualise transconjugants using a simple, cell permeable/impermeable stains. Thus, FISH assays were being created to aid in the detection of enterococcal transconjugants in this system.

5.5.17. Microscopic visualisation of *in situ* conjugation in biofilm using selection, Live/dead imaging and fluorescent *in situ* hybridisation.

It is well known that enterococci have high level resistance to lysozyme (>62mg/ml) (Le Jeune *et al*., 2010). The enterococci used in the triple selection assay were at the lower end of the lysozyme resistance spectrum with a maximum MIC of 16mg/ml. The effect of lysozyme was compounded when added as a third agent for the killing and destruction of cells. Lysozyme can elicit bactericidal degradation of the cell wall of enterococci (Varahan *et al*., 2013). The action of lysozyme functions to digest glyosidic bonds within peptidoglycan on Gram positive bacteria regardless of cell metabolic activity (Davis and Weiser, 2011). The effects of all three antimicrobial selection agents used in this experimentation with sufficient time, would be able to degrade susceptible enterococci to such an extent that cellular morphology would be rendered unrecognisable compared to resistant cells.

This triple selection assay has revealed for the first time transconjugants existing *in situ* biofilm that were created in the system. Whilst the assay has limitations (potential human error in the perception of normal/abnormal cellular morphology), it remains a cost effective, low technology, viable method, to investigate the novel HGT interactions that are now known to naturally occur in biofilms with little external interference.

Live dead imaging is a useful tool for determining cell permeabilization, especially with vancomycin (Chau *et* al., 2011). Increases in red staining does correspond to cell death (Berney *et al*., 2007). However, it is still possible to visualise dead cells as false positive (live) cells, therefore a comprehensive FISH protocol was devised to stain transconjugants *in situ*. As one of the transconjugants was the result of an interspecies reaction, FISH probes to select for the recipient *E. faecium* would immediately exclude all donor isolates. Applying selection conditions to eliminate the remaining recipients

would leave only the transconjugant inside the biofilm. The reasoning for this selection comes from the work by Malic *et al*. (2009), where they used FISH probes in combination with a general DNA stain (DAPI) to differentiate *E. coli* (DAPI staining minus probe fluorescence) from *S. enterica* (red probes) and *L. monocytogenes* (green probes) in multi-species biofilms.

Applying the ENU 1470 *E. faecium* FISH probe to a conjugal biofilm of ST01109^{Rif} and MF06036 after double selection with tetracycline and lysozyme yielded a positive result (figure 5.4.18 (e + f). Sub-figure (e) showing total cellular staining of surviving enterococci (MF06036 and transconjugant T3) and in sub-figure (f) transconjugant T3 is highlighted with the *E. faecium* FISH probe. The blue Hoechst staining shows all cells organised in clusters, clumped together and with the overlay of red FISH staining, the transconjugants can be identified and appear to be in contact with the donor cells.

Utilising FISH, in tandem with the selective killing of parents, freshly created enterococcal transconjugants were visualised inside biofilm for the first time. This FISH assay is wholly adaptable, and if specific sequences are known to exclusively lie within an isolate of interest, then fluorescent visual selection filters can be applied to this system in a similar fashion more specific and cost effective than immunofluorescence.

Chapter 6

General Discussion

From the mid-20th century bacterial infection of humans had been well controlled with the development and use of antibiotics (Aminov, 2010). Resistance would emerge, but so too would new antibiotics. Synthetic antimicrobial compounds were created as an effective replacement therapy for the 'older' antibiotic resistant infections that were becoming more prevalent (Walsh, 2003). As time passed the healthcare and pharmaceutical sector evolved their strategies to keep up with bacterial antibiotic resistance evolution (Davies and Davies, 2010). Mortality associated with bacterial infection in the developed world practically disappeared as infection control was highly successful (Dowling, 1977). Examples such as the mass fear of death from tuberculosis ("consumption") that plagued western society in the late 19th and early 20th century vanished (Bloom and Murray, 1992). Generations of people were raised in the golden age of antibiotics with little to no first-hand experience of serious bacterial infection (Levy and Marshall, 2004).

The significance of the evolution of antibiotic treatments went mostly unnoticed to the public. Antibiotics became synonymous with mild afflictions such as head colds and other minor self-clearing infections (Hawker *et al.*, 2014). These generations of people had limited understanding of the lifesaving origins of antibiotics. As western society modernised throughout the golden age of antibiotics, these drugs were essentially repurposed, overused prophylactically, and their use spread into various non-therapeutic functions such as growth supplementation in livestock farming (Dibner and Richards, 2005, English and Gaur, 2010). This format of introduction, control of disease and then misuse of antibiotics has followed every society during proliferating prosperity (Levy and Marshall, 2004). Efforts to create new antibiotics by pharmaceutical companies were practically abandoned and now the cycle of new antibiotics to combat old antibiotic resistance has been essentially broken (Bassetti *et al.*, 2013; Livermore, 2004; Fernandes, 2015). Whilst efforts are being undertaken in the developed nations of the world to curtail

the antibiotic crisis and prevent further development of totally drug resistant bacteria; developing nations are decades behind this rationale as they continue to facilitate the emergence of these completely untreatable bacterial infections (Carlet *et al.,* 2014; Gough *et al.,* 2014).

Antibiotics and bacterial antibiotic resistance co-evolved as a means of competition for space and nutrients between prokaryotic organisms, and as such they existed for a long time before the exploitation of antibiotics by humans (Wright, 2014). Humans are responsible for creating selective pressures conducive for the evolution and survival of antibiotic resistance as a ubiquitous phenotype of bacteria (Laxminarayan *et al.,* 2013). Horizontal gene transfer of genetic information including antibiotic resistance genes is the primary way that bacteria pass on antibiotic resistance traits (Cabezón *et al.,* 2015; Polz *et al.,* 2013). The evidence for this process is overwhelming as bacteria can easily and repeatedly conjugate DNA under *in vitro* laboratory conditions (Ravenhall *et al.,* 2015; Soucy *et al.,* 2015). In the natural environment, empirical identification of horizontal gene transfer events has been difficult to obtain (Woolhouse *et al.,* 2015). In the majority of cases these events are assumed based on identifiers such as the existence of specific genes and organisms associated with HGT such as *E. faecalis* and *E. faecium* (Woolhouse *et al.,* 2015; Rizzo *et al.,* 2013, Gillings, 2017).

The aim of this thesis was to assess conjugation of antibiotic resistance genes amongst *E. faecalis* and *E. faecium* to establish if these bacteria can propagate antibiotic resistance in the natural environment. Research has mostly focused on the prevention of enterococcal biofilm rather than to focus on the processes which go on within (Gilmore *et al.,* 2014). There are some reports which discuss the fundamentals of enterococcal biofilm (Leuck *et al.,* 2014; Dunny *et al.,* 2014; Barnes *et al.,* 2012).

All enterococci used in this thesis were isolated from an agrarian waterway ecosystem and had the potential to encounter each other in the environment. They also possessed representative AMR phenotypes based on initial identifications. The biggest limitation on research investigations into HGT processes in the environment is the great number and variation of bacteria that exist in these environments (Aminov, 2011; Thomas and Nielsen, 2005). As enterococci are listed as an identifier for high potential for conjugation to occur (Palmer *et al.*, 2010), selecting for a subgroup of unique individuals based on the criteria of AMR profile, biofilm formation ability and clumping phenotype allowed for the most efficient screening of the potential for capture of HGT of AMR between enterococci.

Where possible this thesis was designed to allow conjugation of antibiotic resistance genes to occur as naturally as possible to emulate the typical conditions present to the bacteria during HGT processes. There was only one manipulation placed on the enterococci in this study: spontaneous generation of rifampicin resistance in three potential recipient isolates: MW01105, ST01109 and MF02043 denoted with ^{Rif}. This kind of spontaneous mutation created in a recipient *Enterococcus* for the purposes of capturing conjugation has been accomplished by Dunny *et al*. (1978) with a rifampicin and fusidic acid mutant *E. faecalis* OG1RF. Jacob and Hobbs, (1974) identified another spontaneous enterococcal rifampicin mutant JH2-2. Kristich *et al.* (2007) created a spectinomycin resistant spontaneous mutant *E. faecalis* OG1Sp.

Several conjugation experiments were attempted using isolates with unique AMR phenotypes, with varied success (as discussed in chapter four) before creating the rifampicin resistant mutants. Arguments can be made that genetic manipulation of conjugal plasmids may diminish conjugation efficiency during HGT processes as

evidenced by Chen *et al.* (2008). Whereby, mutations and deletions of the putative ATPase related to Gram negative type 4 secretion systems (*pcfc*) in the pCF10 plasmid, reduced conjugation efficiency by at least 24-fold (max: 580,000-fold). However, Enterococcal conjugation systems are regulated within plasmids and self-transferable mobile genetic elements. Rifampicin mutations occur in the *RpoB* gene which lies within the genomic DNA of enterococci and encodes the beta subunit of RNA polymerase (Drancourt *et al.,* 2004). Both OG1RF and JH2-2 are derived from clinical isolates and they have served the same function, as a recipient for enterococcal conjugation of antibiotic resistance genes for 40 years without issue. These two conjugation strains are functionally the same as MW01105^{Rif}, ST01109^{Rif} and MF02043^{Rif}, both being created from susceptible isolates from differing sources. Therefore, usage of the three rifampicin mutants in this study continued without reservations of reduced or ineffective conjugation possibilities.

The rifampicin mutants displayed no notable reductions in growth as compared to previous growth with the original isolates (data not shown). Enne *et al.* (2004) described no disadvantages to the rifampicin mutants beyond the laboratory with minimal affliction to fitness. With the minimal effects of spontaneous rifampicin generation described by Enne *et al*. (2004) and the repeated use of rifampicin mutants from Dunny *et al*. (1978) and Jacob and Hobbs, (1974) to carry out conjugation studies, the three rifampicin mutants generated in this study were used for conjugation testing. This rendered all 15 isolates compatible for conjugation testing, as explained in chapter four.

An interesting finding from gathering gene information from chapter three was that gene nomenclature for enterococci varies. The aggregation gene, has several names: *agg*, *prgB* and *asa1* (Daniels, 2011; Vankerckhoven *et al.,* 2004; Chung and Dunny, 1995). Research investigations have treated these genes as unique and even attribution of

different functions to them has occurred (Choi and Woo, 2015). When assessing the primers from Choi and Woo, (2015), it was identified that the primer sets amplify the same gene. The conclusion from this finding is that published primer sequences should be cross checked from exact specificity to the gene that it will amplify.

There are several investigations whereby enterococcal biofilm studies comprise inevitable biofilm destruction to analyse experimental results (Leuck *et al.,* 2014, Cook *et al.,* 2011). The identification and enumeration of conjugation inside biofilm carried out by Cook *et al.* (2011) relied on EDTA removal of enterococcal biofilm cells into suspension, and plating on selective agar, destroying the biofilm in the process. This is a valuable assay for rapidly identifying conjugation inside biofilm and was utilised in chapter five to initially identify conjugation inside enterococcal biofilm. However, there remains a lot of ambiguity in the method, as to whether conjugation occurred before, during or after establishment of biofilm. Whilst this end-point assay has proven to be invaluable in the pursuit of knowledge on the inner processes of enterococcal biofilm communities in the literature and in this study, the visualisation of such interactions would provide definitive results on the transfer of antibiotic resistance from one isolate to another. The different results obtained in chapter 5 between the standard bacterial biofilm assays and the GBA optimised for the laboratory enterococci highlighted the importance of biofilm morphology. Any assay that required manipulation of the biofilm will, by definition, destroy the morphology and overlook potentially important aspects of cellular interactions. Visualisation *in situ* would minimise disturbance of biofilm morphology and cellular localisation, similarly to fixation of tissues as observed in the investigations of extracellular structure production during enterococcal biofilm formation by Barnes, (2012). This had never been accomplished before and was therefore investigated in this

study. Time was taken to devise the gene frame biofilm apparatus (GBA) (discussed in chapter five), to accomplish potential *in situ* investigations. One of the major advantages of the GBA was that the enterococci formed an even biofilm coating over the entire surface area of the GBA chamber.

The GBA was devised primarily as a visualisation aide for microscopic investigations. Fluorescence based tools were essential for distinguishing *E. faecalis* and *E. faecium* species, extracellular polymeric substance, DNA content of individual cells and even the *vanA* vancomycin resistance gene inside biofilm. Fluorescent *in situ* hybridisation was designed for resolving the transconjugants from any conjugation events inside biofilm, versus other fluorescence assays. The advantage with FISH was that the probes were small (typically 10-15bp) and could be 5' tagged with a range of fluorophores, making colocalization studies possible in a microorganism based protocol. Additionally, probes could be targeted to any gene desired. FISH has been well established in detecting specific bacteria in infected tissues and biofilms for several years, utilising probes devised from primer sequences used in 16S or 23S rRNA PCR identifications of bacteria (Harmsen *et al.*, 1999; Bezirtzoglou *et al.*, 2011; Swidsinski *et al.*, 2005). Waar *et al*. (2005) used 16S rRNA target oligonucleotide probes to target specifically, *E. faecalis* and *E. faecium* in blood cultures, faecal material and biofilm. Fazli *et al*. (2014) used peptide nucleic acid probes to individually target *Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus sp. and Micrococcus sp* with multiplex probe conditions in multispecies biofilms. FISH is therefore a powerful tool for examination of bacteria inside biofilm. Evidence from Warr *et al*. (2005) and Fazli *et al*. (2014) demonstrate the ability to easily identify bacteria in biofilm by species alone, by adoption of primer sequences that target genes of interest. Application of this assay to enterococcal conjugation inside biofilm had never been published before and could be easily modified to target genes of interest for analysis inside enterococcal biofilm.

Results from the updated Tremblay and Archambault, (2013) conjugation protocol, (where isolates were mixed at a ratio of 1:1 after the donor was induced with pheromone supernatant for 20 minutes and plated on selective media for 48 hours), were varied and inconsistent with literature. Successful isolation of transconjugants, as defined by the selection procedures and controls was achieved after a selection incubation period of 96 hours. When comparing this data to the literature, maximum conjugation selection incubations are 48 hours with higher recovery of transconjugants (CFU's) using the same methodology (Cook *et al.,* 2011; Tremblay and Archambault, 2013). Conjugation was only successful with an extended incubation time in selection (96 hours versus their 48 hours), transconjugant per donor efficiencies were inefficient, $x10^{-10/11}$ compared to their Tremblay and Archambault, (2013) $x10^{-3}$. The donor strain (*E. faecalis* strain 543) used by Tremblay and Archambault, (2013) had similar resistance phenotypes (erythromycin, streptomycin, tetracycline) to the donor used in this study (MF06036) and were both isolated from poultry litter. They used the rifampicin resistant mutant JH2-2, whereas in this study, recipients were selected based on unique resistance traits of selected partners. Whilst experimental controls revealed no contamination, the phenotypic identification tests as described in the general materials and methods were carried out on the transconjugants identifying them as enterococci. Conclusions could therefore be made that these presumptive conjugation selection isolates were likely transconjugants; however, the failings of this protocol to match the observations detailed in the literature could not be ignored.

There were variations in the methodology for conjugation between enterococci, usually the conjugation substrate. The previous method relied on suspensions of conjugation partners to be brought into contact for mating and plated directly on to selection. The second conjugation protocol had an additional step which involved the use of a solid phase growth plate preceding selection, incubated for 24 hours (Cook *et al.,* 2011). The

rifampicin resistant potential recipient mutants were used in this assay as a recipient. We hypothesised that the chances of successful conjugation would be greater with reactions whereby the potential recipients had antibiotic resistance genes located chromosomally, so that potential gene transfer was one directional. This improved protocol eliminated the issues discovered whilst using the initial conjugation method.

Transconjugants were isolated using this approach, one of which was a cross species transfer from *E. faecalis* into *E. faecium*. Conjugation transconjugant donor efficiencies recovered from the four conjugation reactions had maximum efficiencies of $x10^{-1}$, which was congruent with the efficiencies of $x10^{-2}$ and $x10^{-3}$ carried out by Cook *et al*. (2011) and Tremblay and Archambault, (2013) respectively. Donor isolate MF06036 was responsible for the creation of two transconjugants, T1 and T3, with recipients MW01105^{Rif} and ST01109^{Rif}. This suggests that MF06036 contains at least two different mobile elements that were transferred individually in separate reactions to other recipients. Additionally, the interspecies transfer from farm associated *E. faecalis* MF06036 to water catchment-associated *E. faecium* ST01109^{Rif} has been reported for the first time. Enterococci can possess multiple mobile elements with differing sets of AMR genes on them, however this is the first incidence where different phenotypes were transferred to two different recipients from the same donor (Chung and Dunny, 1995, Choi and Woo, 2015, Tremblay and Archambault, 2013). Multiple AMR phenotypes/genotypes were transferred stably, which was a rare discovery from enterococci of animal origins. T1 received four antibiotic resistance phenotypes (vancomycin, erythromycin, trimethoprim and teicoplanin) from MF06036. Vignaroli *et al*. (2011) were one of the few groups to identify the rare co-transfer of multiple (vancomycin and erythromycin) resistance in enterococci from pig isolates to human isolates. The data obtained from these conjugation investigations was published in *BMC Microbiology* (Conwell *et al.,* 2017).

When examining the MIC values for the four transconjugants it was discovered that the MIC values for some of the non-transferred AMR had changed. T1 had not received tetracycline resistance phenotypes, nor genes from the donor MF06036; however, its base MIC increased fourfold. The same was noted with T2 to chloramphenicol, and T4 to vancomycin, with doubling of their base MIC to these antibiotics. Alterations to multidrug efflux could cause changes in resistance to families of antimicrobials. Several of the tetracycline genes (*tetL, tetK*) code for putative efflux proteins (Molale and Bezuidenhout, 2016). Knowledge on *Enterococcus* drug efflux is limited, however Hürlimann *et al*. (2016) identified two presumptive ABC-type multidrug transporters in *E. faecalis* and postulated that these analogues may be capable of upregulated multidrug efflux. At this stage, research investigations are only beginning to investigate multidrug efflux mechanisms.

Rifampicin mutants in other genera of bacteria have been shown to alter the susceptibility of tested strains to other antibiotics. Cui *et al*. (2010) showed increased resistance to vancomycin and daptomycin with rifampicin *rpoB S. aureus* mutants. Louw *et al*. (2011) demonstrated reduced susceptibility to ofloxacin when rifampicin was used on a rifampicin resistant *rpoB531 M. tuberculosis* mutant. These insights share similarities to the conditions exposed to the enterococcal transconjugants used in this thesis. Further investigation would be required to uncover the mechanism of this phenomenon.

Novel gene transfers were observed in the enterococci; however, one of the aims of this thesis was to test the probability of these observed interactions occurring under conditions analogous of the agrarian environment from which the bacteria were isolated. The conjugation reaction between MW01105^{Rif} and MF06036 was successfully replicated under conditions of significant reductions in nutrient availability (starvation) and temperature (4 and 20^{o}C based on unpublished surveillance data acquired from river

catchments across the province of Ulster). The reductions in temperature had a corresponding reduction in conjugation efficiency; however, the positive identification of conjugation at temperature ranges consistent with the typical waterway sites across the local province add merit to the feasibility of opportunistic environmental conjugation. These results agree with the data published by Marcinek *et al*. (1998), whereby enterococcal conjugation was successful in bioreactors exposed to the natural conditions of municipal sewage plants associated with the natural environment. Which include low temperatures (8-25ºC), variations in nutrient availability, oxygen saturation (1.4-10mg/ml) and chemical stress (heavy metals). Our conjugation efficiencies at low temperature (4ºC and 20ºC) and nutrient availability (starvation) were $3.3\pm1x10^{-12}$ and $7.4\pm1.5x10^{-7}$, compared to efficiencies of $x10^{-7}$ to $x10^{-9}$ for conjugative elements at slightly higher temperatures of 8 to 25ºC with limited data on nutrient availability due to the setup of the system.

Conjugation under environmental conditions demonstrated that bacterial HGT could occur if specific circumstances are met: such as two compatible strains coming into contact for long enough; or in sufficient numbers to allow conjugation to occur. Attachment assays and conjugation assays under nutrient deprivation and normal water conditions were carried out in tandem with *S. lacustris* and *E. fluviatilis*. Histological analysis identified MF06036 located through the entirety of the mesohyl of the sponges using the customised FISH protocol with probes specific for *E. faecalis*; additionally, MF06036 conjugated with $MW01105^{Rif}$. Collagen is an important component of sponge mesohyl and it is known that enterococci can bind to collagen (*esp*) and produce gelatinase (*gelE*) (Thurlow *et al.*, 2010). The ability of enterococci to bind to biotic

substrates composed of collagenous material may facilitate their binding into sponge tissue and may even enable them to feed on collagen (Alexander *et al.*, 2015).

The conjugation reactions that occurred in sponge mesohyl had recovered efficiencies of: *E. fluviatilis* - $1.26x10^{-6}$ per sponge, and *S. lacustris* - $1.05x10^{-6}$ per sponge at 20ºC in water. These efficiencies were 6-7-fold higher compared to solid phase conjugation under the same conditions (20ºC under nutrient deprivation). The enterococcal conjugation efficiencies reported by Marcinek *et al*. (1998) in activated sludge basins during summer (20-25ºC) were $3.4x10^{-1}$ for plasmid pAD1, $1.1x10^{-1}$ for plasmid pIP1017, $1.9x10^{-7}$ for plasmid pIP501 and $9.3x10^{-9}$ for transposon *Tn916*. They stated that conjugation under these natural conditions reduced conjugation efficiency by 4 to 6-fold. Their conjugation microcosm that was used in the activated sludge basins varied in length from 15 to 45cm making it a much larger surface for conjugation to occur compared to a single sponge hatchling (0.5 to 48mm^2). Comparing the conjugation of enterococci in sponge to sewage basins under similar temperatures, it appears that sponge offers enhanced conjugation based on higher conjugation efficiencies versus no sponge and solid phase conjugation at a similar temperature. Conjugation results are comparable to those obtained by Marcinek *et al*. (1998) and considering the small size of the sponge hatchlings, enterococcal conjugation efficiency is high (water only controls were on average 600-fold less efficient).

The introduction of enterococci into wastewater treatment by Marcinek *et al*. (1998) provided evidence for the feasibility of conjugation in the natural environment. Wastewater treatment plants are still considered hotspots for horizontal gene transfer due to the concentrating of bacterial cells under antibiotic and chemical stresses (Rizzo *et al.*, 2013). Bacteria from wastewater treatment plants have been detected in receiving waters previously (Ferreira da Silva *et al.*, 2007; Łuczkiewicz *et al.*, 2010). Tetracycline

resistance genes have been previously detected in the groundwater downstream from swine farm processes, positively demonstrating transferrable elements in the environment (Chee-Sanford *et al.,* 2001). These preliminary assays, when factoring the work of Marcinek *et al*. (1998) and published literature demonstrate for the first time an interaction between enterococci and sponges that could occur in the natural environment, downstream of wastewater processes or even agrarian processes (Schwartz *et al.,* 2003; Baquero *et al.,* 2008). This interaction could facilitate the propagation of antibiotic resistance genes in the environment.

In chapter five the successful conjugation partners were subjected to biofilm formation investigations culminating in conjugation assays within biofilm. At the time of this investigation, several biofilm formation assays using simple apparatuses were available. They were sequentially tested revealing inconsistences in facilitating enterococcal biofilm formation, even in control isolates. Quantification and microscopic investigations were limited due to the variations of formation and retention during processing for data collection. Issues with biofilm assays such as the polystyrene microplate assay were reported previously by Leuck *et al*. (2014) and needed to be resolved in this research investigation. Leuck *et al*. (2014) revealed that the polystyrene microplates produced variations in biofilm formation, often weak production, in enterococcal clinical isolates which all could form biofilm on porcine heart valves. They suggested that enterococcal *ex vivo* biofilm formation should be performed on relevant tissue substrates. In our investigations, glass coated with gelatin yielded significant improvements on biofilm formation as compared to polystyrene and glass alone. These results better reflect the biofilm capabilities of the tested isolates, both with adherence to the substrate, and consistency between experiments. The results obtained with gelatin coated glass align

with results from Bukhari, (2013), whereby substrate improvements with tissue components (collagen based) improve enterococcal biofilm formation.

The gene-frame biofilm apparatus (GBA) was a successful resolution to the issues discovered during initial biofilm characterisation experiments. There were many devices in the literature for improving biofilm formation. However, when examined they did not provide the necessary augmentations for analysing biofilm *in situ* without disturbing the macrostructures. The Calgary biofilm device could not be imaged with the microscopes in the laboratory and could only be imaged with glass bottomed microplates (Ceri *et al.*, 1999). Coupon based biofilm apparatuses such as the drip-flow biofilm reactor, rely on an insert that must be removed and processed, increasing chances of damage (Xu *et al.*, 1998). Construction of the GBA was simple, with just three components. It was autoclavable and therefore reusable and it was mounted on a glass microscope slide allowing for detailed analysis of especially weak biofilm both in terms of biomass and attachment strength. This was useful as Leuck *et al*. (2014) stated that enterococcal *ex vivo* biofilm formation can often be weak as compared to using *in vivo* substrates or explanted tissue. The GBA would therefore account for potential diminished biofilm formation in a non-ideal biofilm environment. The GBA excelled in all aspects of biofilm formation and visualisation, as compared to all the tested assays in chapter five. Biofilms, could be imaged without applying mechanical stress. The mechanical stresses applied to biofilm processing such as washing with PBS carried out by Toledo-Arana *et al*. (2001), will apply sheer stress to biofilm cells. This is especially true when biofilm formation assays are carried out on abiotic surfaces (polystyrene), known to facilitate weak biofilm formation as carried out by Nallapareddy *et al*. (2006).

Fixation is known to affect the morphology of bacterial cells as well as surface ultrastructures, such as pili (Chao and Zhang., 2011). Alcohol based fixatives detach surface filaments known to contribute to biofilm formation in enterococci (Nallapareddy

et al., 2006; Chao and Zhang, 2011). Hence a fixative was only used to carry out FISH on enterococcal biofilm for five minutes. All analysis of biofilm using conA and fluorescence microscopy was used on unfixed biofilm. This approach using the GBA revealed novel physical characteristics of examined enterococcal biofilm. These included the identification of a unique branched macro-scaffolding using dark field microscopy, captured on mature, undisturbed biofilm, never reported before in static *Enterococcus faecalis* biofilm. This macro-scaffolding structure likely forms as mature biofilm releases and disperses cells and fragments of biofilm in the static, closed system of the GBA (Costerton and Stewart, 2001). The branches out from the mature biofilms have an appearance of streamers that protrude from mature biofilm colonies (Kanaparthy and Kanaparthy, 2012).

The GBA's ability to preserve biofilm characteristics was used to microscopically catalogue enterococcal biofilm development. Combined with a limited visualisation data in the literature of early set down of enterococcal cells during biofilm formation, data obtained here revealed comprehensive microscopy on the spatial localisation of cells and EPS production during biofilm development. Due to the plethora of factors at play during biofilm formation: such as the aggregation gene, enterococcal surface protein, collagen adhesion and various EPS production modalities, it is unlikely that any given species or even isolate of *Enterococcus* will form biofilm in the same fashion. Seneviratne *et al*. (2017) revealed that 18 isolates of non-disease linked oral enterococci could form varied biofilms, suggesting that biofilm formation ability is directly linked to pathogenicity. However, enterococcal biofilm biomass is unrelated to resistance to antibiotic stress, similarly to certain isolates of *Lactococcus, Streptococcus* and *Salmonella* (Zhang *et al.,* 2013b; Ghasemmahdi *et al.,* 2015; Seneviratne *et al.,* 2017). It appears that specific combinations of virulence genes (*esp, gelE, ace*) as well as specific antibiotic resistance determinants will result in a large degree of variations in enterococcal biofilm formation.

This was observed in the biofilms formed by MF06036 (*vanA, ermB*) versus MF04010 (*tetL, tetM, efaA, ace*). MF06036 biofilms were weaker than MF04010, which contained the endocarditis antigen (*efaA*) and collagen binding (*ace*) genes. However, MF06036 had six antibiotic resistance phenotypes and MF04010 had three.

Biofilm formation studies under nutrient deprivation are beginning to emerge and have used *Enterococcus* species sourced from humans (Gao *et al.*, 2016, Ran *et al.*, 2015). Examination of starved biofilms produced by environmentally isolated enterococci was carried out to assess how they would perform under conditions encountered naturally. The tested isolates retained biofilm formation capabilities. This was regardless of the effects of nutrient deprivation, an observation reported by Liu *et al*. (2010), where *E. faecalis* could form typical biofilms on human dentin under starvation. Starvation models on tissue substrates such as those used by Lie *et al*. (2010) and in this study, exemplify the true nature of enterococcal biofilm formation requiring biotic surfaces to form strong biofilms. Results such as these highlight the inherent obsolescence of polystyrene as a suitable substrate for studying enterococcal biofilm.

The strong biofilm producer MW02102 lost biofilm EPS staining intensity when starved. However, when starvation conditions were applied to the non-biofilm producer MF01028, EPS staining intensity increased. Gao *et al*. (2016) reported that *E. faecalis* can better resist starvation as compared to other bacteria (*Streptococcus gordonii, Actinomyces viscosus, or Lactobacillus acidophilus*). When *E. faecalis* formed biofilms with these other bacteria, biofilms were stronger and more resistant to starvation. This was likely due to the ability of *E. faecalis* to out survive other bacteria and utilise the components of dead cells to continue to resist starvation and form biofilm. It is known that within a species of *Enterococcus* there will be sub populations of cells which do not possess resistance to enterocin, cytolysin or gelatin mediated cell death (SprE). These

cells can be preyed upon by dominant populations containing resistance to bacteriocins and gelatin (Thomas *et al.,* 2009; Gao *et al.,* 2016). In the single species biofilms created in our study it could be that starvation increases cell death, providing "ingredients" to produce biofilm as a protective response to starvation stress that was readily observed in the multispecies biofilms by Gao *et al*. (2016).

The novel analysis of biofilm formation and quantification of EPS production was accomplished using fluorescently tagged concanavalin A. An emerging biofilm stain, it has only recently been adopted to use in enterococcal biofilm, after confirmatory investigations by Anastasiadis *et al*. (2014); Jung *et al*. (2015); Ridan and Benxiang, (2014) provided evidence for the effectiveness of conA as an EPS stain for bacterial biofilms. It was also successfully tested in our microbiology laboratory on a *Burkholderia thailandensis* transposon mutant that was deficient in rhamnolipid production (rhlAB) that showed reduced EPS production as compared to wild type (Funston, 2016).

Applications of vancomycin stress and nutrient deprivation on MF06036 identified that the chain form of cellular organisation, which is lost during biofilm formation, is retained during vancomycin stress. In addition, biofilm formation capability is reduced but not eliminated. This suggests that the enterococci may utilise chain form as a defence mechanism to ensure all clones have partitioned AMR genes. Babic *et al*. (2011) identified the rapid acquisition of the conjugative element ICE*Bs1* down through the entire chain of *B. subtilis* after successful conjugation to a single cell within the chain form. They suggested that that intra-chain spread of a mobile plasmid from a donor or a recent transconjugant could be a standard feature of bacterial conjugation. Cells in chain form are already close enough to stably pass genetic information. This system could be triggered by stress or concentrating of conjugal machinery at the cell pole (Grohmann,

2010). FISH investigations using vancomycin stress and *vanA* probes revealed that chain form MF06036 have variations in *vanA* colocalization, further alluding to the role of chain form under stress. It is known that chain form reduces bacteria surface area for biofilm formation, which may explain the reduction in biofilm formation under vancomycin stress (Qin *et al.,* 1998). Guiton *et al*. (2009) suggested that increased chaining reduced the binding sites of enterococci during initial biofilm formation. Electrostatic charges have been shown to influence bacterial adherence to substrate. Increased cell surface charges could increase the repulsive forces of the substrate on depositing cells during biofilm formation (van Merode *et al.,* 2006).

Another mechanism behind the increased chaining observed under vancomycin stress involves the altered D-Ala-D-lactate mechanism of peptidoglycan cross linking present in *vanA* vancomycin resistant enterococci. Autolysins (AtlA/B) break the glycoside bonds in peptidoglycan during cell division allowing partition of two enterococcal cells (Mesnage *et al.,* 2008). Paganelli *et al*. (2013) identified that autolysin mutants of *E. faecium* formed long chains of cells, which could be resolved with the addition of autolysin ($AtlA_{EFM}$). These mutants had an 80% reduction in biofilm formation which was attributed to reduced initial adherence due to chaining of the bacterial cells. Impaired biofilm growth was also linked to the association of autolysin in the clumping of enterococcal cells and lack of autolysin reduces eDNA presence in enterococcal biofilm. Vancomycin tolerance and vancomycin stress were associated with inhibition of autolytic systems in *S. aureus* (Sieradzki and Tomasz, 2006). It is possible that glycopeptides such as vancomycin applied, in a sub-MIC or to resistant isolates with altered peptidoglycan synthesis, interfere with the activity of autolysins responsible for cleavage of the cell septum during division. This would increase the frequency of chain form as well as the ability of cells to attach to substrate and form biofilm (Guiton *et al.,* 2009).

The Success of the GBA allowed for the development of a tandem FISH protocol for easy analysis of individual members of multispecies biofilms. It also permitted examinations of AMR genes from an unknown perspective with minimal autofluorescence inside enterococcal biofilms. FISH assays have been previously used to monitor the presence of enterococci in faecal matter and activated sludge from wastewater treatment plants using genomic identifiers such as 16S or 23S rRNA (Harmsen *et al.*, 1999, Roller *et al.*, 1994). FISH has been a useful tool to identify and monitor specific species of bacteria, as it has the sensitivity to target DNA sequences unique to a single species of bacteria amongst a community of several species (Moter and Göbel, 2000). Something that could not be accomplished as extensively or as cost effectively as using immunofluorescence approaches. Additionally, FISH can rapidly identify pathogenic strains of bacteria as compared to the gold standard of cultivation on growth plates, where not all bacteria can be cultivated (Rhode *et al.*, 2015). The novelty of this protocol is the ability to identify low copy number AMR mobile genes without molecular amplification steps. Modifications to the protocol have been reported such as introducing reporter genes into plasmids and replicating modified plasmid into a strain of *Enterococcus* (Cook *et al.*, 2011). This was accomplished by simply designing multiple probes to the same gene, in this case the *vanA* vancomycin resistance gene (Gilmore *et al.*, 2014). This approach was effective, with no modifications required after optimisation of the probes as they were designed to work at the same annealing temperature, much like multiplex PCR (Oliveira and de Lencastre, 2002). Single probe oligonucleotide FISH provided insufficient signal to noise ratio to allow observations of genes from the cellular background. Therefore, approaches such as RING FISH were designed to maximise emitted fluorescent signal with multiple fluorophores conjugated to a large probe (Zwirglmaier *et al.*, 2004). These methods are complex, requiring generation of specific probes and conjugation of fluorophores in-house. The multi-probe approach used in our study was simple and had

the quality control from integrated DNA technologies and Invitrogen to ensure high quality probe construction. The application of multi-probe FISH was entirely novel for examination of environmentally isolated enterococcal biofilms containing mobile resistance (*vanA*) genes.

The subsequent GBA enterococcal conjugation in biofilm reactions were carried out with MW01105^{Rif} and MF06036 and their T1. Since the GBA was a novel device, updated conjugation protocols were created to accommodate the changes and confined space of the chamber. This took the form of creating a 24-hour single conjugation partner biofilm, removing planktonic cells and then adding the conjugation partner for a further 24 hours. This modification was an essential step to ensure that any observed conjugation could only occur from within biofilm, as one of the partners had created and existed in biofilm. This protocol was successful at isolating T1 under normal and starved biofilm growth conditions. This is the second instance of biofilm conjugation within the *Enterococcus* genus (Cook *et al.,* 2011); however, this research group utilised plasmids that were genetically modified, carrying reporters and incubated both conjugation partners at the start of their biofilm conjugation experiments. When comparing the conjugation efficiencies of our protocol in the GBA versus the efficiencies of Cook *et al*. (2011), planktonic conjugation within their system could have occurred. Their efficiencies were calculated per membrane and ours were calculated per GBA well, and their protocol was the basis for our protocol in terms of donor and recipient ratios. In their mixed biofilm, they achieved an efficiency of $1:2.2x10^{-5}$, our mixed biofilm efficiency was $3.04x10^{-3}$ following their protocol exactly. Comparing our mixed biofilm efficiency to our modified protocol accounting for planktonic conjugation from simultaneous co-incubation of donor and recipients: MW01105^{Rif} based biofilm was $2.01x10^{-3}$ (1.5-fold less efficient) and

MF06036 based biofilm was $1.01x10^{-3}$ (3-fold less efficient). The reduction in efficiency can be explained by thorough removal of planktonic cells as well as allowing a single partner biofilm to form prior to attempting conjugation.

This thesis collates data of natural conjugation of unmodified MGE's containing AMR inside the biofilm of either conjugation partner upon the addition of the other partner. Success was also gained when biofilms were grown under starvation. The significance of this finding was that the lack of nutrients limited the ability of any subsequent transconjugants to grow post conjugation. Therefore, this report is the first to describe enterococcal transconjugant recovery from *in vitro* biofilm conjugation where the only manipulation was selection for spontaneous rifampicin resistance. More complex models of studying AMR transfer exist, such as gut models, the previously mentioned activated sludge model and aquatic bed models (Hellweger *et al*., 2011; Hirt *et al*., 2018). The natural progression of experimentation on the enterococcal isolates used in this thesis would be to examine their conjugation proficiency in the gut model used by the Dunny enterococcal research laboratory. Additionally, to further examine sponge mediated enterococcal conjugation in an aquatic bed model system to mimic the source environment.

The GBA was designed for *in situ* visualisation, therefore the FISH protocols, fluorescence microscopy and the GBA conjugation reactions were combined with increased selective pressures of antibiotics and lysozyme to visualise newly created T1, inside an undisturbed conjugation biofilm. After extensive optimisation to ensure 'selective' killing of the conjugal pairs post conjugation, fluorescent imaging would reveal the surviving transconjugants.

Usage of rifampicin, vancomycin and lysozyme at concentrations only newly created T1 could survive, combined with live/dead imaging provided for the first-time fluorescent

micrographs of T1 inside biofilm. However, the non-specificity of the live/dead imagining of T1 could be argued as refutable. Therefore, A second conjugation experiment was carried out using the interspecies conjugal pair: *E. faecium* ST01109^{Rif} and *E. faecalis* MF06036. Post conjugation double selection using tetracycline and lysozyme would eliminate any remaining *E. faecium* ST01109^{Rif} recipients. Fluorescent staining using Hoechst to stain all intact cells and FISH imagining with the ENU 1470 *E. faecium* FISH probe was used to select for the *E. faecium* transconjugants T3, excluding any *E. faecalis* MF06036 donors. This protocol undisputedly demonstrates for the first time: enterococcal biofilm conjugation by way of visualisation of transconjugants, ensured to be only created inside biofilm.

6. 1. Conclusion

The overarching aim of this thesis was to demonstrate that environmentally isolated *E. faecalis* and *E. faecium* form natural biofilms, subsequently inducing the transfer of antibiotic resistance genes. This thesis collates data showcasing:

1. *E. faecalis* and *E. faecium* isolated from an agrarian environment had prolific and diverse AMR profiles
2. A selection of these isolates created four transconjugants transferring vancomycin, erythromycin and tetracycline genes, published in *BMC Microbiology*
3. Multiple gene transfers were observed in single reactions
4. Gene nomenclature and association to published genes for enterococci can be inaccurate
5. *E. faecalis* MF06036 could transfer two different AMR phenotypes during separate conjugation reactions
6. MIC values for certain antibiotics that were not involved in conjugation were increased in transconjugants T1, T2 and T4, suggesting synergy between the resistance mechanisms to certain antibiotics
7. Reductions to temperature and nutrient availability limit but do not inhibit enterococcal conjugation
8. *E. faecalis* MF06036 could bind to *Spongilla lacustris* and conjugation reactions were successful using *Spongilla lacustris* and *Ephydatia fluviatilis* as a substrate
9. Gelatin coated glass substrate was essential in the formation of reliable enterococcal biofilm.
10. The gene-frame biofilm apparatus (GBA) was created to successfully image fragile enterococcal biofilm where standard published assays could not

11. The GBA helped reveal novel macro-scaffolding, development, EPS characteristics, spatial density, chain forming characteristics, granularity and size of enterococcal biofilm under normal and stressed conditions using florescence and the newly adapted conA stain
12. A newly created FISH protocol allowed for rapid, straightforward visual examination of mixed species biofilm in the GBA using species probes
13. This FISH protocol was adapted to work on whole mounts of entire sponge hatchlings so that rapid identification of bacteria could be carried out without significant histological interventions
14. Multiprobe FISH targeted to *vanA* on mobile elements demonstrated for the first-time vancomycin staining inside enterococcal biofilm
15. Enterococci can form biofilm, have a compatible conjugation partner introduced, and subsequent intra-biofilm conjugation will occur even under nutrient deprivation
16. This novel conjugation reaction was also visualised using selective killing of the conjugation partners and imaging the transconjugants with live/dead staining and FISH probes targeted to the surviving transconjugant species

6.2. Future directions

Future work would focus on further elucidation of enterococcal biofilm conjugation characteristics. Additionally, investigations to:

- Identify and characterise the mobile elements transferred during conjugation would reveal any additional AMR genes at play as well as the specific composition of the elements themselves. Investigations focusing on this would uncover how MF06036 could transfer two sets of AMR phenotypes
- Identify what kind of efflux pumps if any, the transconjugants possessed, and compare them and their activity to the conjugal parental isolates to further elucidate the observed shifts in non-transferred MIC changes to antibiotics
- Assess additional environmental factors at play during transfer, up to working in the environment itself. Assessing alterations to pH and chemical composition of agrarian waterways would be an essential step
- The mechanism at play during the attachment of MF06036 to sponge mesohyl and assess transcriptional activity of specific tissue attachment virulence factors *ace*, *esp*, asc1 and *gelE* during sponge adhesion and conjugation in tandem with the environmental conditions discussed previously
- Analyse (using molecular and transcriptional methods) the expression of *vanA* under vancomycin stress to assess why there were significant death rates on the VRE MF06036 upon the introduction of a sub inhibitory concentration of vancomycin
- Further develop the FISH protocols and assess other genes of interest in biofilm using the GBA

- Utilise an rpm sigma factor mutant strain to assess the true proficiency of enterococcal biofilm formation versus a non-biofilm producing mutant with conA staining.
- Carry out chemical and transcriptional characterisation studies of the components of EPS and how they may change under biofilm starvation experiments
- Assess transcriptional activity of the conjugation machinery at play during biofilm conjugation and evaluate any differences associated with the stress observed in the extra enteric environment
- Identify unique markers that could only exist in a transconjugant without using artificial inclusions and apply them to the FISH visualisation protocol for conjugal biofilms

6.3. Concluding remark

Environmentally isolated enterococci can form biofilm of their own construction, subsequently conjugating antibiotic resistance within. This also occurs under the influence of temperature and nutrient conditions akin to the environment from which they were isolated.

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