Investigation of the environmental effect on the Regulation and Transfer of Conjugative Transposon Tn*916*

Thesis submitted by

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Declaration

I hereby certify that the work embodied in this thesis is the result of my own investigations, except where otherwise stated.

Abstract

Antibiotic resistance is a major global concern, as it has spread rapidly and prevents the treatment of infectious diseases. One of the main reasons for this is the acquisition of resistance genes carried on mobile genetic elements (MGEs). There are many different types of MGEs, including transposons, plasmids, insertion sequences, pathogenicity islands, bacteriophages and gene cassettes.

Tn*916* is a conjugative transposon that contains the tetracycline-resistance gene *tet*(M). This transposon transfers to a broad range of bacteria. In this study, the research aimed to determine the behaviour of Tn*916* in the human oral cavity, which is the portal to the digestive system. *Bacillus subtilis* (BS34A) has been used as a Tn*916* donor, and six *Streptococcus* spp. (*S. oralis, S. pyogenes, S. salivarius, S. mutans, S. sanguinis* and *S. parasanguinis*) and *Enterococcus faecalis* as recipients. The donor *B. subtilis* is considered as a transient bacterium within the oral cavity, normally inhabiting soil, and the recipients are all considered normal oral and gastrointestinal bacteria. Filter-mating assays were carried out in pairs between donor–recipients under conditions that mimic those found in the mouth.

It was observed that *B. subtilis* was able to transfer Tn*916* into *S. oralis, S. pyogenes* and *E. faecalis* under anaerobic conditions at frequencies ranging from 10^{-9} to 10^{-7} transconjugants per recipient within 1 min on solid and liquid media. In contrast, no transfer was observed aerobically or when mating with *S. salivarius, S. mutans, S. sanguinis* and *S. parasanguinis* as recipients.

However, after a mutant strain of *B. subtilis* onTn*916* (Δ*orf12* terminator of Tn*916*) was developed, transfer of Tn*916* occurred from *B. subtilis* into *S. oralis, S. pyogenes* and *E. faecalis* aerobically at frequencies ranging from10^{-9} to 10^{-8} transconjugants per recipient and anaerobically only into *S. pyogenes*.

Using qPCR, the copy number of circular Tn*916* in different conditions (aerobic and anaerobic) with and without a recipient (*E. faecalis* JH2-2) was determined. There were10^{7}copies/µl in the absence of a recipient in both conditions. However, in the presence of the recipient, the copy numbers increased significantly to 10^{8} copies/µl anaerobically compared to 10^{7} copies/µl aerobically.

Furthermore, the role of the *orf12* terminator in aerobic and anaerobic conditions was determined by quantifying the expression level using an enzymatic reporter assay. The transcription level from p*tet*(M) is controlled by the terminator on *orf12*, and it was almost the same in both conditions. However, the transcription level was significantly increased when *orf12* terminator mutated and the condition was switched from aerobic to anaerobic.

In conclusion, transient bacteria in the oral cavity may have ample opportunities to disseminate their DNA to the oral microbiota.

Impact statement

A fundamental understanding of the evolutionary pressures, which select for resistance, is necessary to design strategies to stop the spread of antibiotic-resistant genes (ARGs). The conjugative transposon Tn*916* spreads tetracycline resistance to a broad range of bacteria found in soil, oral cavity and gut. New insights into the evolution of the conjugative transposon Tn*916* within conditions that mimic the oral condition are needed.

The work in this study has shown that Tn*916* transfer was enhanced in the absence of oxygen. In addition, it has shown that the presence of the recipient increases the excision of Tn*916* in oxygen-deficient conditions.

This work has also helped with understanding the effect of environmental conditions on the transfer of Tn*916*, which is highly important, as this transposon is one of the most common elements found in oral bacteria. The data have proven that the terminator on *orf12* could play an important role in modulating the transfer of Tn*916* in different conditions.

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Abbreviations

AACs	Chloramphenicol acetyltransferases
AGEs	Aminoglycoside-modifying enzymes
AMR	Antimicrobial resistance
ATP	Adenosine tri-phosphate
BHI	Brain heart infusion
bp	Base pair
CAP	Catabolite activator protein
CCC	Covalently closed circle
CDC	Centers for Disease Control and Prevention
CI	Circular intermediate
DNA	Deoxyribonucleic acid
DprA	DNA processing protein A
dsDNA	Double-strand DNA
ESBL	Extended spectrum beta-lactamase
ECL	Enhanced chemiluminescence
FNR	Fumarate Nitrate Reductase
gRNA	guide RNA
GSR	General stress response
Hfr	High frequency recombinant
HGT	Horizontal gene transfer
ICEs	Integrated conjugative elements
IM	Inner membrane
Int	Integrase

LB	Luria-Bertani
MDR	Multi-drug resistance
MGEs	Mobile Genetic Elements
MLS	Macrolides, lincosamides and streptogramins
Mpf	Mating-pair formation
MRSA	Methicillin-resistant *Staphylococcus aureus*
MVs	Membrane vesicles
OM	Outer membrane
OMT	Outer-membrane-enclosed tube
OMVs	Outer-membrane vesicles
ORFs	Open reading frames
oriT	Origin of transfer
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
QPCR	Quantitative Polymerase Chain Reaction
QS	Quorum sensing
R-M	Restriction-Modification system
RNA	Ribonucleic acid
RNAP	Ribonucleic acid polymerase
RT-PCR	Reverse-transcriptase polymerase chain reaction
sgRNA	Synthetic single guide RNA
ssDNA	Single-strand DNA
SSO	Single-strand origin
T4CP	Type IV coupling proteins
T4SS	Type IV secretion system

TNTs	Tunnelling nanotubes
T-strand	DNA transfer strand
VRE	Vancomycin-resistant enterococci.
WHO	World Health Organisation
Xis	Excisionase
^{o}C	Degrees Celsius
g	Gravitational force
h	Hour
kb	Kilobase
λ	Bacteriophage lambda
µg	Microgram
µl	Microlitre
µm	Micrometre
ml	Millimetre
µM	Micromolar
mM	Millimolar
min	Minute
nm	Nanometre
OD	Optical density

Chapter 1

General Introduction

Chapter 1 General introduction

The antibiotic pollution of soil and water is caused by the widespread use of antibiotics in agriculture as an organic fertilizer, in livestock industries as a breeding inducer and as therapy. This has resulted in the distribution of antibiotic resistance gene (ARG) via mobile genetic element (MGE) through horizontal gene transfer (HGT), which varies with the host and environmental factors. Antibacterial resistant genes have been detected in many human commensal and pathogenic bacteria.

1.1 Antibiotic resistance threats

An antibiotic is a compound that either kills bacteria (bactericidal) or prevents their growth (bacteriostatic) with limited side effects on the host (Waksman and Woodruff, 1940). Different types of antibiotics target different sites in the bacterial cell, including the cell wall, cell membrane, ribosomes and enzymes such as RNA polymerase or DNA gyrase which is required for nucleic acid synthesis (Kapoor et al., 2017) (**Figure 1.1**).

Figure 1.1: Action mechanisms for different groups of antibiotics

Reprinted from "Action and resistance mechanisms of antibiotics: A guide for clinicians," by G. Kapoor, S. Saigal and A. Elongavan, 2017, *Journal of anaesthesiology, clinical pharmacology, 33*, p. 301. © 2017 by Wolters Kluwer Medknow Publications.

Penicillin was, the first antibiotic discovered in 1929 by Sir Alexander Fleming when a fungus (*Penicillium notatum*) showed antibacterial activity against *Staphylococcus* (Fleming, 1929). However, the antimicrobial sulphanilamide was prepared and patented first in 1908-1909 (Sharma and Anand, 1997). Then in 1935, a commercial sulphanilamide drug (KI-730, Prontosil) was developed and tested for its antibacterial activity against many infections (Aminov, 2010). However, the easy accessibility and wide usage of this drug eventually caused an increase in dissemination of the sulphonamide resistance gene, which is expressed by class 1 integrons and carried within gene cassettes (Enne et al., 2004). Despite this, many

synthetically modified derivatives are still used in therapy, causing an increase in the spread of resistance genes as a result of their selective pressure (Walsh, 2003).

Tetracycline was widely used in the 1950s and the 1960s in the United States (O'Brien, 1987). It inhibits protein synthesis by irreversibly binding to the 30S bacterial ribosome subunit, blocking the A site and inhibiting aminoacyl tRNA from binding (Goldman et al., 1980, Epe et al., 1987). However, due to the widespread utilisation of tetracycline in 1980–1990 resistant strains appeared and multiplied (Levy, 1988). Resistance to tetracycline is developed in three ways: efflux, which reduces tetracycline access to ribosomes; inhibiting tetracycline binding by altering the ribosomes; or producing tetracycline-inactive enzymes (Sundqvist et al., 1998). Ribosomal protection is the most widespread mechanism of resistance to tetracycline, which is conferred by *tet*(M), *tet*(O), *tet*(Q), *tet*(S), *tet*(T), *tet*(W), *tet*(32), *tet*(36), *tetB*(P) and *otr*(A) (Burdett et al., 1982, Agerso et al., 2002, Billington et al., 2002, Chung et al., 2002, Miranda et al., 2003, Dugan et al., 2004). *Tet*(M) is frequently found on conjugative transposons in a wide range of bacteria and on plasmids in *Neisseria* spp (Knapp et al., 1988, Roberts, 1990, Franke and Clewell, 1981). *Tet*(M) is regulated by attenuation, which will be described in section 1.3.1.1.3 (Su et al., 1992).

Bacteria have many mechanisms to develop resistance to antibiotics. For example efflux pumps (Džidić et al., 2008), antibiotic inactivation by enzymes such as β-lactamases, aminoglycoside-modifying enzymes (AMEs) and chloramphenicol acetyltransferases (CATs) or modification of target

molecules, such as alteration in ribosomes subunits, penicillin-binding protein (PBP), cell wall precursors, RNA polymerase mutation or DNA gyrase mutation (Tenover, 2006) (**Figure 1.2**).

Figure 1.2: Mechanisms of antibacterial resistance

1) Modification of antibiotic target site, 2) Antibiotic inactivation, 3) modification via enzymes or 4) Elimination of antibiotic accumulation in the cell via an efflux pump. Adapted from ‘’Mechanisms of action’’ by Gavalda, 2017, (https://twitter.com/gavalda_j/status/875986659799703552).

After 1970, the discovery of new classes of antibiotics decreased substantially, and the development of resistance against existing antibiotics increased. Therefore, a new strategy for modifying antibacterial agents was established in the search for new treatments that could provide better activity and less toxicity (Chopra et al., 2002). However, bacteria can acquire resistance to modified antibacterial drugs through HGT of resistance genes on MGEs due to the presence of selective pressure (Davies, 1994, Read and Woods, 2014).

Antibiotics play a major role in the advancement in medicine and surgery by eliminating or treating infections which can develop in patients with chronic diseases, such as diabetes, rheumatoid arthritis and end-stage renal disease, or in patients undergoing chemotherapy treatments or complex surgeries, e.g. organ transplants, joint replacements or cardiac surgery (Gould and Bal, 2013, Wright, 2014, Solomon and Oliver, 2014, Rossolini et al., 2014). In addition, antibiotics have been used extensively as growth promoters in agriculture and in livestock nutrition, and unsurprisingly often end up in the soil, sediment and ground water (Phillips et al., 2004). Also, the wide use of antibiotics by humans either in hospital or at home means that they are introduced into the environment through the municipal wastewater treatment system into the water supply (Richardson and Bowron, 1985). Moreover, disinfectants that are widely used in the food and medicine industries may also select for antimicrobial resistance (AMR) due to co-linkage of genes for resistance.

Antibiotic-resistant infections are now spread across the world, and mortality rates are increasing as a result (**Figure 1.3**) (Golkar et al., 2014). The Centers for Disease Control and Prevention (CDC) has classified a number of bacteria as urgent, serious and concerning threats; they are listed in Table 1.1 (CDC, 2013). In addition, the World Health Organisation (WHO) has listed and focused on *Enterococcus* spp., *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. as serious bacteria that cause resistant infections and urgently require new antibiotics (Golkar et al., 2014, Solomon and Oliver, 2014, Lushniak, 2014, Rossolini et al., 2014, WHO, 2017, Tacconelli et al., 2018). It has been estimated that by 2050, 10 million deaths per year could result globally from antibacterial resistant infections at a cost of £66 trillion (**Figure 1.3**) (O'Neill, 2015, TheWorldBank, 2017). In the United Kingdom from 2013 to 2017, the estimated total number of septicaemia cases caused by pathogen resistance to one or more antibacterial increased by 35% from 12,250 to 16,504 (**Figure 1.4**). *Escherichia coli* and *K. pneumoniae* are responsible for most of the antibiotic resistant infections in the bloodstream with a high incidence rate (84.4% in total) (PHE, 2018).

Table 1.1: CDC list and categories of 18 threats of bacteria and fungi based on human health: urgent, serious and concerning.

Urgent threats
Clostridium difficile
Carbapenem-resistant *Enterobacteriaceae*
Drug-resistant *Neisseria gonorrhoeae*
Serious threats
Multidrug-resistant *Acinetobacter*
Drug-resistant *Campylobacter*
Fluconazole-resistant *Candida* (fungus)
Extended spectrum beta-lactamase-producing *Enterobacteriaceae* (ESBLs)
Vancomycin-resistant *Enterococcus* (VRE)
Multidrug-resistant *Pseudomonas aeruginosa*
Drug-resistant *nontyphoidal Salmonella*
Drug-resistant *Salmonella typhimurium*

Drug-resistant *Shigella*
Methicillin-resistant *Staphylococcus aureus* (MRSA)
Drug-resistant *Streptococcus pneumoniae*
Drug-resistant tuberculosis
Concerning threats
Vancomycin-resistant *Staphylococcus aureus* (VRSA)
Erythromycin-resistant Group A *Streptococcus*
Clindamycin-resistant Group B *Streptococcus*

Note: Adapted from "Antibiotic resistance threats in the United States," by CDC, 2013.

Figure 1.3: The predicted number of deaths caused by AMR every year by 2050 worldwide

The mortality rate of AMR in Africa and Asia per 10,000 persons would be almost twice the mortality in North America, Europe and Australia by 2050. Reprinted from " Review on Antibacterial Resistance," by J. O'Neill (Ed), Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations, 2014, CC by 4.0.

Figure 1.4: Estimated number of bloodstream infections caused from antibiotic resistant pathogens in UK from 2013 to 2017

Reprinted from "English Surveillance Programme for Antimicrobial Utilisation and Resistance (ESPAUR)," by PHE, 2018, London: PHE. © 2018.

1.1.1 Types of antibiotic resistance

1.1.1.1 Intrinsic resistance

Intrinsic resistance is a trait, which exists in the genome of all members of bacteria from the same species (Cox and Wright, 2013). An example is resistance to the activity of vancomycin, which acts on the cell wall (peptidoglycan) of Gram-positive bacteria and is inhibited in Gram-negative bacteria via outer membrane (OM) barrier impermeability (Arthur and Courvalin, 1993, Hong et al., 2002).

1.1.1.2 Acquired resistance

Acquired resistance is achieved by mutation in a specific gene in the host chromosome or by acquiring a resistance gene via HGT (Blair et al., 2014). Acquired resistance causes a serious threat to individual health.

1.2 Horizontal gene of transfer (HGT)

HGT is the process of transferring a segment of DNA from one bacterium to another, which may then acquire a new gene that potentially contributes to functional and phenotypic changes. This transfer can occur via transformation, conjugation or transduction. In addition, another HGT mechanism has been discovered involving the fusion of vesicles containing DNA, which then can be transferred into bacteria (Yaron et al., 2000). Furthermore, the transfer of non-conjugative plasmid DNA from *Bacillus subtilis* to *B. subtilis*, *S. aureus* or *E. coli* through nanotubes has also been observed (Dubey and Ben-Yehuda, 2011). A recent mode of transfer

called lateral transduction was discovered with *S. aureus* phage, which transfers a large section of the host chromosome including any elements, adjacent to the integrated phage at high frequency in a single lytic event (Chen et al., 2018).

Figure 1.5: A schematic presentation of HGT pathways

The donor is represented in blue and the recipient in green. In section **A)** conjugation is the transfer of a single strand from donor to recipient via cell contact, **B)** transformation is the uptake of DNA from the surrounding environment, **C)** transduction is a transfer mediated by phage, **D)** membrane vesicles containing DNA released (budding) from the donor cell mediate transfer via fusion with the cytoplasmic membrane, and **E)** a nanotube extended between cells mediates the transfer of proteins through the nanotube. Reprinted from “Horizontal gene transfer: building the web of life,” by S. M. Soucy, J. Huang and J. P. Gogarten, 2015, *Nature review Genetics.* © 2015 by springer Nature. Reprinted with permission.

1.2.1 Transformation

Transformation is the uptake of extracellular DNA from the surrounding environment by a bacterium into its genome (Griffith, 1928). It was first observed in *S. pneumoniae* when a non-virulent strain became virulent after being mixed with a heat killed virulent strain (Griffith, 1928). This finding was later confirmed by Avery when a non-virulent strain was converted to a virulent strain after being mixed with at least 0.01 µg of extracted DNA from a virulent strain (Avery et al., 1944).

Transformation occurs when a group of competence proteins in bacteria form a pore-like structure in the cell membrane of the bacteria and allows DNA to enter the cell (Lorenz and Wackernagel, 1994). In *Streptococcus* spp., this process begins with the induction of the competence genes (Com) which are required for DNA binding, uptake and processing (Peterson et al., 2004). It has been suggested that Gram-positive bacteria have a structure that is similar to that of type IV pili in Gram-negative bacteria, called a competence pseudopilus (Dubnau, 1999). However, the protrusion of competence pseudopilus from the cell surface is limited owing to the cell wall thickness (Dubnau, 1999, Laurenceau et al., 2013). Competence pseudopilus drives the exogenous double-strand DNA (dsDNA) to the receptor, ComEA (Provvedi and Dubnau, 1999, Laurenceau et al., 2013). The ComEA receptor delivers the dsDNA to the endonucleases (Coykendall, 1977), thereby causing one strand of the DNA to degrade while the other enters the cytoplasm in a 3'-5' orientation through the membrane pore (ComEC) (Puyet et al., 1990, Berge et al., 2002) (**Figure 1.6**).

Once the single-strand DNA (ssDNA) enters the cell, the DNA processing protein A (DprA) attaches to the ssDNA and assists in loading the recombinase (RecA) onto ssDNA (Mortier-Barriere et al., 2007). Thus, RecA facilitates the search for homology in the host genome. The DNA can either be completely homologous or can have heterologous regions that continue in single strand form, flanked by homologous regions (Johnston et al., 2014) (**Figure 1.6**).

Figure 1.6: A summary of the transformation mechanism in both Gram-negative and Gram-positive bacteria

The exogenous dsDNA binds to the competence pilus, which consists of the ComGC subunit in Gram-positive bacteria and the PilE subunit in Gram-negative bacteria. dsDNA is transferred into cytosol after being internalised into ssDNA through transmembrane channels ComEC in Gram-positive bacteria and PilQ and ComA in Gram-negative bacteria. ssDNA is bound by DNA processing protein A (DprA) and recombinase RecA promoting homologous recombination. Reprinted from “Bacterial transformation: distribution, shared mechanisms and divergent control,” by C. Johnston, B. Martin, G. Fichant, P. Polard and J.P. Claverys, 2014, Nature Reviews Microbiology, 12, p. 181.

1.2.2 Conjugation

Conjugation is the transfer of DNA from one bacterium (donor) to another (recipient) through direct-contact pili (Lawley et al., 2003) or through pores via surface-encoding adhesins (Tatum and Lederberg, 1947, Grohmann et al., 2003). Conjugation was discovered when two strains of mutant *E. coli* (auxotrophic) requiring different additional nutrition than the wild-type nutrition requirement, such as biotin, threonine, methionine, leucine, and thiamine, were incubated together and a new isolate acted as a wild-type that was able to grow in the absence of a nutritional supplement. This finding has suggested that a recombination of genes occurred between the two strains (Lederberg and Tatum, 1946, Tatum and Lederberg, 1947). Later, an investigation was conducted using a U-shaped tube divided into two sections by porous glass filter to allow only the movement of the medium; it showed that no isolates were grown when the two auxotrophic strains were inoculated separately in each side of the tube (Davis, 1950). This finding suggests that physical contact between the two strains was required for a recombination event to occur. The conjugation transfer mechanism operates in the presence of conjugative plasmids or integrated and conjugative elements (ICEs) as they encode genes required for the conjugative transfer of DNA to occur (Goessweiner-Mohr et al., 2013, Cabezon et al., 2015, Ilangovan et al., 2015, Johnson and Grossman, 2015).

The conjugation process is initiated by signalling events secreted by the recipient e.g. pheromones, which trigger the transfer of DNA and involves the assembly of multiple-proteins and the mating-pair formation (Mpf) (Frost et

al., 2005). Conjugative DNA transfer requires a type IV secretion system (T4SS), which is a membrane-associated transporter complex (Goessweiner-Mohr et al., 2013, Cabezon et al., 2015, Ilangovan et al., 2015). The T4SS in Gram- negative bacteria is subdivided into four parts: pilus (VirB2 and VirB5), outer membrane (OM) components (VirB7, VirB9 and C-terminal of VirB10), periplasmic components (VirB8,VirB10,VirB6 and VirB2) and inner membrane (IM) components (VirB6, VirB8 and VirB10) (Sun, 2018) (**Figure 1.7**). Conjugation starts with connecting the donor cell to the recipient via pilus, which is formed by the assembly of pilin (VirB2) and adhesin (VirB5). In the donor cytoplasm, the relaxase protein binds and nicks one strand at origin of transfer (*oriT*) to form an open circular molecule and remains covalently bound to the resulting 5' end of the ssDNA known as transfer strand (T-strand or T-DNA). The relaxosome (DNA-protein complex) is recognised by VirD4 type IV coupling proteins (T4CP) which link the T-strand to the T4SS translocation channel to facilitate transfer to the recipient (Alvarez-Martinez and Christie, 2009). VirD4, with the help of two additional ATPases, energizes VirB4, and hexameric ATPase VirB11 pumps the DNA through the membrane translocation channels across the IM, periplasm and OM (Leonetti et al., 2015, Sun, 2018) (**Figure 1.7**).

Figure 1.7: Model of the structure of T4SS in Gram-negative and Gram-positive bacteria

T4SS consists of 12 components divided into four groups: the pilus, which is formed of VirB2 and VirB5 in Gram-negative bacteria and pores which are formed by VirB6 in Gram-positive bacteria; the IM in Gram-negative bacteria, which consists of VirB3, VirB6, VirB8 and VirB10; cell membranes in Gram-positive bacteria, which consist of VirB6; periplasm, which consists of VirB8, VirB10 and VirB6 in Gram-negative bacteria; and the OM, which consists of VirB7, VirB9 and the C-terminal of VirB10 in Gram-negative bacteria. VirB4, ViB11 and VirD4 attach to the IM in Gram-negative bacteria, and VirB4 and VirD4 attach to the cell membrane in Gram-positive bacteria to provide energy for DNA transfer. Reprinted from "Type IV secretion systems," (http://www.secretion.org/navigateHome.action).

1.2.3 Transduction

Transduction is the transfer of DNA by a virus (bacteriophage) from a phage infected bacterial cell to another (Thierauf et al., 2009). Transduction was discovered by Zinder and Lederberg (1952) when one strain of *Salmonella typhimurium* transferred part of its chromosomal DNA into another strain without cell-to-cell contact.

There are two types of transduction: generalised and specialised (Soucy et al., 2015). Generalised transduction is the transfer of a piece of the host DNA

packaged in the phage particles before cell lysis due to the phage lytic cycle (the lytic pathway) (Canchaya et al., 2003). With cell lysis, phage particles bind to the recipient cells via specific receptors and insert DNA into the cells (Lindsay, 2014) (**Figure 1.8**).

Specialised transduction is the excision of a prophage from the host genome that includes some of the flanking host DNA (Soucy et al., 2015). Generally, the integration of a bacteriophage (prophage) in the host chromosome is stable, and the prophage is transferred to the daughter cells through bacterial DNA replication. Under stress, a prophage is induced, excised with the host flanking DNA and replicated; phage heads and tails are synthesised, and collected into active phage particles. The phage particles are released during bacterial cell lysis. The infectious phage particles binds and inserts its DNA to a new host (Xia et al., 2011) (**Figure 1.8**).

Recently, a new mode of transfer called Lateral transduction was discovered by Chen et al. (2018) in a *S. aureus* phage. The prophage did not excise until late in its life cycle. While it is still in the host genome, a bidirectional replication starts forming a multiple integrated prophage (**Figure 1.8**). This allows the continuation of both the lateral and normal phage maturations in parallel. In this mode of transfer, a large segment of the host chromosome, adjacent to the prophage, was transferred at a high frequency in a single lytic event. Any DNA element located within the prophage boundaries becomes a hypermobile platform for transfer at a high frequency.

Figure 1.8: Generalised, specialised and lateral transduction

Once phages infect a host cell, the DNA of the lytic phage takes over the cell molecular machinery in order to replicate their own DNA and develop descendent virions, which are released through cell lysis. The DNA from previous bacterial hosts inserted instead of the viral DNA into the recipient and recombines into the genome (generalised). In specialized transduction, the life cycle of viruses goes through the lysogenic sequence of viral infection. A specific section of the virus DNA is inserted into the host chromosome to replicate (prophage). Then, under specific conditions, the prophage activates and is excised from the bacterial genome, including flanked pieces from the host DNA, and undergoes a lytic cycle to transfer the DNA to another bacterial host (Canchaya et al., 2003). In lateral transduction, the integrated prophage replicates within the host DNA and packaged including a large segment of the host DNA to transfer the DNA to another bacterial host. Reprinted from "Broadband' Networks of Viruses May Help Bacteria Evolve Faster," by L. Jonathan, *Quanta Magazine,* 2018.

1.2.4 Membrane vesicles mediate gene transfer

Membrane vesicles are spherical particles (25–500 nm in size), which are released from the bacterial surface (Perez-Cruz et al., 2013, Fulsundar et al., 2014, Domingues and Nielsen, 2017). In Gram-negative bacteria, membrane vesicles consist of the OM and the peptidoglycan layer enclosed with periplasmic components, which are released by plugging outward (Mashburn-Warren et al., 2009, Kulp and Kuehn, 2010, Schertzer and Whiteley, 2012, Haurat et al., 2015, Roier et al., 2016). In Gram-positive bacteria, MVs consist of the cytoplasmic membrane, which is released by a budding mechanism (Brown et al., 2015) or by endolysin-trigger cell death (Shingaki et al., 2003, Toyofuku et al., 2017) (**Figure 1.9**). Outer-membrane vesicles (OMVs) Gram-negatives are important for nutrient acquisition, pathogeneses, quorum sensing and biofilm formation (Li et al., 1998, Liao et al., 2014).

It has been demonstrated in several studies that the membrane vesicles are a mechanism of HGT (Deich and Hoyer, 1982, Kolling and Matthews, 1999). Liao et al. (2014) reported that *Streptococcus mutans* releases exogenous DNA in the membrane vesicles within the oral biofilm. In addition, virulence factors, such as protease, autolysin, phospholipase, Shiga toxin and haemolysin, have been isolated from the vesicles (Kolling and Matthews, 1999). Moreover, it was discovered that DNA containing vesicles are released from *Pseudomonas aeruginosa*, *Acinetobacter baylyi* and *Neisseria gonorrhoeae* (Dorward et al., 1989, Kadurugamuwa and Beveridge, 1995, Fulsundar et al., 2014). The mechanism of delivering DNA into the recipient

is considered to be dependent on the recipient species. For example, the OMVs could attach to the recipient surface and lyse, releasing the DNA, or the OMVs could be fused into the host (Fulsundar et al., 2014). It has been proposed that the membrane vesicles pass through the thick membrane via pores or protein channels (Beveridge and Kadurugamuwa, 1996, Ellen et al., 2009, Deatherage and Cookson, 2012).

Figure 1.9: Structure and composition of Gram-negative and Gram-positive Membrane Vesicles (MVs)

Structure and composition of Gram-negative and Gram-positive MVs. Gram-negative MVs consist of the OM, which is embedded with lipopolysaccharides and outer-membrane proteins, and periplasmic contents including peptidoglycan, enzymes, toxins, cytoplasmic proteins and nucleic acids. Gram-positive MVs are comprised of the cytoplasmic membrane containing lipoproteins, cytoplasmic proteins, enzymes, toxins and nucleic acids. Reprinted from "The Therapeutic Benefit of Bacterial Membrane Vesicles," by N.J. Bitto and M. Kaparakis-Liaskos, 2017, *International Journal of Molecular Sciences, 18*. P. 1287. CC by 4.0.

1.2.5 Tunnelled nanotubes

Recently, an exchange of DNA between an adjacent cell grown on a solid surface (agar) via a small protruding tube was observed. A nanotube was formed and enabled to acquire a nonhereditary antibiotic resistance

(chloramphenicol and lincomycin) between two *B. subtilis* strains on an agar plate. In addition, nanotubes can transfer a nonconjugative plasmid from *B. subtilis* to *B. subtilis, S. aureus* or *E. coli* (Dubey and Ben-Yehuda, 2011) (**Figure 1.10**).

Nanotubes or tunnelling nanotubes (TNTs) are very small tunnels ranging from 20 to 500 nm, which can reach adjacent cells with a varying length. TNTs are highly fragile and sensitive to light exposure. These TNTs allow sharing the intracellular components, which are required for development, immune response or the regeneration process between distant cells (Rustom et al., 2004). TNTs are formed within a few minutes and last for minutes or up to several hours (Rustom et al., 2004, Bukoreshtliev et al., 2009).

TNTs were first discovered in 2004 by Gerdes and colleagues when they observed thin F-actin membranous transient tubes in cultured rat pheochromocytoma PC12 cells connecting between cells (Rustom et al., 2004). The exchange of cellular content is typical of multicellular organisms. In mammalian cells, neurons or immune cells are connected via gap junctions and synapses (Dustin et al., 2010); in plants, they are connected via a cytoplasmic tube (plasmodesmata), which mediates the exchange of nutrients, signals, proteins and transcripts (Heinlein and Epel, 2004, Lucas et al., 2009). In addition, nanotubes can develop in prokaryotes, such as *Shewanella oneidensis* (Pirbadian et al., 2014), *Francisella tularensis* (McCaig et al., 2013), *Vibrio vulnificus* (Hampton et al., 2017) and *B. subtilis* (Dubey and Ben-Yehuda, 2011, Dubey et al., 2016). However, it has been discovered that nanotubes' development in prokaryotes consists of

continuous chains of enclosed membranes (McCaig et al., 2013, Remis et al., 2014, Wei et al., 2014, Dubey et al., 2016) (**Figure 1.10** and **Figure 1.11**).

Figure 1.10: Overview of various dimensions and patterns of nanotube networks

In section **A)** Different lengths (336 nm, 894 nm and 482 nm) of nanotube connections between *B. subtilis* cells, **B)** patterns of nanotubes connections between *B. subtilis* and *S. aureus* (indicated by green arrows) and between *S. aureus* cells (indicated by blue arrows), and **C)** nanotubes connecting *B. subtilis* and *E. coli* (circled). Adapted from "Intercellular nanotubes mediate bacterial communication," by G. P. Dubey and S. Ben-Yehuda, 2011, *Cell,144*. © 2011 by Elsevier. Adapted with permission.

Figure 1.11: *Myxococcus xanthus* forms a chain-like outer membrane-enclosed tubes (OMT) extending to connect neighbouring cells

Adapted from **A)** "Bacterial Social Networks: Structure and composition of *Myxococcus xanthus* outer membrane vesicle chains," by J. P. Remis, D. Wei, A. Gorur, M. Zemla, J. Haraga, S. Allen, H. E. Witkowska, J. W. Costerton, J. E. Berleman and M. Auer, 2014, *Environmental Microbiology*. © 2013 by John Wiley and Sons. **B)** "*Myxobacteria* produce outer membrane-enclosed tubes in unstructured environments," by X. Wei, C. N. Vassallo, D. T. Pathak and D. Wall, 2014, *Journal of Bacteriology* © 2014 by American Society for Microbiology. Adapted with permissions.

1.3 Mobile Genetic Elements (MGEs)

MGEs are segments of DNA that encode the enzymes and other proteins necessary for movement within the genome (intracellular transposition) or between two bacterial cells (intercellular conjugation) (Frost et al., 2005). These elements may contain different genes that are necessary for the host adaptation mechanisms. The genes can confer virulence factors or resistance to antibiotics, biocides, disinfectants and metals. Such MGEs are important to the evolution of new populations that can then adapt to new environmental niches (Lindsay, 2010).

There are many different types of MGEs, such as insertion sequences and transposons, bacteriophages, pathogenicity islands, gene cassettes, conjugative plasmids and conjugative transposons (**Figure 1.12**) (Johnson and Grossman, 2015). Here, only conjugative transposons will be considered.

Figure 1.12: Locations of MGEs in the cell

Locations of MGEs in the cell. 1) Plasmid, represented in blue, present as a free circular DNA, 2) Transposon, represented in green, can integrate into the plasmid or the genome, 3) Insertion sequences or gene cassettes, represented in yellow, can insert into the genome or the plasmid and 4) Prophage, represented in red can be packaged into phage heads and released via cell lysis.

1.3.1 Conjugative transposons

Conjugative transposons, which are also known as integrative and conjugative elements (ICEs), are elements that encode transposition and conjugation systems, which catalyse their intracellular transposition and intercellular conjugative transfer and subsequent integration into the host chromosome. In addition, they carry accessory genes, which encode proteins conferring e.g. antibiotic resistance, virulence factors and heavy metal resistance. Also, some ICEs confer the ability to utilise an alternative

carbon source or sucrose fermentation, e.g. ICE*clc* (Ravatn et al., 1998), ICE CTn*Scr94* (Hochhut et al., 1997), and ICE Tn*5276* (Rauch and De Vos, 1992). These elements are composed of functional modules, where genes responsible for specific functions are grouped together, e.g. conjugation, recombination, regulation and accessory genes (Beaber et al., 2002, Juhas et al., 2007, Wozniak et al., 2009, Guerillot et al., 2013).

Most genes, such as the transfer genes, are repressed when the transposon is within the host chromosome, as this keeps it stable, thus, it replicates with the host chromosome (Johnson and Grossman, 2015). However, transcription of these genes is de-repressed under certain conditions. These include, the presence of a selective advantage encoded by the accessory genes (e.g. in Tn*916*-like family, CTnDOT-ERL family and ICE*clc*) (Franke and Clewell, 1981, Doucet-Populaire et al., 1991, Showsh and Andrews, 1992, Sentchilo et al., 2003, Wang et al., 2004, Wang et al., 2005, Minoia et al., 2008), the activation by the SOS response (e.g. in SXT, ICE*Bs1* and ICE*St3*) (Burrus and Waldor, 2003, Beaber et al., 2004, Auchtung et al., 2005, Auchtung et al., 2007, Bellanger et al., 2007), the host growth phase (e.g. in ICE*clc*, ICE*M1*Sym^{R7A} and ICE*St3*) (Rajeev et al., 2009, Ramsay et al., 2009, Carraro et al., 2011, Miyazaki et al., 2012) and the signalling molecules secreted by the recipient, such as quorum sensing (e.g. in ICE*M1*Sym^{R7A} and ICE*Bs1*) (Auchtung et al., 2005, Ramsay et al., 2006, Auchtung et al., 2007, Bose et al., 2008, Ramsay et al., 2009). Thus, conjugative transposons are excised from the host chromosome and form a dsDNA circular intermediate (CI) molecule (Scott et al., 1994). Proteins encoded by conjugative transposons (relaxases) recognise the origin of

transfer (*oriT*) site and generate a linear ssDNA protein complex (transfer DNA or T-DNA). The mating apparatus drives the transfer of the DNA into the recipient cell, and after re-circularisation, it forms dsDNA, which is integrated via encoded integrase in a site-specific recombination in the recipient chromosome (Scott et al., 1994, Johnson and Grossman, 2015) (**Figure 1.13**).

Figure 1.13: Outline of ICE conjugative transfer

In section **(a)** Donor, cell with integrated ICE in the chromosome (shown in grey). **(b)** Under specific environmental conditions, the ICE genes are induced and excised from the donor chromosome, forming a dsDNA CI molecule, and assemble ICE gene products into the mating pore. **(c)** Relaxases recognise *oriT* and nick one strand of the ICE circular dsDNA and attach covalently to the 5' end, thus generating transfer DNA (T-DNA). **(d)** When the recipient cell (shown in green) is close to the donor cell, the conjugation machinery transfers the T-DNA into the recipient. **(e)** In the recipient, the ICE recirculates and second DNA strand is synthesised (complementary strand). In the donor, the ssDNA generates a dsDNA CI molecule via rolling circle replication. **(f)** The ICE integrates and reintegrates in the recipient and donor chromosome, respectively, catalysed by integrase. Reprinted from "Integrative and Conjugative Elements (ICEs): What They Do and How They Work," by C. M. Johnson and A. D. Grossman, 2015, *Annual Reviews Genetics, 49*.

1.3.1.1 Tn*916*

The conjugative transposon Tn*916* was discovered by Franke and Clewell (1981) when they observed the transfer of tetracycline resistance from *Enterococcus faecalis* DS16 to *E. faecalis* JH2-2 in the absence of a plasmid. Tn*916* is divided into four functional modules: conjugation, regulation, recombination and accessory genes (**Figure 1.14**) (Senghas et al., 1988, Flannagan et al., 1994, Roberts and Mullany, 2009). It is 18 kb in size and has 24 open reading frames (*orfs*), which encode putative proteins (Flannagan et al., 1994).

Figure 1.14: Tn*916* functional modules

Conjugation in blue, regulation in green, accessory gene in grey and recombination in red. Arrow boxes represent the *orfs* and the orientation of the genes. The black triangle represents the location of the *oriT.* Reprinted from “A modular master on the move: the Tn*916* family of mobile genetic elements,” by A. P. Roberts and P. Mullany, *Trends in Microbiology, 17*, P. 251-8, 2009. © 2009 by Elsevier. Reprinted with permission.

1.3.1.1.1 Accessory genes of Tn*916*

An antibiotic resistance gene *tet*(M) which confers resistance to tetracycline is present in Tn*916* (Flannagan et al., 1994). This encodes a 72KDa protein, which is a ribosomal protection protein (RPP) (Su et al., 1992). Different members of the Tn*916* family have different resistance genes such as *erm*(B) (encodes for erythromycin resistance) (Trieu-Cuot et al., 1990), *mef*(E) (encodes for macrolide efflux pump protein) (Gay and Stephens, 2001), *mer*(A) (encodes for mercury resistance) (Soge et al., 2008), *aphA* (encodes kanamycin resistance) (Caillaud et al., 1987) and ABC transporters (Brouwer et al., 2011) (**Figure 1.15**).

Figure 1.15: Structure of various members of the Tn*916*/Tn*916*-like elements

Functional modules are represented as shown in the key. The organisms from which the elements were isolated are listed in the left column. Mobility of the elements is denoted by a capital M on the right. Reprinted from “A modular master on the move: the Tn*916* family of mobile genetic elements,” by A. P. Roberts and P. Mullany, *Trends in Microbiology, 17*, P. 251-8, 2009. © 2009 by Elsevier. Reprinted with permission.

1.3.1.1.2 Recombination of Tn*916*

Tn*916* integration and excision is mediated by two proteins, integrase (Int) and excisionase (Xis), their genes are located at one end of the transposon (Jaworski et al., 1996, Rudy et al., 1997) (**Figure 1.14**). In the excision step, both Int and Xis are required, whereas for the integration step, only Int is required (Storrs et al., 1991). Both proteins bind to the ends of Tn*916* (Rudy et al., 1997, Jia and Churchward, 1999). Int is a member of the tyrosine recombinase family, which performs highly directional recombination reactions (Scott et al., 1988, Storrs et al., 1991, Abbani et al., 2005). Xis is a 67 amino acid protein which controls recombination by either facilitating or preventing the binding of Int to sites needed for recombination (Rudy et al., 1997, Marra and Scott, 1999, Hinerfeld and Churchward, 2001, Abbani et al., 2005).

Conjugative transposition of Tn*916* starts with staggered endonucleolytic cleavages at each end of the transposon, forming 5-6 bp single strand regions protruding at 5'-hydroxyl ends which are called coupling sequences (Scott et al., 1988, Caparon and Scott, 1989, Rudy and Scott, 1994, Rice and Carias, 1994, Manganelli et al., 1996, Taylor and Churchward, 1997, Manganelli et al., 1997). Subsequently, the sequences are linked covalently together via Int forming a CI molecule with a heteroduplex or a homoduplex at the circle joint (Scott et al., 1988, Caparon and Scott, 1989, Lu and Churchward, 1994, Rice and Carias, 1994, Manganelli et al., 1997) (**Figure 1.16**). A single strand of the circularised transposon transfers to the recipient after it is cleaved (nicked) at the *oriT* site (Jaworski and Clewell, 1995). In the recipient cell, the complementary strand is synthesised (Scott et al., 1994)

(**Figure 1.13**). Tn*916* integrates into an AT-rich region, which is catalysed by Int (Jaworski and Clewell, 1994, Lu and Churchward, 1994, Lu and Churchward, 1995, Taylor and Churchward, 1997, Wang et al., 2000b, Cookson et al., 2011). Integration of Tn*916* into the target site results in the formation of heteroduplex sequences on either side of the conjugative transposon, which resolves during replication or by DNA repair (Manganelli et al., 1997) (**Figure 1.16**).

Studies have postulated that a conserved sequence of Tn*916* target sites in examined multiple mutants is TTTTTnnnnnnAAAAA, (Scott et al., 1994, Nelson et al., 1997). In addition to this, a study by Cookson et al. (2011) has shown that the consensus sequence TTTTT*TATATA*AAAAA (the italicised hexanucleotides are variable and form the coupling sequence) is used by Tn*916* for integration in 123 insertion sites in the *Butyrivibrio proteoclasticus* strain B316T. Furthermore, Mullany et al. (2012) have indicated that Tn*916* has preferentially inserted the genome of *Clostridium difficile* strains 630 and R20291 at intergenic regions, with a consensus sequence of 5'TTTTTA[AT][AT][AT][AT]AAAAA.

Figure 1.16: Schematic image showing the recombination reactions of Tn*916* (excision and insertion)

The thick lines represent Tn*916*, and the thin lines are the flanking genomic DNA. Nucleotides in yellow represent the nucleotides originally connected with the inserted copy of Tn*916*. Nucleotides in green indicate the nucleotides of the new target site. Nucleotides in red indicate the product of DNA repair through replication or mismatch repair. **(a)** A hexamer-staggered cleavage at the coupling sequence results in the excision of Tn*916* with non-complementary overhangs, which are, ligated together forming a heteroduplex. The heteroduplex site in the vacated target site is resolved by DNA replication. **(b)** A staggered cleavage at the 3′ end of the new target site and the heteroduplex at the joint of the circular intermediate result in molecules that are subsequently ligated together, causing insertion of Tn*916* with heteroduplexes at each end. Again, these heteroduplex nucleotides are resolved by DNA replication resulting in two copies, each flanked by different DNA sequences. Reprinted from "Tn*916*-like genetic elements: a diverse group of modular mobile elements conferring antibiotic resistance," by A. P. Roberts and P. Mullany, 2011, *FEMS Microbiology review, 35*, p. 856-871.

1.3.1.1.3 Tn*916* regulatory region

The regulatory region of Tn*916* consists of *orf12*, *orf9*, *orf7* and *orf8* (**Figure 1.14**) this is conserved in nearly all Tn*916*-like elements, and the essential purpose of this region is transcriptional regulation of these elements (Liu et al., 2008, Roberts and Mullany, 2009).

The *orf12* located upstream of *tet*(M) encodes a 28 amino acid peptide (leader peptide) and contains a number of inverted repeat sequences which can form hairpins followed by uracil residues when transcribed into RNA. In the absence of tetracycline, transcription from p*tet*(M) is terminated at *orf12* due to the attenuation at the leader sequence (*orf12*) by the formation of a strong terminator (Su et al., 1992, Celli and Trieu-Cuot, 1998). However, in the presence of tetracycline, transcripts from *orf12* read-through reaching *tet*(M) and downstream genes (Su et al., 1992) (**Figure 1.17**).

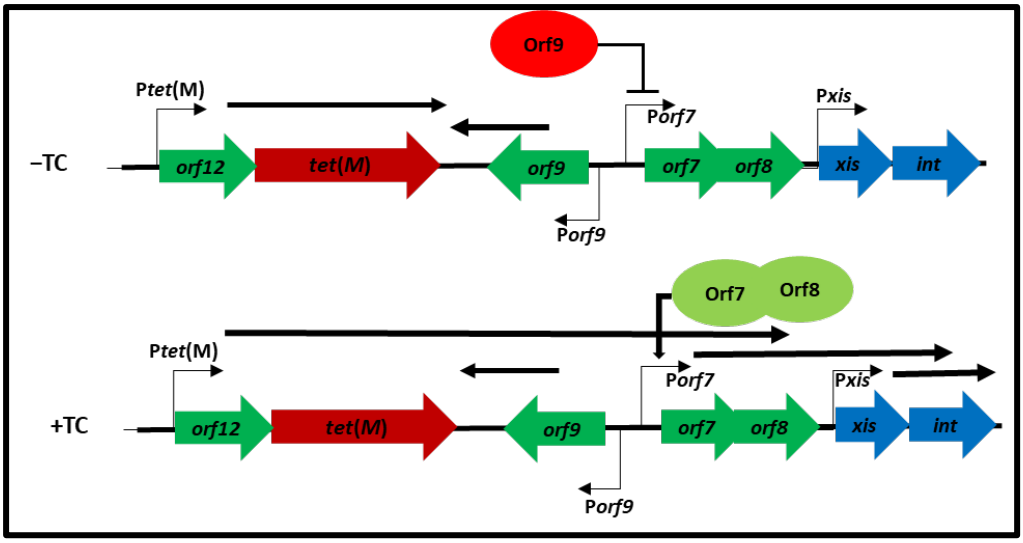

Figure 1.17: Regulation of expression of the transfer genes within Tn*916*

Regulation of expression of the transfer genes within Tn*916*. *Orf12*, *orf9*, *orf7* and *orf8* are represented by green arrows, *tet*(M) is represented by the red arrow and *xis* and *int* are represented by blue arrows pointing towards the direction of transcription. The transcription level is represented by the thick and thin arrows, which denote the higher and lower transcription, respectively. In the absence of tetracycline (–Tc), most of the transcripts initiated at P*tet*(M) terminate at *orf12*. P*orf9* transcribes the *orf9* efficiently, where a low level of transcription reaches P*orf7* causing insufficient transcription of *orf7* and *orf8*. In these conditions, P*orf7* and P*xis* direct a low level of transcription through the transposition and downstream genes.

In the presence of tetracycline (+Tc), the transcripts initiated at the P*tet*(M) read-through the *orf12, orf7* and *orf8*. This condition decreases transcription of *orf9* from the P*orf9* and increases the transcription of *orf7* and *orf8* from P*orf7*. The resulting overexpression of Orf7 and Orf8 stimulates the activity of P*orf7*, thus leading to an increased transcription of downstream genes *xis* and *int*.

Most ribosomes are inactivated in the presence of tetracycline via reversible binding of tetracycline, which causes a delay in the protein synthesis, resulting in the accumulation of charged tRNA molecules. A few ribosomes remain active via the protection from low-level Tet(M). Due to the accumulation of a high level of charged tRNA molecules, rapid *orf12* translation results from the protected ribosomes, which catch up with the RNA polymerase (RNAP) and inhibit or destroy the terminator structure formation designated 5S:6S and 7:8 (**Figure 1.18**). Therefore, transcription starts from the P*tet*(M) promoter

upstream *orf12* and continues into and through *tet*(M) to the downstream genes (**Figure 1.17**).

In the absence of tetracycline, the ribosomes pause on *orf12* due to the low level of charged tRNA molecules and the rare codons present in *orf12*. Thus, the ribosome pauses behind the extending RNAP, allowing the formation of the predicted large 5S:6S terminator and/or the small 7:8 terminator (**Figure 1.18**). Most of the transcripts terminate at *orf12* terminators, resulting in a low expression of *tet*(M) and downstream genes.

Figure 1.18: The alternative secondary structures that are predicted to form in the mRNA of Tn*916*

The stem-loops 1:2, 3:4 and 5S:6S are mutually exclusive of structure 5L:6L. The free energy values of each structure are shown in kcal/mol. The red-shaded area represents the *orf12*. The Shine-Dalgarno sequences (ribosomal binding site) are underlined. The start codon of *tet*(M) is shaded yellow. Section **A)** shows the stem loop structure, which formed in the presence of tetracycline where the ribosomes continue following RNAP. Sections **B)** and **C)** represent the stem loop formed in the absence of tetracycline where the ribosomes stalled behind RNAP and terminate at 5S:6S and 7:8. Reprinted from "A modular master on the move: the Tn*916* family of mobile genetic elements," by A. P. Roberts and P. Mullany, *Trends in Microbiology, 17*, P. 251-8, 2009.

Tn*916*, in addition to the P*tet*(M) promoter, contains other promoters, P*orf7*, P*orf9* and P*xis* (**Figure 1.14**). In normal cell growth where no tetracycline is present, transcription from P*orf9* through *orf9* produces sense RNA, which is translated to Orf9 which represses transcription from P*orf7* and reduces transcription of *orf7*, *orf8* and downstream genes. However, in the presence of tetracycline, transcription from P*tet*(M) stops the translation of *orf9* by antisense *orf9* and thus lowers the Orf9 repression activity on P*orf7*. This results in a high transcription of *orf7*, *orf8* and downstream genes *xis* and *int*, leading to excision. Orf7 and Orf8 activate their transcription from P*orf7* (Celli and Trieu-Cuot, 1998) (**Figure 1.17**). When Tn*916* is excised and circularised, transcription continues through the joint of the ends into the conjugation genes (*orf24* to *orf13*) which are present at the other end of the element (Celli and Trieu-Cuot, 1998) (**Figure 1.19**).

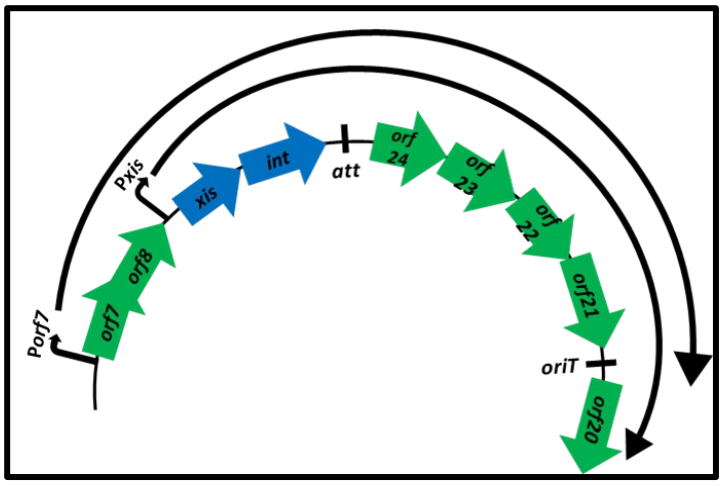

Figure 1.19: The regulation of expression of circular form of excised Tn*916* from the host chromosome

A read-through transcription from P*orf7* promoter to the downstream P*xis*, *xis* and *int* enhances Tn*916* excision. The ends of Tn*916* are indicated by the small black rectangles and *att* represents the insertion site. Blue arrows represent the *int* and *xis* genes, and green arrows represent the other genes in Tn*916*. The black arrow represents the direction of the transcription.

Once most of the ribosomes are protected by Tet(M), the translation rate will decrease due to the consumption of the charged tRNA in the translation process. Therefore, the ribosomes translating *orf12* will stall behind the transcribing RNAP, allowing the formation of the terminator structures and reducing *tet*(M) transcription. This system permits the cell and the transposon to react quickly by downregulating or upregulating Tn*916* transcription and translation in the presence or absence of tetracycline (Su et al., 1992, Celli and Trieu-Cuot, 1998).

The regulation of Tn*916* does not just require the presence or absence of tetracycline but depends on a change in the transcriptional rate, which is linked to the level of charged tRNA (Roberts and Mullany, 2009). When the amount of charged tRNA is increased, this will likely result in the upregulation of Tn*916* genes. Based on this, any malfunction in the cell's transcriptional apparatus will cause a drop in the transcriptional rate, thus building up the amount of charged tRNA. This event is likely to be harmful to the cell, and Tn*916* could sense this cellular distress and activate its own transcription and movement.

1.3.1.1.4 Conjugation of Tn*916*

Conjugation of Tn*916*/Tn*916*-like elements contributes to the dissemination of antibiotic resistant genes among various bacteria. The *orf*s from 24 to 13 are necessary to the conjugation process (Senghas et al., 1988) (**Figure 1.14**). As the conjugation genes are promoterless, they are poorly transcribed when integrated into the host genome except if it inserted downstream of a strong promoter. However, in the circular excised transposon, the conjugation genes are transcribed from P*xis* and P*orf7* (Senghas et al., 1988, Celli and Trieu-Cuot, 1998) (**Figure 1.19**).

Conjugation of Tn*916* starts with an Orf20 relaxase protein nick at the *oriT* site (Jaworski and Clewell, 1995, Rocco and Churchward, 2006) (**Figure 1.13**). Jaworski and Clewell (1995) have localised the *oriT* sequence of Tn*916* which is 466 bp in length, containing six sets of inverted repeats within the intercistronic space between *orf21* and *orf20*. The activity of Orf20 on the *oriT* sequence was demonstrated in the presence of Int producing an endonucleolytic cleavage at the TGGT motif on the transferred strand (Rocco and Churchward, 2006).

In addition, the *orf18* in the conjugation region encodes an anti-restriction protein (ArdA) that could protect the transposon DNA from the host restriction modification system (Serfiotis-Mitsa et al., 2008, Fulsundar et al., 2014).

1.3.1.1.5 Replication of Tn*916*

The excised Tn*916* can autonomously replicate by a rolling circle mechanism. This process starts from the binding of relaxase (ORF20) to *oriT*, which acts as the origin of replication with the help of helicase processive factors Orf22 and Orf23 (HelP homologous) to enable processive unwinding of the nicked strand. After that, the unwound strand is re-circularised and, with the help of a single-strand origin (SSO) of replication, primes the lagging strand DNA synthesis (complementary strand) (Wright and Grossman, 2016) (**Figure 1.20**).

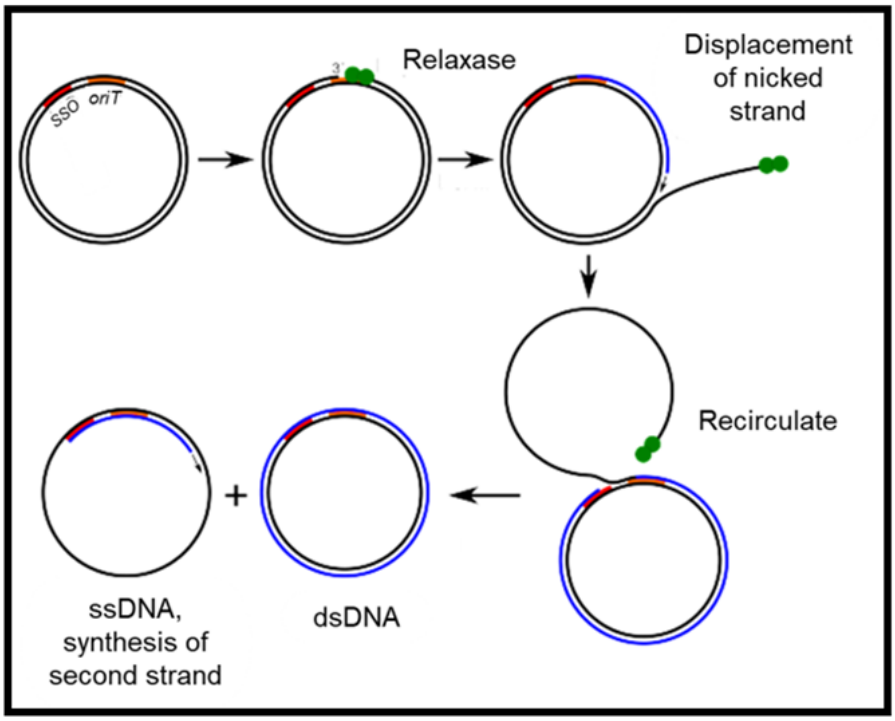

Figure 1.20: Tn*916* autonomous rolling circle replication

The complementary strand is represented in blue. Relaxase binds on *oriT* and nicks one strand. The nicked strand unwinds, and the complementary strand is synthesized while the first strand is displaced. After re-circularising the displaced strand, the complementary strand is synthesized from the SSO.

1.4 The oral microbiome

The oral cavity is the second most complex of the bacterial communities found in the human body (Topazian et al., 2002, Dewhirst et al., 2010, Human Microbiome Project, 2012, Santoro et al., 2014). Because the oral cavity is composed of various surfaces such as the teeth, tongue, gingival crevice, hard palate and buccal mucosa, it allows colonisation by different specific microbiomes listed in **Figure 1.21** (Marsh et al., 2009). The oral microbiome obtains its nutrition from saliva, gingival crevicular fluid or foods.

The oral cavity environmental conditions determine the population at each site (**Figure 1.21**). It includes a chemical environment (e.g. the nutrients, antibiotics and host diet) and a physical environment (e.g. hard or soft tissue surface, pH, temperature, continuous flow of saliva, humidity, O_2 and CO_2 concentration, host smoking habits, hygiene and immunity) (Marcotte and Lavoie, 1998, Marsh et al., 2009). Some members of the oral microbiome have the ability to ferment sugar and turn it into acid, resulting in the development of dental caries (Takahashi and Nyvad, 2011). Numerous beverages have also been shown to significantly affect the oral bacterial community. Coffee and wine have been reported to significantly reduce the number of oral bacteria (Signoretto et al., 2010). In addition, as the oral cavity is an open system, it is always exposed to non-oral (transient) bacteria from the air, food, water, human-human and human-animal interactions or to bacteria on any other objects, which find their way into the mouth. Therefore, the commensal oral bacteria help maintain systemic and oral health by inhibiting the colonisation of exogenous pathogens (Ahn et al., 2012).

Sometimes, transient bacteria cannot colonise the oral cavity as most of the mouth surface is colonised by commensal microorganisms (Wade, 2013).

Figure 1.21: The predominant microbiomes, which colonised different oral cavity sites and oropharyngeal

Commensal oral bacteria need to overcome several challenges, such as temperature fluctuations, different carbohydrate sources and interactions with non-oral bacteria (transient bacteria). Therefore, they may acquire a selective advantage encoded by MGE via HGT. Several studies have demonstrated an ability of HGT to occur among a wide range of oral bacteria *in vitro* (Roberts et al., 1999, Roberts et al., 2001, Ready et al., 2006,

Hannan et al., 2010) and reviewed in (Santoro et al., 2014, Roberts and Kreth, 2014).

1.4.1 Tn*916* in the oral cavity

The conjugative transposon Tn*916* is a very common mobile element in the oral cavity and is responsible for tetracycline resistance encoded by *tet*(M). For example, a study involving 26 children aged 2– 4 years who had never received tetracycline treatment showed that 15 children harboured tetracycline-resistant oral bacteria encoded by *tet*(M), which is located on a Tn*916*-like element in *Streptococcus, Granulicatella, Veillonella* and *Neisseria* (Lancaster et al., 2005). Moreover, Ready et al. (2006) isolated the oral bacterium *Veillonela dispar* that has Tn*916* in its genome and demonstrated its ability to transfer to four tetracycline sensitive oral *Streptococcus* spp. via conjugation. The *tet*(M) is the most predominant gene in tetracycline-resistant isolates in root canals. A tetracycline-resistant *Neisseria* sp. isolated from endodontic patients transferred the *tet*(M) on Tn*916* to *E. faecalis* (Rossi-Fedele et al., 2006).

A study that analysed metagenomic DNA of oral and faecal human samples taken from 20 healthy volunteers (age ranged 21-65) from six different European countries showed that *tet*(M) was the most common tetracycline-resistant gene in the oral cavity and the presence of Tn*916* was inferred by detecting integrase gene (Seville et al., 2009). Roberts et al. (2001) isolated four oral *Streptococcus* spp. harbouring Tn*916*-like elements and demonstrated their ability to transfer the conjugative transposon Tn*916* into

another four oral *Streptococcus* spp. A study on saliva samples of 19 healthy volunteers showed by PCR and sequencing that Tn*916*-like elements were present in oral *Streptococcus* (Ciric et al., 2012b).

1.4.2 Oral streptococci

Oral streptococci are the most prevalent bacteria in the healthy human mouth (Rosan and Lamont, 2000, Bik et al., 2010). *Streptococcus sanguinis*, *Streptococcus oralis* and *Streptococcus gordonii* form 80% of the initial biofilm (a group of bacteria species attached to the tooth surface and/or each other) (Diaz et al., 2006). Streptococci are Gram-positive cocci, arranged in chains or pairs, non-motile and they are facultative anaerobes whose growth often enhanced with CO_2 (Zhou and Li, 2015). They have proteolytic activity that assist their growth in dental plaque in independent to the host diet e.g. breakdown of salivary mucin peptide backbone (Bradshaw et al., 1994, Cowman and Baron, 1997, Wickstrom et al., 2009), salivary acidic proline-rich-proteins (PRPs) (Li et al., 2000), and human immunoglobulin A1 (IgA1) molecules (Reinholdt et al., 1990, Homer et al., 1990).

1.4.2.1 *Streptococcus* classification

Streptococci were classified based on their haemolytic activity into three types: beta, alpha and gamma (Sherman, 1937).

Beta (β) haemolysis is characterised by a complete haemolysis of red blood cells (RBCs) present in blood agar medium developing a clear zone, e.g. *Streptococcus pyogenes* (group A) and *Streptococcus agalactiae* (group B)

(**Figure 1.22**). These streptococcal groups can cause pharyngitis, tonsillitis, impetigo, endocarditis, toxic shock syndrome, pneumonia, scarlet fever, wound and urinary tract infection, acute rheumatic fever and acute glomerulonephritis (Whiley et al., 1999, O'Brien and Nohynek, 2003, Dermer et al., 2004, Carapetis et al., 2005, Berardi et al., 2007, Ralph and Carapetis, 2013).

Alpha (α) haemolysis is characterized by a greenish zone around the colonies due to partial haemolysis of RBCs present in in blood agar medium (**Figure 1.22**). This type of streptococci include for example, *Streptococcus virdians* which is part of the normal microflora in the gastrointestinal and respiratory tracts (Facklam, 2002).

Gamma (ɣ) is a non-haemolytic as RBCs are not haemolysed by these bacteria (**Figure 1.22**), exemplified by, enterococci (group D), *Streptococcus salivarius* and *Streptococcus bovis*. However, the genus *Enterococcus* has been differentiated from *Streptococcus* on the basis of DNA-DNA and 16S rRNA studies (Schleifer and Kilpper-Bälz, 1984, Ludwig et al., 1985). Therefore, *Enterococcus* will be described in more detail in section 1.5.

Figure 1.22: The haemolysis types of *Streptococcus* spp.

The haemolysis types of *Streptococcus* spp. Adapted from “Haemolysis of Streptococci and its types with examples,” by A. Sagar, 2018 (https://microbiologyinfo.com/haemolysis-of-streptococci-and-its-types-with-examples/). © 2019 by Microbiology Info.

In addition to the haemolysis type, Streptococcus species were classified into A, B, C, D, F, G and H via Lancefield serological groups, which is based on specific antigens on the cell wall (T proteins) (Lancefield, 1933). Later, the oral streptococci were divided into five different groups: the Sanguinis group (*Streptococcus sanguinis* and *S. gordonii*), Anginosus group or also known as Milleri group (*Streptococcus anginosus*, *Streptococcus intermedius*, and *Streptococcus constellatus*), Mitis group (*Streptococcus mitis* and *Streptococcus oralis*), Salivarius group (*S. salivarius*) and Mutans group (*S. mutans* and *Streptococcus sobrinus*) (Ruoff, 1988, Whiley and Beighton, 1998, Richards et al., 2014, Jensen et al., 2013).

1.4.2.2 *Streptococcus* pathogenesis

Oral *Streptococcus* spp. are mostly non-pathogenic microorganisms. However, some species, such as *S. mutans*, are the primary oral pathogens responsible for causing dental caries (Hamada and Slade, 1980, Loesche, 1986, Lemos and Burne, 2008, Russell, 2008). In addition, *Streptococcus* spp. are associated with other species in root canal infections with periapical disease (Chávez de Paz et al., 2003). Huyghe et al. (2013) found that in noma (a gangrenous disease that affects the maxillary tissue of immunocompromised children) patients with acute necrotising gingivitis, *S. sanguinis* and *S. pyogenes* predominated in their gingival microflora when compared with healthy controls. Oral *Streptococcus* could cause endocarditis when introduced into the blood stream, e.g. *S. gordonii* and *S. sanguinis* (Herzberg, 1996, Herzberg et al., 1997). In addition, 14 out of 47 cases of endocarditis, were caused by *S. oralis* (Douglas et al., 1993).

1.4.2.3 Evidence for MGE and HGT occurring within oral *Streptococcus*

The predominant genus in the oral biofilm is *Streptococcus* and is naturally competent (Davidson et al., 1976, Rosan and Lamont, 2000, Cvitkovitch, 2001, Martin et al., 2006, Havarstein, 2010). Genome sequencing and analysis have revealed the presence of genes acquired via HGT (Richards et al., 2014). The conjugative plasmid pIP501 and pRE25 were detected in oral streptococci, and transferred with high frequency to *E. faecalis* or *E. coli* (Kurenbach et al., 2003). In addition, a novel conjugative transposon Tn*6087* containing *tet*(S/M)) was discovered in the oral *Streptococcus* species *S. intermedius* and showed the ability to be excised from the plasmid pSI01 with one of the two flanked IS1216 elements (Lancaster et al., 2004, Roberts et al., 2006, Brouwer et al., 2010, Ciric et al., 2011, Ciric et al., 2014). Genome sequencing of *S. mutans* UA159 revealed a 40 kb putative conjugative transposon (Tn*Smu2*) containing the remains of insertion sequence (IS) flanked genes that potentially encode gramicidin and bacitracin syntheses (Ajdic et al., 2002). In addition, *S. mutans* under scanning electron microscopy was observed to release eDNA via a membrane vesicle into the biofilm (Liao et al., 2014).

1.4.2.4 *In vitro* investigation on the presence and transfer of Tn*916* within oral *Streptococcus*

The tetracycline resistance gene (*tet*(M)) is common in oral streptococci. Sequence homology to Tn*916* was identified in 3 out of 17 oral streptococci and in 2 out of 18 enterococci strains (Bentorcha et al., 1992). Lacroix and Walker (1995b) observed, after screening subgingival plaque samples collected from 68 adult periodontal patients, that 55% of the oral streptococci carried *tet*(M). The conjugative transposon Tn*916* was detected in seven strains of the oral streptococci, which were isolated from patients treated with tetracycline. One of the strains (*S. sanguinis*l I141) had the ability of conjugal transfer of Tn*916* to three another oral streptococci and also to enterococci (Hartley et al., 1984).

In addition, from human saliva, a microcosm dental plaque grown in a constant depth film fermentor (CDFF) contained Tn*916*-like elements in four streptococcal species. This element was transferred to four other streptococcal species in filter mating and in a model oral biofilm (Roberts et al., 2001). Fitzgerald and Clewell (1985) have detected the presence of a Tn*916*-like element in an oral *Streptococcus* (*S. sanguis* FC1) and its transferral to other oral streptococci (*E. faecalis* FA2-2, *S. sanguis* and *S. lactis*) via filter mating. Moreover, a survey of saliva samples of healthy volunteers showed that Tn*916*-like elements were present in 25 different oral *Streptococcus* spp. (Ciric et al., 2012b).

1.5 *Enterococcus faecalis*

Enterococci are considered as transient bacteria in the oral cavity (Razavi et al., 2007, Zehnder and Guggenheim, 2009). Enterococci have been isolated from the root canals of endodontic patients and show the ability to resist endodontic treatment due to high resistance through forming a biofilm (Sundqvist et al., 1998). In addition, enterococci have been linked to other oral diseases e.g. caries and periodontitis (Kouidhi et al., 2011, Dahlen, 1993, Rams et al., 2013). Therefore, in this investigation, *E. faecalis* has been included as a recipient in conjugation experiments.

The genus *Enterococcus*, comprises of species that are Gram-positive cocci arranged in pairs or short chains, catalyse negative and facultative anaerobes (Ciftci et al., 2009). Enterococci are known as gut bacteria and can be found in faeces (Thiercelin, 1899). In addition, enterococci are present in the environment, e.g. in soil, wastewater and sediments (Song et al., 2005, Hasannejad Bibalan et al., 2015). Enterococci have an intrinsic resistance to several antibacterial agents, such as clindamycin, aminoglycosides and cephalosporin and are able to acquire resistance genes encoded by mobile genetic elements, such as the vancomycin resistance gene *vanB*$_2$ carried on Tn*5382* and Tn*1549* or *vanA* on Tn*1546* (Rice, 2001, Bonten et al., 2001, Maschieto et al., 2004, Launay et al., 2006, Schjørring and Krogfelt, 2011, Boehm AB, 2014).

E. faecalis and *Enterococcus faecium* are the predominant species that cause serious diseases in humans, including, septicaemia, urinary tract

infections (UTIs), abdominal and pelvic abscess, wound infection, endocarditis and meningitis (Colodner et al., 2006, Chirouze et al., 2013, Flores-Mireles et al., 2015). Enterococci are considered globally as nosocomial pathogens (Talebi et al., 2008, Silva et al., 2012, Gilmore et al., 2013, Chowdhury et al., 2014, Mittal et al., 2016).

Genomic analysis for a hospital strain of *E. faecalis* showed a significant amount of MGEs in the genome (Solheim et al., 2011). Moreover, *E. faecalis* has been isolated from volunteer saliva, where 7 out of 31 samples showed a bacteriophage, which was specific to *E. faecalis* (Bachrach et al., 2003). A study was carried out on marginal periodontal patients, and screening *E. faecalis* strains showed 93 out of 106 of the *E. faecalis* strains harbouring a large number of plasmid replicons (120 plasmids) with seven replicons having different tetracycline and erythromycin resistance genes (Song et al., 2013). In addition, the conjugative element Tn*916* was identified for the first time in a *E. faecalis* DS16 dental isolate (Franke and Clewell, 1981).

1.6 *Bacillus subtilis* as environmental bacteria

In this work, *B. subtilis,* a non-oral bacterium was considered as a donor in conjugation experiments. *B. subtilis* has the ability to transfer the conjugative transposon Tn*5397* into oral streptococci (*S. acidominimus*) growing within a biofilm *in vitro* (Roberts et al., 1999). *B. subtilis* is a Gram-positive rod bacterium that forms endospores and grows aerobically or facultative anaerobically (Nakano et al., 1997). Bacteria of the genus *Bacillus* are generally soil inhabitants, but can be isolated from air, water, human and animal gut, vegetables and other foods (Tidjiani Alou et al., 2015, Kotb, 2015). *B. subtilis* has the ability to follow diverse developmental pathways in response to environmental stress and to achieve a competence state for DNA uptake, motility, biofilm formation, chemotaxis and endospore formation (Maughan et al., 2004). As *Bacillus* spores have the ability to survive in the extreme acidity of the stomach and tolerate bile salts and other hostile conditions of the gastrointestinal tract, it is often used as a probiotic microorganism (Rao et al., 2015, Elshaghabee et al., 2017).

1.7 The aim of the study

The aim of this study was to investigate the effect of different environmental conditions that mimic the oral cavity on Tn*916* transfer from an environmental bacterium, such as *B. subtilis*, into oral and gut bacteria, such as streptococci and enterococci. These aims will be met by focusing on the transfer and the transcriptional regulation of Tn*916*. The objectives in detail were:

1. Investigate the effect of different environmental conditions (aerobic and anaerobic), media (liquid and solid) and time required for Tn*916* transfer to occur.
2. Investigate the copy number of Tn*916* transferred in aerobic and anaerobic conditions.
3. Determine the activity of a putative Fumarate and Nitrate reductase (FNR) binding sequence and the transcriptional activity on the *orf12* terminator in different conditions.
4. Investigate the transfer of Tn*916* aerobically and anaerobically after introducing a mutation on the *orf12* large terminator.

Chapter 2

Materials and Methods

Chapter 2 Material and Methods

This chapter outlines the materials and methods that were used throughout the project. Specific materials and methods are also identified at the beginning of each relevant chapter.

2.1 Bacterial strains, plasmids and growth conditions

All the bacterial strains and plasmids used in the current work (listed in Table 2.1) were grown at 37 ºC aerobically, however the streptococci and enterococci strains were grown in the presence of 5% Co_2.

Table 2.1: Bacterial strains and plasmids used in this study.

Bacterial strains/Plasmids	Genotype and/or phenotype	Reference/source
***S. mutans* ATCC 700610**	Tet^s recipient for filter mating	(Ajdic et al., 2002)
***S. mutans* NCTC 10449**	Tet^s recipient for filter mating	(Coykendall, 1977)
***S. mutans* UA 159**	Tet^s recipient for filter mating	(Ajdic et al., 2002)
***S. sanguinis* NCTC 7836**	Tet^s recipient for filter mating	(Xu et al., 2007)
***S. parasanguinis* NCTC 55898**	Tet^s recipient for filter mating	(Sabharwal et al., 2015)
***S. oralis* NCTC 11427**	Tet^s recipient for filter mating	(KILPPER-BÄLZ et al., 1985)
***S. salivarius* NCTC 8618**	Tet^s recipient for filter mating	(KILPPER-BÄLZ et al., 1985)

S. pyogenes erm TR	Tet^S recipient for filter mating	(Seppala et al., 1998)
***E. faecalis* JH2-2**	Tet^S recipient for filter mating	(Jacob and Hobbs, 1974)
***B. subtilis* BS34A**	Tet^R donor of Tn*916*	(Roberts et al., 2003)
***S. oralis* SO03**	Tet^R transconjugant isolated after 3 min containing Tn*916* element	This study
***S. pyogenes* SP03**	Tet^R transconjugant isolated after 3 min containing Tn*916* element	This study
***E. faecalis* JH2-2 EF03**	Tet^R transconjugant isolated after 3 min containing Tn*916* element	This study
***S. oralis* SO0T1**	Tet^R transconjugant isolated after 1 min in tube mating containing Tn*916* element	This study
***S. pyogenes* SPT1**	Tet^R transconjugant isolated after 1 min in tube mating containing Tn*916* element	This study
***E. faecalis* JH2-2 EFT1**	Tet^R transconjugant isolated after 1 min in tube mating containing Tn*916* element	This study

***S. oralis* SOT5**	Tet^R transconjugant isolated after 5 min in tube mating containing Tn*916* element	This study
***S. pyogenes* SPT5**	Tet^R transconjugant isolated after 5 min in tube mating containing Tn*916* element	This study
***E. faecalis* JH2-2 EFT5**	Tet^R transconjugant isolated after 5 min in tube mating containing Tn*916* element	This study
***S. oralis* SO001**	Tet^R transconjugant isolated after 1 h in tube mating containing Tn*916* element	This study
***S. pyogenes* SP001**	Tet^R transconjugant isolated after 1 h in tube mating containing Tn*916* element	This study
***E. faecalis* JH2-2 EF001**	Tet^R transconjugant isolated after 1 h in tube mating containing Tn*916* element	This study
***S. oralis* SO002**	Tet^R transconjugant isolated after 2 h in tube mating containing Tn*916* element	This study

***S. pyogenes* SP002**	Tet^R transconjugant isolated after 2 h in tube mating containing Tn*916* element	This study
***E. faecalis* JH2-2 EF002**	Tet^R transconjugant isolated after 2 h in tube mating containing Tn*916* element	This study
***S. oralis* SO024**	Tet^R transconjugant isolated after 24 h in tube mating containing Tn*916* element	This study
***S. pyogenes* SP024**	Tet^R transconjugant isolated after 24 h in tube mating containing Tn*916* element	This study
***E. faecalis* JH2-2 EF024**	Tet^R transconjugant isolated after 24 h in tube mating containing Tn*916* element	This study
***B. subtilis* BS34AΔ*orf12*(T_1)**	Δ*orf12*(T_1) mutant derived from BS34A	This study
***E. coli* α-select silver**	Competent Cells	Bioline (UK)
pGEM®-T easy	Cloning vector (Amp^R)	Promega (UK)
pGEMT-Tn*916CJ*	pGEMT-T with 136bp fragment of circular joint of Tn*916* (Amp^R)	This study

pGEMT-Tn*916CJ-GyrB*	pGEMT-T-Tn*916CJ* with128bp fragment of *B. subtilis gyrB* gene (Amp^R)	This study
pHCMC05-p*tet*(M)-*orf12-gus*A	Shuttle vector (Amp^R,Cm^R), containing the *tet*(M) promoter and *orf12* upstream *gusA*	(Seier-Petersen et al., 2014)
pHCMC05-p*tet*(M)-Δ*orf12-gus*A	Shuttle vector (Amp^R,Cm^R). containing Δ*orf12* mutant drived from pHCMC05-p*tet*(M)-*orf12-gus*A	This study
pHCMC05-p*tet*(M)-Δ*orf12*(T_1)-*gus*A	Shuttle vector (Amp^R,Cm^R). containing Δ*orf12*(T_1) mutant drived from pHCMC05-p*tet*(M)-*orf12-gus*A	This study
pHCMC05-p*tet*(M)-Δ*orf12*(T_2)-*gus*A	Shuttle vector (Amp^R,Cm^R). containing Δ*orf12*(T_2) mutant drived from pHCMC05-p*tet*(M)-*orf12-gus*A	This study
pHCMC05-p*tet*(M)-s*orf12*(T_1)-*gus*A	Shuttle vector (Amp^R,Cm^R). containing s*orf12*(T_1) mutant drived from pHCMC05-p*tet*(M)-*orf12-gus*A	This study
pJOE8999	CRISPR-Cas9 system (Km^R)	(Altenbuchner, 2016)

pJOE8999 Ω sgRNA	sgRNA fragment cloned into pJOE8999 (Km^R)	This study
pJOE8999 Ω sgRNA-HA	Homologous arms cloned into pJOE8999 Ω sgRNA (Km^R)	This study

Note: Tet^s, Tet^R, Amp^R, Cm^R and Km^R indicate tetracycline-sensitive, tetracycline-, ampicillin-, chloramphenicol- and kanamycin-resistant, respectively.

2.2 Molecular biology methods

2.2.1 Oligonucleotide synthesis

All the used oligonucleotides (primers) in the current work were synthesised by Sigma-Aldrich (UK) (Table 2.2).

Table 2.2: List of primers used in this study.

Primer	Sequence (5'-3')	Amplification product	Reference
TetM-F1	ACCAAAGCAACGCAGGTATC	*tet*(M) of Tn*916*	This study
TetM-R1	TCGGACAATAGAGGGGGAAT		
Intxis(F)	GGCTGATTATGAATGGCAATCGTATTC	Integration and excision region of Tn*916*	This study
Intxis(R)	GCTGTAGGTTTTATCAGCTTTTGCAAAG		
27F	AGAGTTTGATCMTGGCTCAG	16S rRNA gene	(Wang et al., 2000a)
1492R	TACGGYTACCTTGTTACGACTT		
Tn*916CJ*-F1	GGCTTTACGAGCATTTAAGAAAA	Circular joint of Tn*916* (136 bp)	

Tn*916CJ*-R1	AAATTGAGTGGTTTTGACCTTGA		This study
GyB-NsiI-F1 **GyB-NsiI-R1**	GCGGATGCATGGGCTGACAGCGATTATTTC GCGGATGCATAAATGTTTCCATCGCCGTAG	*gyrB* of BS34A (128bp) with restriction site of NsiI	This study
FNR-F	CGCGCATATGAATTTTCTCTCTGTTCGACCATC	*fnr* of BS34A (240bp) with restriction site of Nde1	This study
FNR-R	CGGCGGATCCGTCAATATTGCAAATCTCCAGC	*fnr* of BS34A (240bp) with restriction site of BamH1	This study
Veg-F **Veg-R**	TGGCGAAGACGTTGRCCGATATTA AAAATATCAGCATAACTGTATGAAACTC	*Veg* of BS34A	This study
Δ*orf12*(T_1)-F **Δ*orf12*(T_1)-R**	GGGATTTTTATGCCCTTTTG GGGATTTTTATGCATAACCATAG	Portion deletion of *orf12* large terminator (T_1) from pHCMCO5-P*tet*(M)-*gusA*	This study
s*orf12*(T_1)-F **s*orf12*(T_1)-R**	TTTAATGGGGGATTTTTATGCCCTTT CTTACCATTGGGATTTTTATGCATAAC	Substation of *orf12* large terminator (T_1) on pHCMCO5-P*tet*(M)-*gusA*	This study
Δ*orf12*(T_2)-F **Δ*orf12*(T_2)-R**	GAATGGAGGAAAATCACGAAT ATAAAAATCCCAGTGATAAATACTCTTATC	Deletion of *orf12* small terminator (T_2) from pHCMCO5-P*tet*(M)-*gusA*	This study
Δ*orf12*-F **Δ*orf12*-R**	TGGAGGAAAATCACGAATTCCTGCAGT ACAGATATTCTCCGGATACTTTAGAATCA	Deletion of *orf12* from pHCMCO5-P*tet*(M)-*gusA*	This study

HA-xbaI-F **HA-xbaI-R**	GATCTAGACCAAAGCAACGCAGGTATCTCAATT GATCTAGAGTGATTTTCCTCCATTCAAAAGCCC	Homology template for *orf12* terminator(T_1) deletion containing restriction site of xbal	This study
sgRNA –F1 **sgRNA – R1**	**tacg**TGATAAGAGTATTTATCACT **aaac**AGTGATAAATACTCTTATCA	Target sequence for deletion *orf12* terminator(T_1)	This study
Orf12-F **Orf12-R**	CAGCAGACCAAAGCAACGCAGGTATC ACATGAGCTAAAACTCCAATATTAATAA	*orf12* of Tn*916*	This study
M12-R	AGTGATAAATACTCTTATCACTGGGA	Binds to *orf12* large terminator(T_1)	This study
M13F **M13R**	GTAAAACGACGGCCAG CAGGAAACAGCTATGAC	sequencing pGEM-T vector	Universal
T7 primer F	TAATACGACTCACTATAGGG	Forward sequencing pJOE8999 vector	Universal
PHCMC05-p*tet*(M)-*gusA*-F **PHCMC05p*tet*(M)-*gusA*-R**	AAAATCGTCTCCCTCCGTTT TCCGAGCTTCGTCCAAAATA	sequencing PHCMC05-p*tet*(M)-*gusA*	This study
pHCMC05-F **pHCMC05_R**	TTTTAACGATCAGTTCGCCG TAACATACGGCGTGACATCG	sequencing PHCMC05-ptet(M)-*gusA*	This study
TS-F2	AAATAACCCCAAAAATCTAAGAAAA	Forward sequencing pJOE8999	This study

TS-F3	CCTTTTAACATTAAAAACCCAATATT	Forward sequencing pJOE8999	This study
TS-F4	TGTAAGTCTTAAAGTAACAGCAACTTTT	Forward sequencing pJOE8999	This study

Note: The grey highlighting indicates the enzyme restriction site and the bold lower-case sequence indicates the plasmid backbone.

2.2.2 Polymerase chain reaction (PCR) and conditions

PCR mixtures with a 25-µl final volume was prepared as follows: 12.5 µl of 2X Biomix Red (Bioline, UK), which contains *Taq* DNA polymerase, 2 µl of each primer (1 µM), 1.5 µl of the DNA template (50-100 ng/µl) and 9 µl of molecular-grade water. The PCR conditions using the Biometra T3000 Thermocycler (Biometra, Netherlands) was started with an initial denaturation at 94 °C for 2 min, and 30 cycles commenced with denaturation at 94 °C for 2 min, annealing at 50–55 °C for 30 sec and ended with an extension at 72 °C for 2 min and finally elongation at 72 °C for 5 min.

2.2.3 Site-directed mutagenesis

A site-directed mutagenesis reaction was prepared using the Q5® Site-Directed Mutagenesis Kit (NEB, UK). The entire plasmid was amplified using specific primers that introduced the desired mutations. A PCR mix was prepared in a 24-µl final volume containing 12.5 µl of the X Q5 master mix, 1.5 µl of each primer (10 µM), 1 µl of the plasmid template (~100ng/ µl) and 9 µl of molecular biology-grade water. The PCR cycle conditions using the

Biometra T3000 Thermocycler (Biometra, Netherlands) were as follows: 98 °C for 30 sec (initial denaturation) and 35 cycles starting at 98 °C for 10 sec (denaturation), 50–60 °C depending on the primer melting temperature for 30 sec (annealing) and ending with 72 °C for 5 min (extension). In addition, the final extension step condition was 72 °C for 2 min.

The amplified PCR product was re-circularised and the template DNA was removed via the kinase ligase DpnI (KLD) enzyme in a 10-µl total reaction. The reaction was prepared containing 1 µl of the PCR product, 1 µl of the 10X KLD enzyme mix (NEB, UK), 5 µl of 2X KLD buffer and 3 µl of molecular biology-grade water. The reaction mixture was incubated at room temperature for 5 min and transferred to *E. coli* α-select silver, as described in section 2.2.14.1.

2.2.4 Agarose gel electrophoresis

DNA products were visualised by 1% agarose gel electrophoresis. One percent (w/v) of agarose (Bioline, UK) gel was prepared with 1X tris-acetate-EDTA (TAE) buffer and 0.5 µg/ml ethidium bromide stain (EtBr; Life Technology, UK). DNA products were mixed with 5X loading buffer (Bioline, UK) except for PCR products which amplified via Biomix Red and loaded on to the gel. To reference the size, 1 µl of HyperLadder 1Kb (Bioline, UK) was also loaded to the gel. The gel was electrophoresed in 1X TAE at 80V/cm² for 60 min. DNA bands were visualised under a UV illuminator via Alpha Imager (Alpha InnoTech, UK), and the image was captured by the Alpha View software version 1.0.1.10 (Alpha InnoTech, UK).

2.2.5 PCR product purification

PCR purification was performed on PCR products and other enzymatic reactions to clean DNA using the QIAquick PCR Purification Kit (Qiagen, UK). All centrifugation steps were done using the Eppendorf centrifuge 5415 D at 14,680 × *g* (13,000 rpm). One volume of the PCR product was mixed with 5x volumes of buffer PB (Appendix 1) and transferred to a QIAquick spin column. After centrifugation for 1 min, the flow-through was discarded and 700 µl of buffer PE (Appendix 1) was added. Then spin column was centrifuged for 30 sec and the flow-through was discarded. Wash buffer residuals were removed after additional centrifugation for 1 min. The spin column was transferred to a 1.5-ml microcentrifuge tube, and 30 µl of molecular-grade water was added to the centre of the QIAquick membrane and left to stand for 1 min to elute the DNA after 1 min of centrifugation.

2.2.6 DNA sequencing reaction and analysis

Sequencing plasmids (100 ng/µl) and PCR products (50 ng/µl) were prepared by sending 10 µl of DNA with the appropriate primer to GeneWiz (Beckman Coulter Genomics, UK).

The sequencing results were compared with the nucleotide database using the National Centre for Biotechnology Information (NCBI) online tool (Altschul et al., 1990) and for sequence alignment used the Clustal Omega online software (https://www.ebi.ac.uk/Tools/msa/clustalo/).

2.2.7 DNA gel extraction

The interested separated DNA fragments on gel were cut under UV light and transferred to a 1.5-ml microcentrifuge tube. Using the QIAquick Gel Extraction Kit (Qiagen, UK), 3x volumes of buffer QG (Appendix 1) were added to 1x volume of gel and incubated at 50 °C using a heat block until the gel dissolved. One volume of isopropanol was mixed with the dissolved gel and transferred to a QIAquick spin column. All centrifugation steps were done in the Eppendorf centrifuge 5415 D at 14,680 × *g* (13,000 rpm). After 1 min of centrifugation, the flow-through was discarded and 700 µl of buffer PE was added. The spin column was centrifuged for 30 sec and the flow-through was discarded. The wash buffer residual was removed after an additional 1 min of centrifugation. The spin column was transferred to a 1.5-ml microcentrifuge tube, and 30 µl of molecular-grade water was added to the centre of the QIAquick membrane and left to stand for 1 min to elute the DNA after 1 min of centrifugation.

2.2.8 Plasmid extraction

Plasmid extraction was achieved using the QIAprep Spin Miniprep Kit (Qiagen, UK). A single colony of *E. coli* was cultured aerobically at 37 °C with shaking (200 rpm) for 16 h in 5 ml of LB broth supplemented with appropriate selective antibiotics. The bacterial cells were harvested by centrifugation in the Eppendorf centrifuge 5804 R at 4,500 × *g* (5,000 rpm) for 15 min at 4 °C. Then, the supernatant was discarded and the pellet re-suspended in 250 µl of buffer P1 (Appendix 1). Subsequently, the 250 µl volume was transferred

to a 1.5-ml microcentrifuge tube and mixed gently with 250 µl of buffer P2 (Appendix 1). Thereafter, 350 µl of buffer N3 (Appendix 1) was added and gently mixed by inverting the tube six times. All centrifugation steps were done using a table-top centrifuge at 14,680 × *g* (13,000 rpm, Eppendorf centrifuge 5415 D). The mixture was centrifuged for 10 min and the supernatant was transferred to a QIAprep 2.0 spin column by pipetting. After 1 min of centrifugation, the flow-through was discarded and 500 µl of buffer PB was added to the QIAprep spin column. The column was then centrifuged for 1 min and the flow-through was discarded. Seven hundred and fifty microliters of buffer PE was added to the column and centrifuged for 1 min. The flow-through was discarded and centrifuged for an additional 1 min to remove the remaining buffer from the column. The QIAprep spin column was transferred into a 1.5-ml microcentrifuge tube to elute DNA. Thirty microliters of molecular biology-grade water was added to the centre of the membrane and left to stand for 1 min. The tube was centrifuged for 1 min to elute DNA.

2.2.9 Genomic DNA Extraction

Genomic DNA extraction was performed using the Puregene Yeast/Bact Kit for Gram-Positive bacteria (Qiagen, UK). Five hundred µl of the overnight bacterial culture was pipetted into a 1.5-ml microcentrifuge tube and centrifuged for 1 min in the Eppendorf centrifuge 5415 D at 14,680 × *g* (13,000 rpm). The cell pellet was re-suspended with 300 µl of cell suspension solution and 1.5 µl of lytic enzyme solution. For mixing, the tube was inverted 25 times and incubated for 30 min at 37 °C. After 1 min of centrifugation, the supernatant was discarded by pipetting, and 300 µl of cell

lysis solution was added. The tube was incubated for 5 min at 80 °C, followed by adding 1.5 µl of RNase A solution. The tube was inverted 25 times for mixing and incubated for 60 min at 37 °C. After cooling the sample on ice for 1 min, 100 µl of protein precipitation solution was added and mixed vigorously. After centrifugation for 3 min, the supernatant was pipetted into a clean 1.5-ml microcentrifuge tube containing 300 µl of 100% isopropanol and mixed gently by inverting the tube 50 times. After 1 min of centrifugation, the supernatant was discarded and the tube was inverted to drain on absorbance paper. The DNA pellet was washed by the addition of 300 µl of 70% (v/v) ethanol and mixed gently by inverting the tube for 50 times. After 1 min centrifugation, the supernatant was discarded and the pellet was air-dried. The DNA pellet was dissolved in 100 µl of DNA hydration solution and incubated for 1 h at 65 °C. Finally, with gentle shaking, the DNA was incubated overnight at room temperature.

2.2.10 Restriction endonuclease reactions

DNA digestion was performed using a restriction enzyme (NEB, UK) according to the manufacturer's protocol. A final volume of 10 µl of the digestion reaction was prepared containing 1 µl of 10X digestion buffer, 1 µl of the restriction enzyme (20 units), 1–3 µl of DNA sample and made up to volume with molecular biology-grade water. The reaction was incubated in a 37 °C water bath for 1–1.5 h, unless specified otherwise. The digested reaction was purified either via the QIAquick PCR Purification Kit or via QIAquick gel extraction, as performed in section 2.2.7.

2.2.11 Dephosphorylation reaction

Linearised plasmid (~200 ng/µl) was dephosphorylated to inhibit self-ligation prior to cloning. This was performed via the addition of 1 µl of calf intestinal alkaline phosphatase (CIAP) (1 U/µl), 2 µl of 10X CIAP reaction buffer (Promega, UK) and made up to a final volume of 20 µl with molecular biology-grade water. The reaction was incubated for 30 min in a 37 °C water bath. Later, dephosphorylation was stopped at 80 °C for 2 min and purified using the QIAquick PCR Purification Kit prior to ligation, as performed in section 2.2.5.

2.2.12 DNA ligation reactions

The ligation reaction was prepared using T_4 DNA ligase (NEB, UK) following the manufacturer's protocol with some modifications. The volume of the vector and insert DNA was added based on the molar ratio 1:3 of the vector to the insert. The ligation reaction (with a 10-µl total volume containing 1 µl of T_4 DNA ligase (400u/µl), 1 µl of 10X ligation buffer, the vector, the insert DNA and molecular biology-grade water) was incubated at 4 °C overnight. T_4 DNA ligase was deactivated at 65 °C for 10 min.

2.2.13 Preparation of *B. subtilis* competent cells

B. subtilis competent cells were prepared following a previously described protocol (Hardy, 1985). In a 200-ml conical flask containing 10 ml SPI broth (Spizizen's minimal medium) (Appendix 1), one colony of *B. subtilis* BS34A

was inoculated and incubated with shaking (200 rpm) at 30 °C overnight. Then, in a 1-L flask containing 100 ml fresh pre-warmed SPI broth, 10 ml of the overnight culture was transferred and incubated with shaking (200 rpm) at 37 °C until the OD_{600} reading stopped increasing indicating the end of logarithmic phase (~4–5 h). Then, 10 ml was transferred to 90 ml fresh pre-warmed SPII broth (Appendix 1) and incubated at 37 °C under shaking (200 rpm) for 1.5 h. Cells were harvested via centrifugation for 10 min at 3,000 × *g* (5,000 rpm) at 20 °C. Cells were re-suspended in a 10-ml mixture containing 9 ml of a supernatant and 1 ml sterile glycerol. On ice, aliquots of 1 ml were dispensed and stored at -80 °C until needed.

2.2.14 Transformation protocols

2.2.14.1 Transformation of *E. coli*

E. coli transformation using α-select silver efficiency competent cells (Bioline, UK) was performed. Five microliters of ligation reaction was mixed with 50 µl of thawed competent cells on ice. The mixture was mixed gently by tapping the tube and incubated on ice for 1 h. Then, the cells were heat shocked via incubation in a 42 °C water bath for 45 sec and immediately incubated on ice for 2 min. Nine hundred and fifty microliters of SOC broth (NEB, UK) (Appendix 1) was added to the cells and incubated at 37 °C with shaking (200 rpm) for 1.5 h. On LB agar supplemented with appropriate antibiotics, 100 µl of the mixture was spread. The remaining mixture was centrifuged for 1 min at 4,000 × *g* (8,000 rpm, Eppendorf centrifuge 5415 D), and 100 µl was spread on LB

agar supplemented with appropriate antibiotics after the supernatant was discarded. All plates were incubated at 37 °C for 16 h.

2.2.14.2 Transformation of *B. subtilis*

B. subtilis transformation was carried out using BS34A strain. One millilitre of frozen *Bacillus*-competent cells was thawed at 37 °C and quickly transferred into a 50-ml tube. Five microliters of ligation reaction was added with gentle flicking and incubated at 37 °C for 1 h with gentile agitation (50 rpm). After that, 5 ml of LB broth was added and incubated for another 1.5 h with vigorous agitation (200 rpm). Then, the mixture was centrifuged and some of the supernatant was discarded. For transformant isolation, 200 µl was plated on LB supplemented with appropriate antibiotics and incubated aerobically at 37 °C overnight.

2.2.15 Blue/white colony screening

The pGEM®-T easy vector containing the DNA insert was transformed into *E. coli* α-select silver efficiency competent cells and isolated on LB agar supplemented with 100 µg/ml ampicillin, 40 µg/ml 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal) and 0.1 M isopropyl-β-D-thiogalactoside (IPTG). After 16 h of incubation at 37 °C, white colonies, which indicate the existence of recombinant plasmids containing a DNA insert, were selected for further verification via colony PCR or plasmid extraction using the QIAprep Spin Miniprep Kit (Qiagen, UK).

2.3 Southern blot and hybridisation

Southern blot hybridisation and probe labelling were performed using the ECL Direct Nucleic Acid Labelling and Detection System (Amersham, UK) following the manufacturer's instructions.

2.3.1 Gel processing

As described in section 2.2.4, agarose gel electrophoresis run was carried out until reaching the appropriate distance. Then, the gel was placed in a plastic container containing depurination solution (Appendix 1) and gently agitated on a shaker until the Bromophenol blue dye changed completely to yellow (this step opens or widens the gel pores). Then, the depurination solution was discarded and the gel was rinsed with distal water. The gel was covered with denaturation solution (Appendix 1) and agitated gently on the shaker for 25 min until the Bromophenol dye returned to blue colour. Again, the solution was discarded and the gel was rinsed with distilled water. The gel was completely covered with neutralisation solution (Appendix 1) and gently agitated twice after changing the solution for 30 min and 15 min.

2.3.2 Capillary blotting

Capillary blotting was prepared using 20X saline sodium citrate (SSC) (Appendix 1) and the blot was set up as shown in **Figure 2.1**, avoiding in all steps trapped air bubbles. In a suitable container containing 20X SSC, a high platform was prepared. Then, 3MM Whatman filter paper saturated with 20X

SSC was used to cover the platform. The gel was placed onto the 3MM filter paper, and the edges were covered with film to prevent the absorbance of the SSC directly via the filter paper. A sheet of Hybond ECL nitrocellulose membrane (Amersham, UK) was cut to the gel size and placed on top of the gel after wetting with 20X SSC. Another three sheets of 3MM filter paper were cut to the gel size and placed on top of the ECL membrane after soaking with 10X SSC. A stack of absorbent paper towels (7 cm) was placed on top of the 3MM filter paper. Then, a glass plate and a heavy weight were placed on top of the paper towels. The blotting stack was left overnight at room temperature (25 °C).

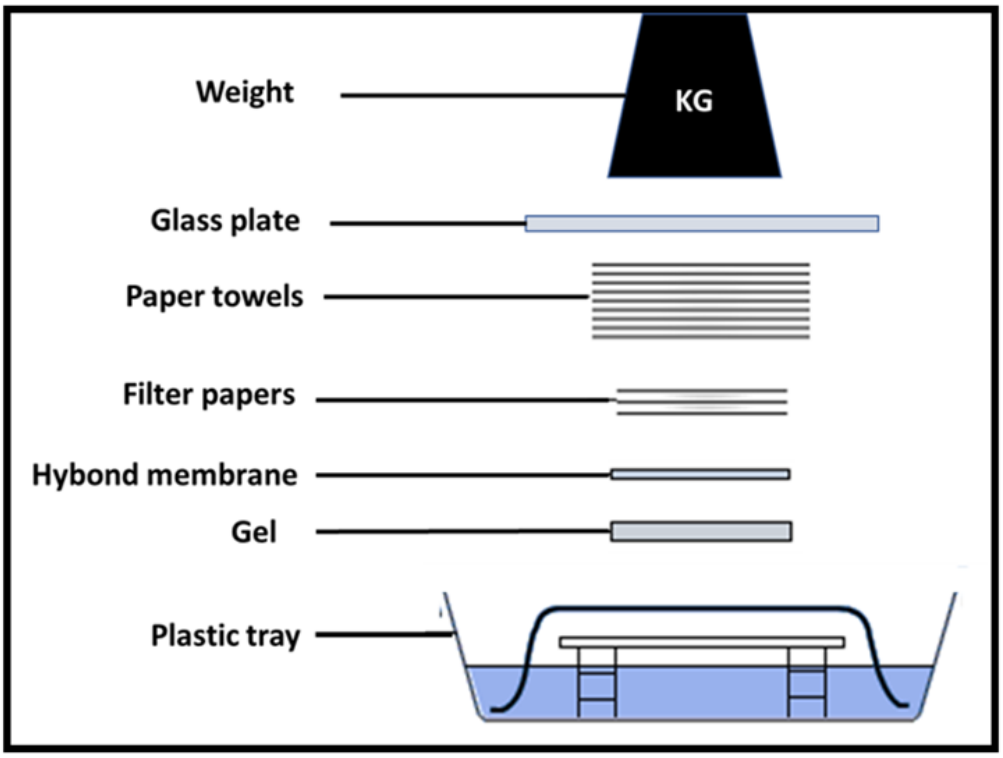

Figure 2.1: Setup of the gel blotting procedure through capillary transfer

2.3.3 Processing the blot

The blotting stack was pulled apart and the hybrid membrane was removed and was placed with side up on a clean sheet of 3MM paper. The DNA was fixed to the membrane by UV cross-linking in the UV Stratalinker 1800 (Stratagene, Netherlands) according to the manufacturer's instructions.

2.3.4 Labelling with DNA probes

Labelling with DNA probes was undertaken according to the manufacturer's instructions (Amersham, UK). Ten microliters of DNA (10 ng/µl) was heated in boiling water for 5 min (denatured) and cooled on ice for 5 min. After the tube was centrifuged, 10 µl of glutaraldehyde solution (labelling reagent) was added and mixed gently. The tube was then centrifuged again to collect the contents at the bottom, and it was incubated for 10 min at 37 °C.

2.3.5 Hybridisation and stringency washes

The hybridisation buffer (Appendix 1) was heated at 42 °C prior to the hybridisation step (pre-hybridisation). The ECL membrane was wetted with 5X SSC, then loosely rolled and placed in a glass hybridisation tube. The appropriate volume of hybridisation buffer was added after pouring off the 5X SSC and incubated in a rotatory oven at 42 °C for 15 min. A small volume of the buffer was transferred into a 15-ml tube for mixing with the labelled DNA probe (10ng/µl) and returned to the glass tube containing the ECL membrane. The hybridisation was re-incubated in the rotatory oven at 42 °C overnight.

The hybridisation buffer was poured off and 50 ml of 5X SSC was added to the tube and replaced to the rotatory oven at 42 °C for 5 min. Then, 5X SSC was replaced with a pre-warmed primary wash buffer (Appendix 1) for 5 min and returned to the rotatory oven for 20 min at 42 °C. The primary wash buffer was repeated twice for less incubation time in the rotatory oven for 10 min. After that, the ECL membrane was removed from the hybridisation tube and placed in a plastic container soaked with the secondary wash buffer (Appendix 1). The wash was obtained with gentle agitation using a rotary shaker for 5 min at room temperature (25 °C).

2.3.6 Signal generation and detection

Ten millilitres of detection reagents 1 and 2 from ECL Direct Nucleic Acid Labelling and Detection kit (Amersham, UK) was mixed and the mixture covered the ECL membrane for 1 min at room temperature, after draining the secondary wash buffer. The excess detection reagents were drained and the membrane was wrapped in saran wrap. In a film cassette, the membrane was placed with the DNA side up, and the autoradiography film was placed over the membrane. Immediately, the film cassette was closed and the film was removed under low light conditions and developed. A second film was exposed for an appropriate length of time if required.

Chapter 3

Investigation of Tn*916* transfer on exposure to different environmental conditions

Chapter 3 Investigation of Tn*916* transfer on exposure to different environmental conditions

3.1 Introduction

Horizontal gene transfer is the process involving the transfer of MGEs between bacterial cells (Griffith, 1928, Zinder and Lederberg, 1952, Tatum and Lederberg, 1947, Canchaya et al., 2004, Brüssow et al., 2004). MGEs carry different accessory genes that confer resistance to antibiotics (Hughes and Datta, 1983), or heavy metals (Lindstrom et al., 1991), encode enzymes for metabolic pathways (Morrison et al., 1984, Sullivan et al., 2002, Crossman, 2005) and virulence factors (Betley and Mekalanos, 1985, Bayles and Iandolo, 1989). However, in some cases these elements can be considered parasitic on their host and selection acts to preserve genes that give limited host advantage, but mainly contribute to element rather than host survival (Jalasvuori and Koonin, 2015, Koonin and Starokadomskyy, 2016). As a consequence of replicating an MGE within the host genome, there is a fitness cost to the host due to the requirement for more energy to replicate or kill the host to release the phage (Koonin and Dolja, 2014, Lynch and Marinov, 2015). All MGE which does not contain any genes that give an advantage to the host would be lost over time from the host genome, therefore, the persistence of MGE is limited to the presence of a selective advantage to the host or niche (Gogarten et al., 2002). The most investigated elements are those linked to the spread of antibiotic resistance. This occurs due to the high usage of antibiotics in human health (da Costa et al., 2013, Chang et al., 2015), food, livestock (Aarestrup, 1999, Mellon et al., 2001) and

agriculture production (Acar and Moulin, 2006), resulting in an expression of the antibiotic gene that most of the time allows dissemination amongst bacterial populations as a selective advantage to the host and allows the host to adapt to environmental changes (Mascher et al., 2003).

The most well-known mechanisms of HGT are: transformation, conjugation and transduction. Transformation is the transfer of exogenous DNA from the environment into a competent bacterium (Griffith, 1928), while conjugation is the transfer of DNA from a donor to a recipient cell via close contact through pili (Gram-negative bacteria) (Lawley et al., 2003) or through pores (Gram-positive bacteria) (Lederberg and Tatum, 1946, Tatum and Lederberg, 1947, Grohmann et al., 2003). Transduction is the delivery of genetic material via bacteriophage (Zinder and Lederberg, 1952). Moreover, membrane vesicles have shown the ability to transfer foreign DNA via binding the DNA to the out membrane vesicles (OMV) or by being carried in the lumen of the MV (Yaron et al., 2000, Renelli et al., 2004). Recently, Chen et al. (2018) discovered in *Staphylococcus aureus* a new mechanism called lateral transduction. In this mechanism, the unexcised phage initiates replication within the host genome and produces a multiple integrated phage genome. Then, large sections of the host chromosome, which are adjacent to the integrated phage, are packaged including any genetic elements, e.g. phagocytic islands (PaSaI), and transferred at a high frequency to other bacteria.

It was thought that conjugation was the main mechanism for the spread of resistance genes due to a broad host range (Clewell and Gawron-Burke, 1986, Waters, 1999, Mazel and Davies, 1999, Shoemaker et al., 2001,

Musovic et al., 2006). However, recently, lateral transduction has been shown to be an important mechanism for HGT (Chen et al., 2018).

One of the main mediators of the dissemination of antibiotic resistance genes (ARGs) is the Tn*916*-like family of conjugative transposons. Tn*916* is a conjugative transposon that encodes tetracycline resistance conferred by *tet*(M) and is found widely among streptococci in the oral cavity (Ciric et al., 2012a, Lunde, 2017) and originally in the gastrointestinal bacteria *E. faecalis,* which have been observed as transient bacteria in the oral cavity (Franke and Clewell, 1981, Gawron-Burke and Clewell, 1982). As the oral cavity is the main entrance to the human body, microbial pathogens may be present in close contact with the oral and nasopharyngeal cavities (Endo et al., 2014).

The aim of the work in this chapter was to determine the effect of different conditions (e.g. oxygen availability, medium and time of cells contact) on Tn*916* transfer in the human oral cavity, which is the portal to the digestive system. To carry out this study, different *Streptococcus* spp. and *E. faecalis* were chosen as recipients to conjugate with a *B. subtilis* (soil bacterium) as a donor. *B. subtilis* was selected, as it can be considered an environmental organism and is a transient donor bacterium in the oral cavity. It is also a proven donor of Tn*916* and fully sequenced and genetically tractable (Mullany et al., 1990, Showsh and Andrews, 1992, Seier-Petersen et al., 2014, Browne et al., 2015).

3.2 Materials and methods

3.2.1 Preparation of donor and recipients

3.2.1.1 Preparation of the donor

All bacterial strains used were listed in Table 2.1. One colony of the cultured *B. subtilis* BS34A was used to inoculated in 10 ml of Brain Heart Infusion broth (BHI broth) (Oxoid, UK). The medium contained 10 µg/ml of tetracycline and was incubated aerobically at 37 °C overnight on a rotary shaker at 200 rpm (Orbi-Safe, Sanyo). The culture medium was diluted 1:10 with fresh, pre-warmed 37 °C BHI broth containing 5 µg/ml of tetracycline, and incubated aerobically at 37 °C on the rotary shaker until mid-exponential phase (an optical density of 0.45–0.6 at 600 nm). After that, the tubes were centrifuged at 1968 x *g* for 10 min and the cell pellets were washed twice with 10 ml of fresh BHI broth.

3.2.1.2 Preparation of the recipients

One colony of *Streptococcus* spp. and *E. faecalis* (Table 2.1) were inoculated in 10 ml of BHI broth separately and incubated at 37 °C aerobically supplemented with 5% CO_2 air until they reached the mid-exponential phase.

3.2.2 Conjugation experiments (filter mating)

Conjugation experiments were carried out separately as follows: aerobically and anaerobically to determine the optimum conditions for Tn*916* transfer from *B. subtilis* to *Streptococcus* spp. and *E. faecalis*; different mating incubation periods (1 min, 3 min, 5 min, 1 h, 2 h and 24 h) to test the speed of Tn*916* transfer and, finally, to investigate the preferred mating medium, e.g. broth mating or on filter placed over a solid medium (BHI agar).

To check for spontaneous mutation, 200 µl of recipients and donor mid-exponential cells were spread separately on nitrocellulose 0.45-µm pore-size filters (Sigma-aldrich, UK), which were placed on a BHI agar plate as in filter mating experiment section 3.2.2.1 and were plated on selective media containing 10 µg/ml tetracycline, 10 µg/ml colistin sulphate and 5 µg/ml oxolinic acid (*Streptococcus*-selective supplement). Colistin sulphate used to inhibit the growth of Gram-negative bacteria and 5 µg/ml oxolinic acid to inhibit the growth of Gram-positive bacteria (e.g. *Bacillus*) except for *Streptococcus* spp. and *Enterococcus* (Petts, 1984).

3.2.2.1 Mating in different conditions (aerobic vs anaerobic)

Two conjugation experiments were done in parallel: one was incubated aerobically with 5% CO_2, and the other was incubated anaerobically in a Macs-MG-1000 anaerobic cabinet (Don Whitely, UK), containing 80% N_2, 10% H_2 and 10% CO_2. The donor cell pellet was mixed with the recipient cell pellet and re-suspended in 1 ml of BHI broth and 50 µg/ml DNase I (Sigma, UK). Then 200 µl of the mixture was spread and incubated at 37 °C

aerobically with 5% CO_2 and anaerobically on three nitrocellulose 0.45-μm pore-size filters, which were placed on BHI agar plates for 24h. After incubation, the filters were removed from the agar plates and placed in 25-ml sterile tubes containing 1 ml of sterile BHI broth and were vortexed for 1 min. Next 200 μl of the bacterial suspension was spread on selective medium containing BHI agar supplemented with 5% defibrinated horse blood, 10 μg/ml tetracycline and *Streptococcus*-selective supplement (10 μg/ml colistin sulphate and 5 μg/ml oxolinic acid) (Oxoid, UK). Then, plates were incubated at 37 °C either anaerobically or aerobically supplemented with 5% CO_2 for 72 h.

3.2.2.2 Mating for different incubation periods

Conjugation experiments were done in parallel with four different incubation times during mating: 3 min, 1h, 2 h and 24 h. The donor cell pellet was mixed with the recipient cell pellet and re-suspended in 1 ml of BHI broth and 50 μg/ml of DNase I. Then 200 μl of the mixture was incubated on three separate nitrocellulose 0.45-μm pore-size filters, which were placed on BHI agar plates at 37 °C in an anaerobic atmosphere for 3 min, 1h, 2 h or 24 h. After incubation, the filters were removed from the agar plates and placed in 25-ml sterile tubes containing 1 ml of sterile BHI broth and were vortexed for 1 min. Then 200 μl of the bacterial suspension was spread on the selective medium and anaerobically incubated at 37°C for 72 h.

3.2.2.3 Mating in different media (liquid vs solid)

Two conjugation experiments were done in parallel: one in tubes only and the others on a filter placed on agar plate for mating (**Figure 3.1**). The donor cell pellet was mixed with the recipient cell pellet and re-suspended in 1 ml of BHI broth containing 50 µg/ml of DNase. Then one tube of the mating mixture was incubated in three separate tubes, and the other tube was spread on three nitrocellulose 0.45-µm pore-size filters on BHI agar plates, and both were incubated at 37 °C for 1 min, 5 min and 24 h. After incubation, the tubes mixture or the filters were removed from the agar plates and placed in 25-ml sterile tubes containing 1 ml of fresh sterile BHI broth and vortexed for 1 min. Then 200 µl of the bacterial suspension either from the tubes mating or the filters (plate) mating were spread on the selective medium and incubated anaerobically at 37 °C for 72 h (**Figure 3.1**).

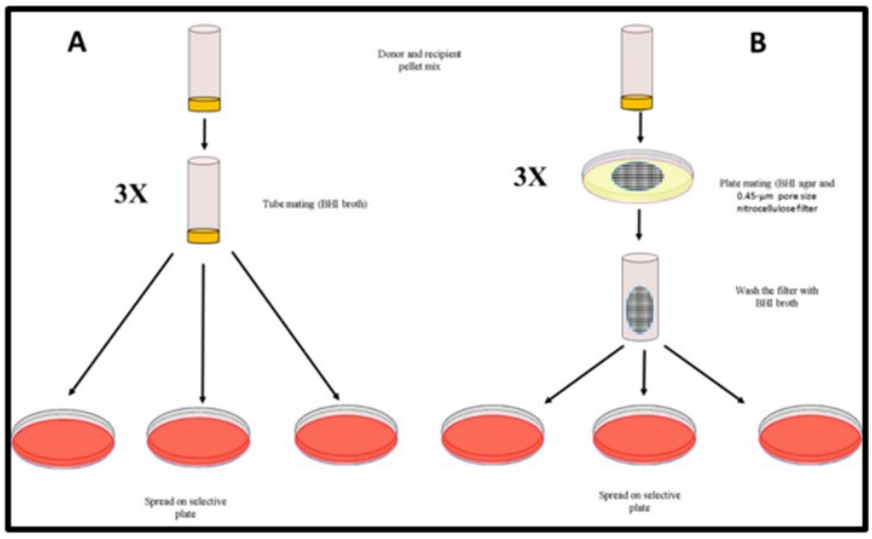

Figure 3.1: Conjugation in tube and on filter (plate)

A) Tube mating: the donor and the recipient pellet in 1 ml BHI broth were incubated in three tubes. **B)** Plate mating: the pellet mix of the donor and the recipient were re-suspended in 1 ml of BHI broth and spread on three separate nitrocellulose filters, which were placed on BHI agar and washed off the filter after incubation. After mating incubation, both **A** and **B** were spread on the selective plates.

3.2.3 Measurement of the frequency of gene transfer

To determine the viable cell count of the recipients serial dilutions of the bacterial suspension after mating were prepared and 100 µl was spread on BHI agar supplemented with 10 µg/ml tetracycline (for donor count) and 100 µl was spread on *Streptococcus*-selective media, which consisted of BHI agar supplemented with 5% defibrinated horse blood, 10 µg/ml colistin sulphate and 5 µg/ml oxolinic acid (for recipient count), and incubated at 37 °C for 24 h anaerobically. The frequency of transfer was calculated using the following equation and imported on GraphPad Prism 7 (GraphPad Software, Inc., USA) for analysis.

Equation 3.1: $$\frac{\text{Number of transconjugants}\left(\frac{\text{cfu}}{\text{ml}}\right)}{\text{Viable cell count of recipient}\left(\frac{\text{cfu}}{\text{ml}}\right)}$$

Colonies of transconjugants were sub-cultured on the *Streptococcus*-selective media containing agar plates, supplemented with 10 µg/ml tetracycline in order to ensure only recipients which had acquired the resistance gene grew.

3.2.4 Identifying and screening transconjugants

All the primers used were listed in Table 2.2. Recipients, donor and transconjugants were identified by amplifying and sequencing the 16S rRNA gene using universal primers (27F/1492R) and screening for the presence of *tet*(M) using (TetM-F1/TetM-R1) as well as the integrase and excisionase genes (*int/xis*) using (intxis(F)/ intxis(R)) of Tn*916* by running PCR tests.

All the sequences were analysed using the Basic Local Alignment Search Tool (BLASTN) (Altschul et al., 1990), which identifies the nucleotide alignments from the nucleotide database, and the Clustal Omega tool (http://www.ebi.ac.uk/Tools/msa/clustalo) was used to conduct the alignments.

3.2.5 Southern blot hybridisation

Southern blots were performed by using an ECL (enhanced chemiluminescence) direct nucleic acid labelling and detection system Kit (Amersham, UK). The genomic DNA of the transconjugants, the donor and recipients were extracted (section 2.2.9) and digested with *Hind*III (section 2.2.10). Following electrophoresis (section 2.2.4) and the transfer of the digested DNA to a nitrocellulose membrane. Then DNA was probed with a labelled PCR product of Tn*916* integrase and excisionase genes. The protocol for southern blot was described in section 2.3.

3.3 Results

3.3.1 Tn*916* transfer experiments

The starting viable cell number of recipients and the donor *B. subtilis* for filter mating experiments was 2.2 $x10^9$ - 4.13 $x10^9$ cfu/ml and 1.6 $x10^9$ - 2.1 x 10^9 cfu/ml respectively (Appendix 2). In three biological repeats there was no spontaneous resistance (no growth) of recipients or donor *B. subtilis* to the selective antibiotics used for transconjugant isolation.

Only *S. oralis*, *S. pyogenes* and *E. faecalis* transconjugants were isolated anaerobically on the selective media with frequencies ranging from 10^{-9} to 10^{-7} transconjugants per recipient (**Figure 3.2**). No transconjugants were isolated when incubated aerobically and when *S. salivarius*, *S. mutans*, *S. sanguinis* and *S. parasanguinis* were used as recipients.

In addition, transconjugants of *S. oralis*, *S. pyogenes* and *E. faecalis* were isolated in both filter (plate) and tube mating. The same species of transconjugants, also have been isolated after 1 min, 3 min, 5 min, 1 h, 2 h and 24 h when mated anaerobically (**Figure 3.2**, **Figure 3.3** and **Figure 3.4**) (Appendix 3, 4, 5).

The number of transconjugants obtained after mating on filters did not vary significantly over a 24-hour period. In **Figure 3.2** the highest transfer of Tn*916* was observed after 3 min incubation at frequencies ranging from 10^{-9} to 10^{-7} transconjugants per recipient. *S. pyogenes* and *E. faecalis* transconjugants showed a 10 to 20-fold decrease in conjugation frequency when incubated for longer period. However, *S. oralis* incubation period showed a stable frequency 10^{-9} - 10^{-8} transconjugants/recipient.

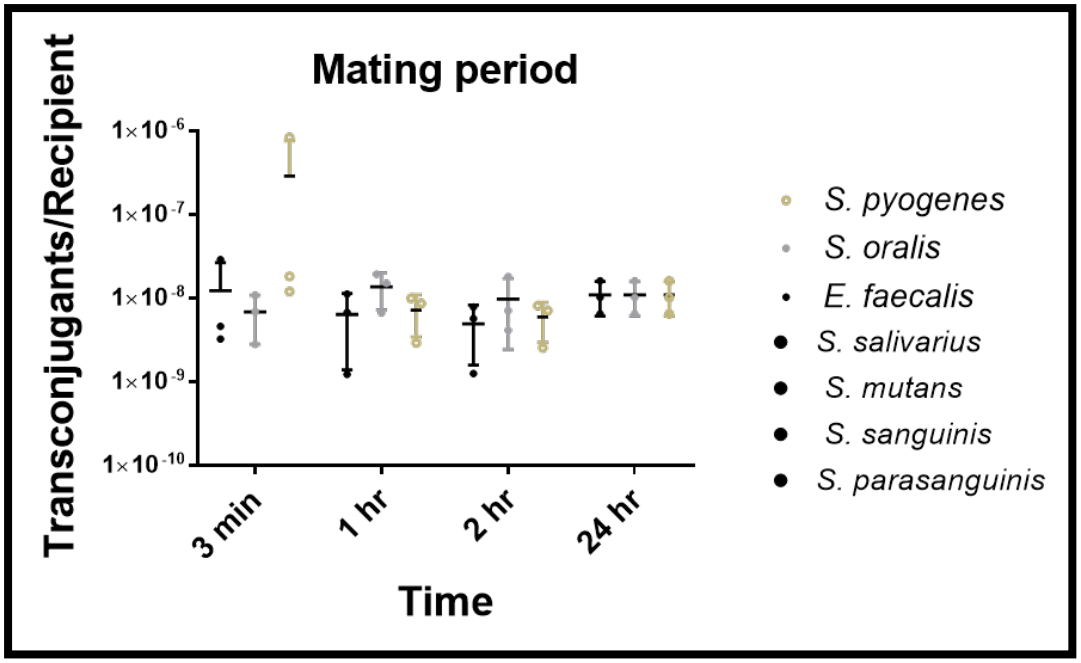

Figure 3.2: The effect of time (3 min, 1 h, 2 h and 24 h) on numbers of transconjugants obtained anaerobically

Mating between *E. faecalis, S. oralis and S. pyogenes* and *B. subtilis* was plotted against the frequency of transfer of Tn*916*. In the column scatter graph plot, the gold, grey and black dots represent *S. pyogenes, S. oralis* and *E. faecalis*, respectively. The error bars correspond to the standard deviation of the mean for three biological repeats. The data was analysed by two-way ANOVA followed by Tukey comparison test. No transconjugants were isolated when mated with *S. salivarius*, *S. mutans*, *S. sanguinis* and *S. parasanguinis* and aerobically. However, no statistically significant differences were observed anaerobically with *E. faecalis, S. oralis and S. pyogenes*. In the scatter plot, every single data that cluster or outlier in the group were visualised. A single reading with *S. pyogenes* was greater than the rest of the data in the same group (3 min) (Appendix 3).

There were no significance differences (p=0.438) in the frequencies of Tn*916* transfer within 3 min, and 1 h on filter into *S. oralis*, *S. pyogenes* and *E. faecalis* in the presence or absence of DNase I, which indicated that the transfer was via conjugation (**Figure 3.3**).

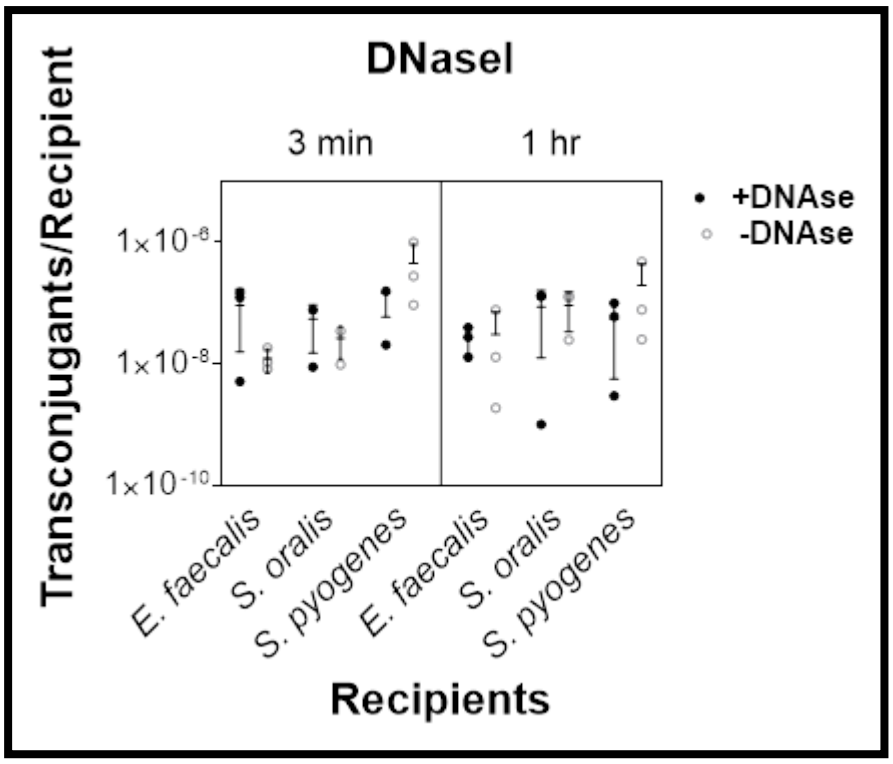

Figure 3.3: The effect of DNase I on mating on filters with different incubation periods on Tn*916* transfer frequency between the recipient and the donor

Mating between *E. faecalis, S. oralis and S. pyogenes* recipients with the *B. subtilis* BS34A donor was plotted against the frequency of the transfer of Tn*916*. In the column scatter graph plot, the black and white dots represent filter mating with and without DNase I, respectively. The error bars correspond to the standard deviation of the mean for three biological repeats. The data was analysed by two-way ANOVA followed by Tukey comparison test. No statistically significant differences were observed (Appendix 4).

In **Figure 3.4**, the number of transconjugants obtained after tube mating showed a non-significant decrease in the transfer of Tn*916,* while the transconjugants of *S. pyogenes* showed a non-significant (p=0.471) increase in mating frequency in liquid media than on filters (**Figure 3.4**).

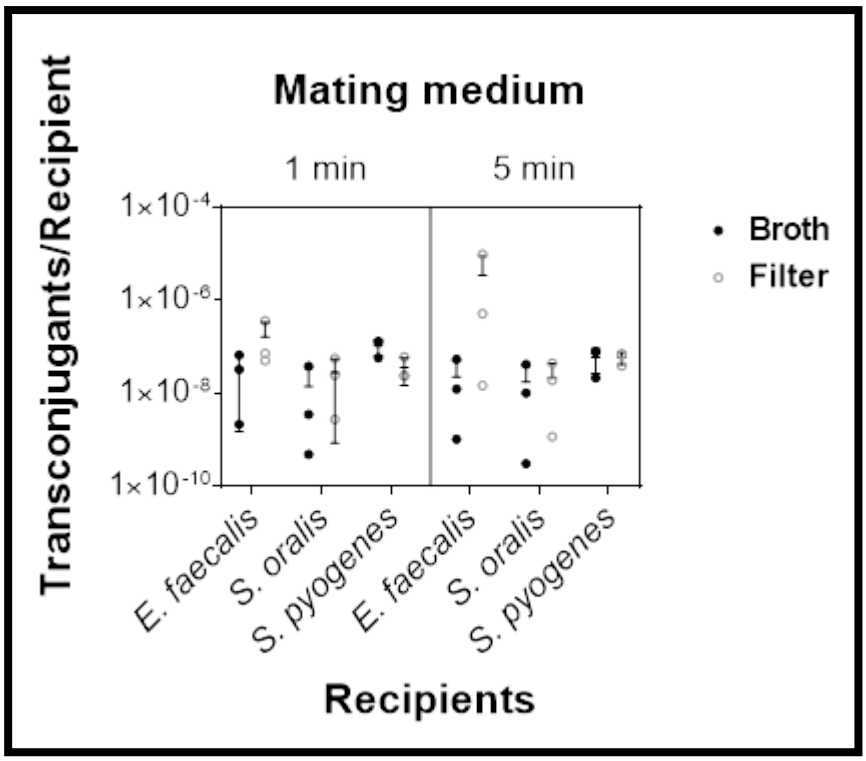

Figure 3.4: The effect of mating in broth vs on filters on Tn*916* conjugative transposition frequency between the recipient and the donor

Mating between *E. faecalis, S. oralis and S. pyogenes* and *B. subtilis* was plotted against the frequency of transfer of Tn*916*. In the column scatter plot, the black and white dots represent the data of mating in broth and on filter (plate), respectively. The error bars correspond to the standard deviation of the mean from three biological repeats. The data was analysed by two-way ANOVA followed by Tukey comparison test. No statistically significant differences were observed. In the scatter plot, every single data that cluster or outlier in the group were visualised. Two data readings of *E. faecalis* in 5 min in broth mating were greater than the rest of the data in the same group (Appendix 5).

3.3.2 Verifying transconjugants containing Tn*916*

PCR analysis of all the putative transconjugants (*S. oralis*, *S. pyogenes* and *E. faecalis*) showed products sized at 2.8 kb for *tet*(M) and 0.8 kb for the integrase and excisionase (*int/xis)* genes of Tn*916*, which were also present in the donor but not in the recipients (**Figure 3.5 B** and **C**). In addition, sequencing of the PCR products sized 1.5 kb of 16S rRNA gene for all the

transconjugants was identical to the recipients' 16S rRNA gene sequence, confirming that Tn*916* had transferred to the recipients (Appendix 6, 7 and 8).

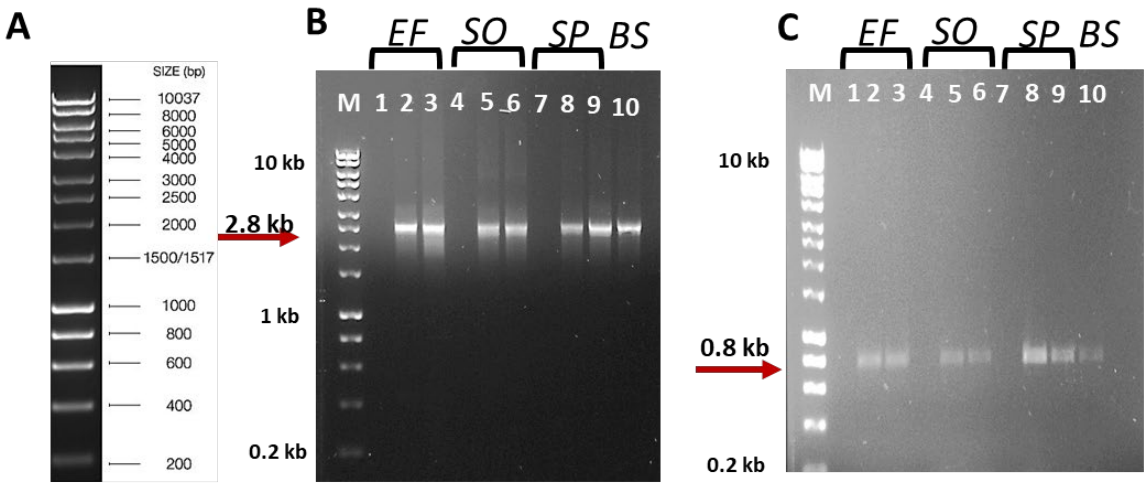

Figure 3.5: PCR results showing the presence of Tn*916* in all transconjugants

A) 1 kb HyperLader marker, B) Tn*916 tet*(M) sized 2.8 kb, and C) the *int/xis* sized 0.8 kb. In all the images B and C, Lane M: 1 kb HyperLader marker; Lane 1: *E. faecalis* (recipient); Lanes 2 and 3: transconjugant of *E. faecalis*; Lane 4: *S. oralis* (recipient); Lanes 5 and 6: transconjugant of *S. oralis*; Lane 7: *S. pyogenes* (recipient); Lanes 8 and 9: transconjugant of *S. pyogenes;* and Lane 10: *B. subtilis* BS34A (donor).

The sequence analysis of the *tet*(M) amplicon for all the putative transconjugants and the *B. subtilis* donor aligned 100%, which demonstrated that the gene was present in all transconjugants and was identical to *tet*(M) in the donor (**Figure 3.6**).

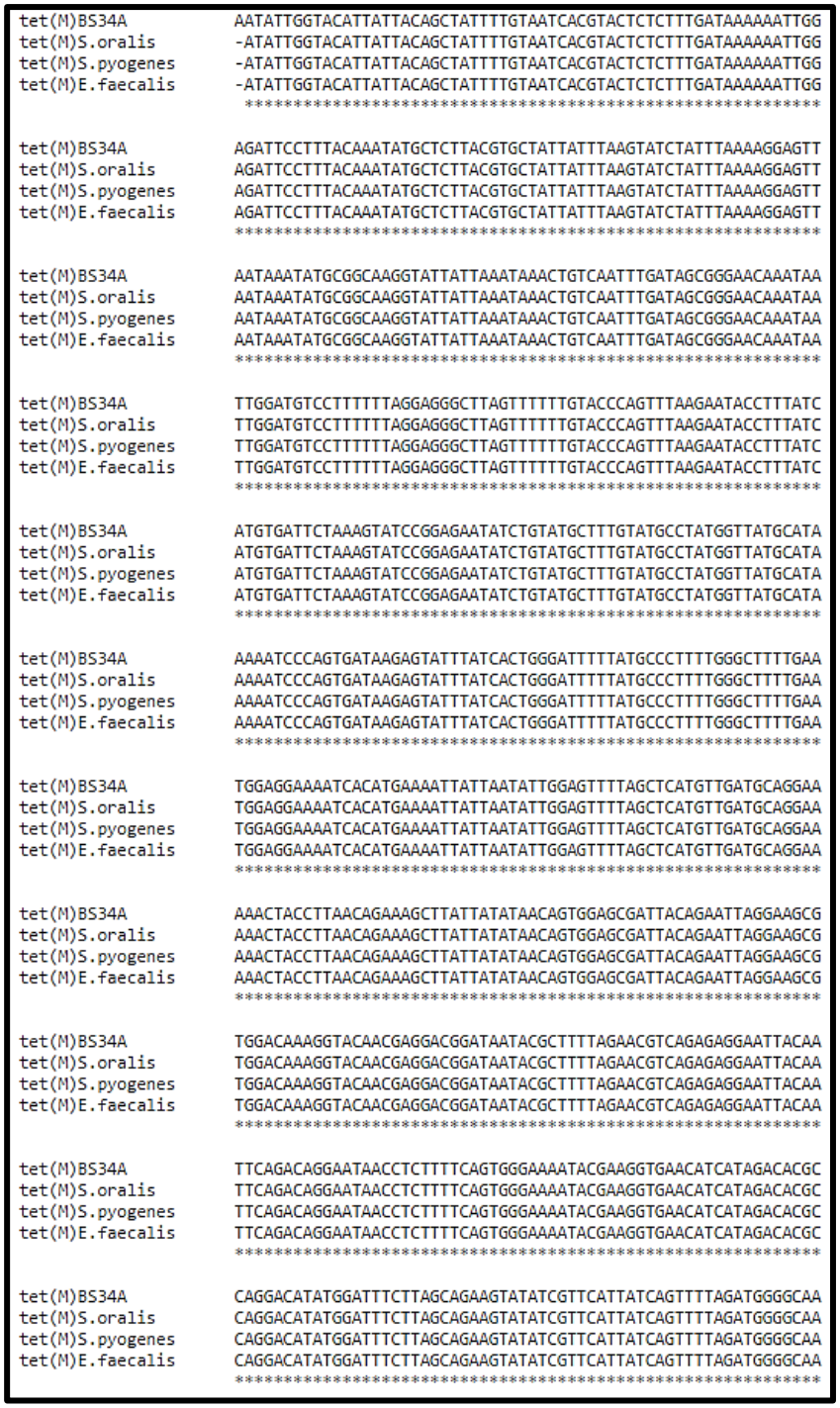

Figure 3.6: Sequences alignment of *tet*(M) in transconjugants (*S. oralis, S. pyogenes* and *E. faecalis*) and *B. subtilis*

3.3.3 Transconjugants contain one copy of Tn*916* (one band)

*Hind*III has only one restriction site within Tn*916*, which is in *tet*(M) (**Figure 3.7**). The southern blot hybridization against the integrase and excisionase genes of Tn*916* showed positive hybridisation for all the transconjugants (*S. oralis*, *S. pyogenes* and *E. faecalis*) and *B. subtilis* (positive control), whereas no hybridization was observed for the recipient DNA (negative control) (**Figure 3.8**).

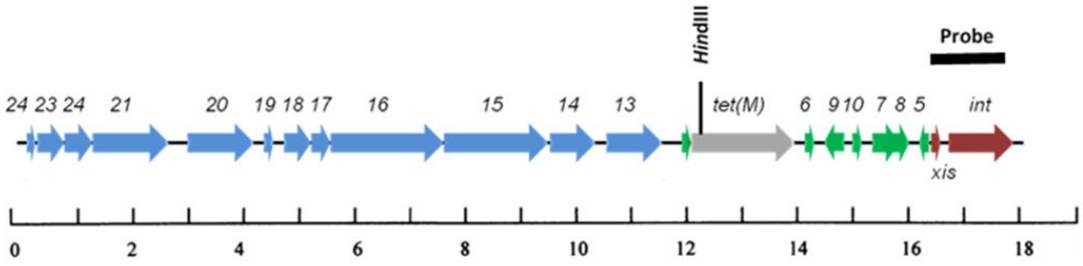

Figure 3.7: Restriction and functional map of Tn*916*

Restriction Enzyme (*Hind*III) Site on *tet*(M) and the position of the probe represented by heavy black line.

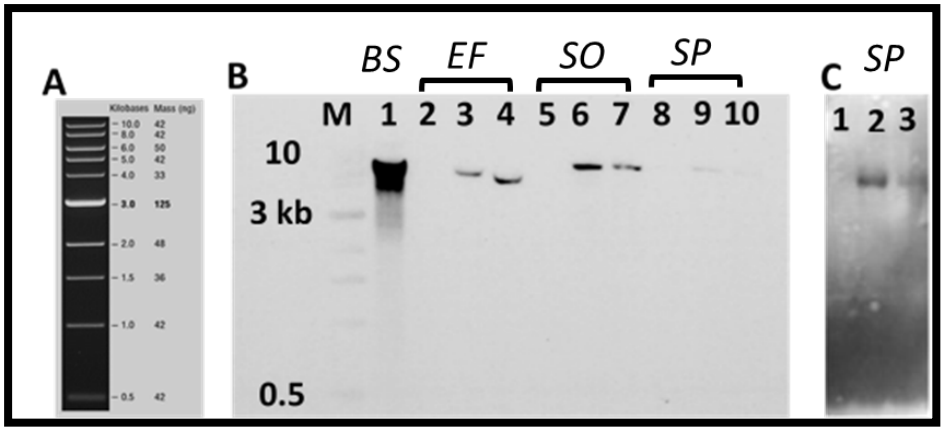

Figure 3.8: Southern blot for the genomic DNA of transconjugants

A) 1kb DNA Ladder Marker **B)** and **C)** Genomic DNA of the transconjugants, recipients and the donor digested with *Hind*III and probed with the labelled Tn*916 int/xis* genes**.** Lane M: 1kb DNA Ladder; Lane 1: *B. subtilis* BS34A (donor); Lane 2: *E. faecalis* (recipient); Lanes 3 and 4: transconjugant of *E. faecalis*; Lane 5: *S. oralis* (recipient); Lanes 6 and 7: transconjugant of *S. oralis*; Lane 8: *S. pyogenes* (recipient); Lanes 9 and 10: transconjugant of *S. pyogenes* (faint bands). In **C)** repeated southern blot for the genomic DNA of *S. pyogenes* (recipient) in Lane 1 and transconjugant of *S. pyogenes* in Lane 2 and 3 (strong bands).

3.4 Discussion

HGT is a process that often leads to microbial adaptation to environmental changes. The naturally competent oral streptococci have a high likelihood of gaining MGEs and thus of having the ability to adapt to environmental stress (Hartley et al., 1984, Fitzgerald and Clewell, 1985, Bentorcha et al., 1992, Lacroix and Walker, 1995a, Burne, 1998, Ciric et al., 2012b). Tetracycline resistance can be conferred to oral streptococci through the transfer of the conjugative transposon Tn*916* either via conjugation or via transformation (Roberts et al., 2001, Hannan et al., 2010). Moreover, streptococci play an important role as donors and in distributing MGEs to other oral bacteria (Roberts et al., 2001). In addition, the gut bacterium *E. faecalis* is considered as a nosocomial pathogen due to the Vancomycin-resistant enterococci

(VRE) outbreaks (Mead, 1978, Noble, 1978, Schaberg et al., 1991). It has been suggested that the oral cavity may act as a reservoir of virulent and antibiotic resistant *E. faecalis* with a high chance of being carried in adults and the elderly (Anderson et al., 2015, Komiyama et al., 2016).

Investigations have been undertaken of HGT in one model that mimics the oral cavity (Roberts et al., 1999, Roberts et al., 2001, Ready et al., 2006). The constant depth film fermentor (CDFF) is a closed vessel that produces dental plaques (biofilms), which are a matrix of multiple bacterial communities attached to the teeth surface and to each other (Peters and Wimpenny, 1988, Costerton et al., 1995). However, these researches have dealt with a multispecies biofilm that is poorly reproduceable. This is due to the variability of the biofilms compared with filter mating, which deals with only two species. The conditions including atmosphere, time and the type of medium required for HGT to occur between environmental organisms and human oral bacteria have not been thoroughly explored. Therefore, this work is focused on the effect of these conditions on the conjugal transfer of Tn*916* from environmental bacteria (*B. subtilis*) into oral (streptococci) and gastrointestinal (enterococci) bacteria.

Streptococci and enterococci are facultative anaerobic bacteria, as the optimal conditions for their growth is an anaerobic atmosphere, but they can also tolerate oxygen (Zhou and Li, 2015). In this study, only transconjugants of *S. oralis*, *S. pyogenes* and *E. faecalis* at frequencies ranging from 10^{-9} to 10^{-7} transconjugants per recipient were isolated only under anaerobic conditions after mating with *B. subtilis* (**Figure 3.2**). Roberts et al. (1999)

demonstrated that the conjugative transposon Tn*5397* transferred anaerobically from the transient microorganism *B. subtilis* BS6A into the oral *Streptococcus acidominimus* within 6 h of its inoculation. However, the mechanism of transfer was not investigated. In the current study, the conjugative transposon Tn*916* has been transferred from the *B. subtilis* BS34A into *S. pyogenes*, *E. faecalis* and *S. oralis* at a frequency of 1.58 × 10^{-7}, 1.24 × 10^{-7} and 7.78 × 10^{-8} transconjugants per recipient, respectively, within 3 min in the presence of DNase I (**Figure 3.3**). Therefore, transfer likely occurred via conjugation only.

Wollman et al. (1956) reported that the transfer of chromosomal alleles from Hfr strain (high frequency recombination) to a F^- cell occurred within 25 min, as the mating was disrupted by mixing the cells for a few seconds in a blender. This supported the current work, as we observed a transfer of Tn*916* to occur after 1 min of mating, and we might have stopped the conjugation after we vortexed the tube. Results in this research highlight that 1 min was an adequate time for gene transfer to occur, meaning that the transient bacteria, e.g. from food, drinks and air, in the oral cavity could play a role in the dissemination of MGEs to oral streptococci and enterococci. Mercer et al. (2001) demonstrated the ability of plasmid DNA to survive in the human mouth for enough time to transform the competent *S. gordonii* DL1 *in vitro.* The role of transient bacteria in the dissemination of Tn*916* has been demonstrated for environmental bacteria (*B. subtilis*) in soil under different conditions e.g. temperature, nutrition and pH (Haack et al., 1996).

Tn*916* was originally found in *E. faecalis,* which inhabits the gut and are found in faeces (Mead, 1978, Noble, 1978, Franke and Clewell, 1981). Thus, *E. faecalis* are introduced to the environment (soil) through sewage waste (Marcinek et al., 1998). In soil, the environmental conditions are variable, e.g. humidity, temperature, oxygen availability, nutrition and pH, compared to the gastrointestinal environment (Haack et al., 1996, Andrews et al., 2004). Therefore, the conjugative element Tn*916* transfers to the resident bacteria in soil, e.g. *B. subtilis* to replicate and persist (Andrews et al., 2004). This explains Roberts et al. (1999) observations, where, the donor *B. subtilis* BS6A, could not be observed after conjugation in a mixed-species biofilm, 24 h after inoculation. In contrast, the conjugative element Tn*5397* has transferred and persisted in oral streptococci. However, there has been no systematic analysis of the survival time of transient bacteria in the human oral cavity. Thus, the role of transient bacteria and the persistence of their DNA in gene transfer in the oral cavity remains to be determined.

The transfer to *S. salivarius*, *S. mutans*, *S. sanguinis* or *S. parasanguinis* have not been observed under aerobic or anaerobic atmospheres. It is possible that, the transfer of MGEs is inhibited by natural conjugation inhibitors, such as the restriction-modification system (Bertani and Weigle, 1953), which is a bacterial defence system where unmethylated foreign DNA is cleaved within recognition sites (Vasu et al., 2012, Oliveira et al., 2014). The Tn*916* sequence was screened for endonuclease restriction sites of *Ssl*I, *SmuE*I and *Sts*I, which are restriction enzymes produced by *S. salivarius*, *S. mutans*, and *S. sanguinis,* respectively (Solaiman and Somkuti, 1990, Benbadis et al., 1991, Molnar et al., 1991, Kita et al., 1992). A single

cleavage site was identified either in the *orf12* or in the *tet*(M) sequence, although Tn*916* encodes an anti-restriction protein known as ArdA (alleviation of restriction of DNA). In addition, Marraffini and Sontheimer (2008) have shown that pG0400 *Staphylococcus* conjugative plasmid transfers by conjugation and transformation was inhibited via the Clustered Regularly Interspaced Short Palindromic Repeats system (CRISPR-Cas). CRISPR-Cas, is an adaptive immune system where foreign DNA is cleaved by Cas nucleases directed by sequences from a previous invading genome (Marraffini and Sontheimer, 2008). The spacer sequences on CRISPR-Cas were identical to the relaxase gene of the plasmid. However, spacer containing Tn*916* sequences have not been detected. Moreover, conjugation could be inhibited by regulatory factors encoded by the host. This has been investigated in IncF plasmid, but not yet in Tn*916*.

B. subtilis can adapt to anaerobiosis by metabolic activity. In an anaerobic environment, it has two pathways to generate energy: through respiration in the presence of nitrate as an electron acceptor and through fermentation in which the pyruvate is transformed into acetoin, 2,3- butanediol, lactate, ethanol and acetate in the absence of an electron acceptor (Nakano et al., 1997, Nakano and Zuber, 1998). However, when the growth conditions of *B. subtilis* are changed from an aerobic to anaerobic environment, a general stress response (GSR) is induced when growth is slowed due to the low ATP level (Volker et al., 1994, Voelker et al., 1995). A regulatory protein, RsbW, senses the cell energy change and activates σ^B by forming a RsbW-RsbV complex and releasing σ^B to bind RNA polymerase and induce transcription (Voelker et al., 1995). Conjugative transposons can respond to cellular

stress. For example, sub-lethal concentrations of compounds such as biocides and antibiotics can promote the conjugation of Tn*916* from *B. subtilis* into another *Bacillus* spp. (Showsh and Andrews, 1992, Seier-Petersen et al., 2014). Therefore, maybe as a response to GSR induction, Tn*916* transposition has been activated, excised forming the covalently closed circular form and transferred to the recipient in the absence of oxygen. This would explain the fact that transconjugants were only found after anaerobic mating. This hypothesis will be tested to understand the transposition of Tn*916* in anaerobic conditions.

Tn*916* containing transconjugants of *S. oralis*, *S. pyogenes* and *E. faecalis* have been isolated only under anaerobic conditions after mating with *B. subtilis*. Previous work, has observed that in microaerobic conditions, the frequency of the conjugative transfer of pSLT from *Salmonella* was higher (10^{-5}/donor) (García-Quintanilla et al., 2008) than under aerobic conditions (10^{-8}/donor) (Ahmer et al., 1999). Also Iuchi and Lin (1991) observed that under anaerobic conditions, the conjugal transfer of F-plasmid starts by a response of two component systems, ArcA (response regulator) and ArcB (sensor histidine kinase), to the oxygen level (**Figure 3.9**). After sensing oxygen level decrease, ArcB phosphorylates and activates AcrA by phosphorylation (Cecchini et al., 2002). ArcA-P then represses the succinate dehydrogenase *sdhCDAB*, which encodes a catalytic enzyme that catalyses succinate to fumarate as an electron transporter. The repressed *sdhCDAB* leads to the transcription of *traJ* (Silverman et al., 1991, Strohmaier et al., 1998, Serna et al., 2010). The TraJ protein under ArcA-P control stimulates *tra* operon expression from *tra*Y promoter (P_Y) (Cecchini et al., 2002, Serna

et al., 2010) (**Figure 3.9**). TraY binds at the *oriT* and stimulates relaxosome activity of nicking and unwinding the plasmid DNA prior to transfer (Nelson et al., 1995, Howard et al., 1995). This work supported Stallions and Curtiss (1972) findings that the frequency of the chromosomal transfer in Hfr strain via conjugation on a rich medium under anaerobic conditions was higher than under aerobic conditions.

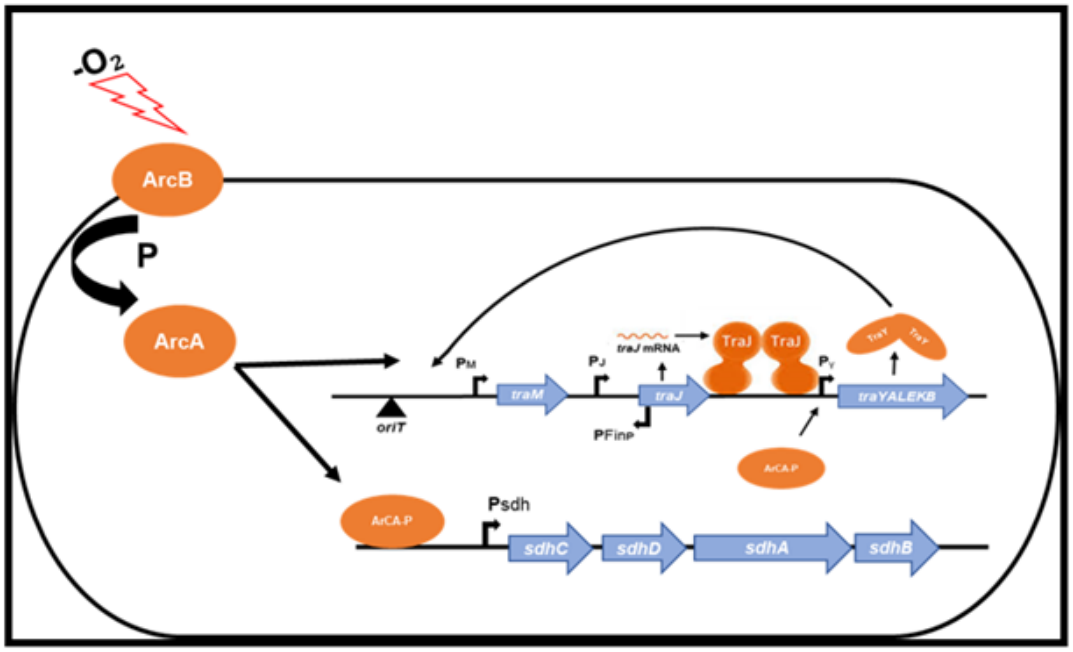

Figure 3.9: Model for the regulation of pSLT in the absence of oxygen

In microaerobic conditions, ArcA-P mediates the repression of *sdhCDAB* by binding upstream of the *sdh* promoter (Psdh) and indirect activation of *tra*Y promoter (P_Y) via increasing TraJ synthesis leading to expression of the *tra* operon from P_Y. TraY binds *oriT* and stimulates relaxosome activity.

An efficient conjugative transfer system during broth mating between Gram-positive bacteria has been discovered in pheromone-responsive plasmids, such as pAD1, pPD1 and pCF10 (Dunny et al., 1978, Clewell, 1981, Clewell, 1993a, Clewell, 1993b, Dunny et al., 1995). Most of these were discovered in

E. faecalis and one in *E. faecium* (Clewell, 1981, Shlaes et al., 1989, Clewell, 1993b). In addition, a non-pheromone-responsive plasmid pMG1 has been found to be transferred with high frequency (10^{-4}/donor) between *E. faecalis* strains, *E. faecium strains*, *E. faecium and E. faecalis* and between *E. faecium and E. hirae* 9790 after 3 hr in broth mating (Ike et al., 1998). Tn*916* usually transfers at ranges from < 10^{-8} to > 10^{-5} transconjugants per donor. It was thought that Tn*916* transferred only on a solid surface (filter mating) when the cells were close to one another. However, in these experiments, Tn*916* containing transconjugants were isolated after broth mating for 1 min or 5 min incubation between *B. subtilis* BS34A and the recipients (*Streptococcus* spp. and *E. faecalis*) at a frequency ranging from 10^{-9} to 10^{-7} transconjugants per recipient (**Figure 3.4**). Jaworski and Clewell (1994) showed that Tn*916* transferred efficiently after 8 hr in liquid mating between *E. faecalis* GO1RF pAM717::Tn*916* (donor) and *E. faecalis* OG1SS pAM717 (recipient). Moreover, the conjugation frequency of Tn*916* into *E. faecalis* was 10-fold lower in liquid than on a solid surface and that this supports the findings of Jaworski and Clewell (1994), as they suggested when a conjugation frequency on solid surface was low (10^{-7}/donor or less), it would be unable to produce transconjugants in broth. In contrast, we isolated transconjugants after 1 min or 5 min incubation in broth mating, but after 24 h incubation, there were no transconjugants. This could be because of the limited nutrients and space in tube mating, leading to a competitive interaction between the parents and transconjugants, as both had the same requirements for growth. For short periods of incubation in broth (1 min or 5 min), there would be less depletion of nutrients and the transconjugants

would not be outcompeted. After plating the mating mixture on selective medium, only transconjugants would persist and parent cells would be killed due to the presence of tetracycline. However, when the incubation period was longer (24 h), the transconjugants were outcompeted and depleted due to the parents maximum nutrient uptake.

The transferred Tn*916* from *B. subtilis* BS34A into *S. oralis*, *S. pyogenes* and *E. faecalis* was confirmed by both PCR and Southern blot hybridisation (**Figure 3.5** and **Figure 3.8**). The latter showed that each of the transconjugants of *Streptococcus* and *E. faecalis* acquired one copy of Tn*916*. This result was in agreement with Roberts et al. (2001), who observed one band digested with *Hind*III in each *Streptococcus* transconjugant using the same probe (*int/xis*).

In conclusion, these results showed that HGT via conjugation of Tn*916* may require 1 min or 3 min in a liquid or on a solid surface to occur. This finding has widened our view of the risk of HGT, as this very brief timeframe is sufficient for microorganisms that pass through the mouth to disseminate their mobile genetic elements. Moreover, the discovery that Tn*916* can transfer in liquid, in anaerobic conditions and in a short time (1 min) coupled with the ability to survive different environmental conditions is concerning.

Chapter 4

Determination of the copy number of circularised Tn*916* in the presence and absence of oxygen

Chapter 4 Determination of the copy number of circularised Tn*916* in the presence and absence of oxygen

4.1 Introduction

The conjugative transposition of Tn*916* starts with excision from donor DNA. There are two transposon-encoded proteins required for this: integrase (Int) and excisionase (Xis). They are located close to one end of the transposon (**Figure 1.12** and **Figure 1.14**). Int is a site-specific recombinase, which belongs to the λ-Int family (Poyart-Salmeron et al., 1989), and Xis is a small protein which binds to both ends of Tn*916* (Rudy et al., 1997). Int cleavage produces 5' single-strand overhangs, consisting of 5 to 6 bp flanking the transposon ends (Scott et al., 1988, Caparon and Scott, 1989, Rice and Carias, 1994, Manganelli et al., 1996, Taylor and Churchward, 1997, Manganelli et al., 1997). The excised transposon ends are ligated, and create a covalently closed circular (CCC) molecule, which consists of 5-6 bp heteroduplex in *E.coli* or homoduplex in *E. faecalis* at a circle joint (Scott et al., 1988, Caparon and Scott, 1989, Rice and Carias, 1994, Manganelli et al., 1997). The Tn*916* circular form is an intermediate of transposition and conjugation transfer. A single strand of Tn*916* can transfer to the recipient cell (Scott et al., 1994) after nicking at the origin of transfer (*oriT*) (Jaworski and Clewell, 1995). After transfer, the complimentary strand is synthesised in both the donor and recipient, and inserts into the target site on the DNA molecule, which includes AAAs followed by TTTs with a static bend (**Figure 4.1**) (Lu and Churchward, 1995).

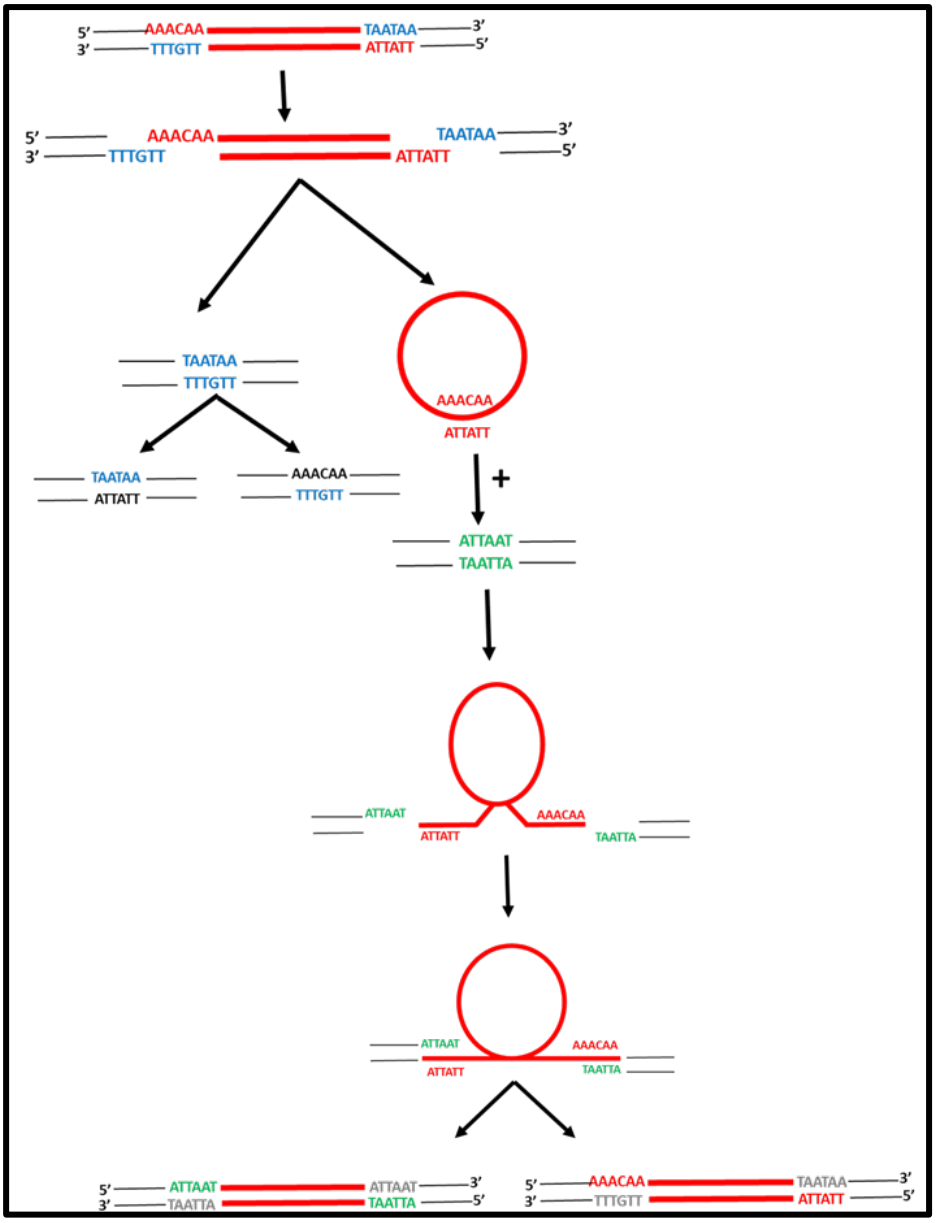

Figure 4.1: A schematic diagram of Tn*916* excision and insertion mechanism

The thick (red) and thin (black) lines represent Tn*916* and the flanking genomic DNA, respectively. The red sequence indicates the bases of the coupling sequence and the blue or green nucleotides indicates the target site. The nucleotides in grey indicates the product of DNA repair by mismatch repair or replication. A staggered cut is established at ends of Tn*916* forming a single-strand overhang containing a non-complementary 6 bp which ligated together forming a covalently closed circle. The bases in the vacated target site are resolved by DNA replication. A staggered cut at the 3' end of the new target site and the coupling sequence at the circular joint leads to the resulting molecules being ligated together. DNA replication resolves the non-complementary bases of the inserted Tn*916* creating two copies, each is flanked by a different DNA sequence. adapted from "Tn*916*-like genetic elements: a diverse group of modular mobile elements conferring antibiotic resistance," by A. P. Roberts and P. Mullany, 2011, *FEMS Microbiology review, 35*, p. 856-871. © 2011 by Oxford University Press. Reprinted with permission.

As previously stated in chapter 3, the transfer of Tn*916* occurred only anaerobically, thus, it was hypothesised that Tn*916* transposition was induced by the changes in growth conditions which agrees with a previous investigation (Showsh and Andrews, 1992, Seier-Petersen et al., 2014). This chapter, aimed to study the relationship between the circular copy numbers of Tn*916* and the growth conditions (aerobic vs anaerobic). To test this hypothesis, the circular form of Tn*916* was quantified using the total DNA of *B. subtilis* BS34A, incubated in different conditions, as a template for qPCR. The joint ends of circular Tn*916* were amplified, and the Ct value compared with the Ct value of a known DNA concentration using the standard curve to calculate the copy number.

The copy number of the circular form of Tn*916* was quantified using Quantitative Polymerase Chain Reaction (qPCR). This is a sensitive and reliable technique that precisely quantifies any target sequence (Ferre, 1992, Burgos et al., 2002, Klein, 2002). The concentration of DNA at each cycle is quantified by a fluorescent dye (e.g., SYBR Green) that binds only to double strand DNA (Wittwer et al., 1997, Ririe et al., 1997, Hernandez et al., 2003). The cycle number in which the fluorescent signal increase above the background is called the threshold value (Hughes and Datta, 1983) and is inversely proportional to the log of the initial amount of input DNA (Ririe et al., 1997).

4.2 Materials and Methods

4.2.1 Bacterial strains, plasmids and culture conditions

All bacterial strains and plasmids used in this chapter were listed in Table 2.1. To determine the copy number of Tn*916*, two separate experiments were conducted. The first involved inoculating *B. subtilis* BS34A that harboured a single copy of Tn*916* (Roberts et al., 2003) into two tubes containing 10 ml of BHI broth supplemented with 10 µg/ml of tetracycline; these were incubated for 18 h at 37 °C aerobically on a rotary shaker at 200 rpm. One of the tubes was transferred into an anaerobic atmosphere for the last hour of incubation with shaking. After overnight culture both tubes were centrifuged at 14680 x *g* for 1 min, and the supernatant was discarded. The second experiment was prepared the same way as the previous one except that in the last step, in 1 ml fresh BHI broth the cell pellet was mixed with *E. faecalis* JH2-2 cell pellets as a recipient. The recipient *E. faecalis* JH2-2 was prepared by inoculation in 10 ml BHI broth and this was incubated for 18 h at 37 ºC in an aerobic atmosphere supplemented with 5% CO_2.

E. coli α-select silver (Bioline, UK) was used as a host for all transformation steps. A pGEM®-T Easy vector (3015 bp) was obtained from Promega (UK) and was used for cloning. *E. coli* α- select silver bearing pGEM®-T was cultured on Luria-Bertani (LB) medium (Becton, UK) or in 10 ml of LB broth, with an addition of 100 µg/ml of ampicillin (Sigma) aerobically at 37 °C on a rotary shaker at 200 rpm overnight.

4.2.2 Preparation of the DNA template for qPCR

The total DNA of *B. subtilis* BS34A from all tubes was extracted during the exponential growth phase, which was determined by the optical density (OD) at 600 nm. The extraction was performed using the Gentra Puregene Yeast/Bact. Kit (Qiagen, UK), following the DNA purification protocol for Gram-positive bacteria, which is described in the manufacturer's instructions and reported in section 2.2.9. To ensure consistency of the qPCR assay, the concentration of the template DNA of *B. subtilis* was normalized to 100 ng/µl using molecular grade water (Sigma, UK). This was to ensure that the starting quantity of DNA molecule was the same.

4.2.3 Construction of pGEMT-Tn*916*CJ-*gyr*B as the standard template for absolute qPCR

Two primer sets specific for amplifying the Tn*916* circular joint containing coupling sequence (Tn*916* CJ) and the gyrase B gene (*gyrB*) of *B. subtilis* BS34A were designed using Primer3 web-based software (https://biotools.umassmed.edu/bioapps/primer3_www.cgi) (Table 2.2). The sequences for the left and the right ends of Tn*916* (accession number: KM516885.1) and *B. subtilis* BS34A *gyrB* (accession number :LN680001.1) were obtained from the public database of the National Center for Biotechnology Information (NCBI) (Altschul et al., 1990).

The amplified circular joint of Tn*916* from BS34A genome obtained using primers Tn*916*CJ-F1 and Tn*916*CJ-R1 was cloned into the open pGEM®-T Easy vector in lacZα site resulting pGEMT-Tn*916*CJ (**Figure 4.2**). After that

the plasmid was transformed into *E. coli* α-select silver, purified using the QIAprep Spin Miniprep Kit (Qiagen, UK) and sequenced using plasmid specific primers M13-F and M13-R (Table 2.2).

GyrB was amplified using primers containing NsiI restriction recognition sites at the 5' end (GyrB-NsiI-F1 and GyrB-NsiI-R1) and cloned to pGEMT-Tn*916*CJ after being digested with NsiI, producing pGEMT-Tn*916*CJ-*gyr*B and transformed into *E. coli* α-select silver (**Figure 4.2**). The plasmid was purified using the QIAprep Spin Miniprep Kit, sequenced and analysed using BioEdit software version 7.2.0 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) in order to confirm that the expected sequences were present. The concentration of the standard DNA (pGEMT-Tn*916*CJ-*gyr*B) was measured using a Nanodrop™ 1000 spectrophotometer (Thermo Scientific, UK).

All the reaction mixture, the PCR conditions, the digestion and purification methods were performed as described in section 2.2.

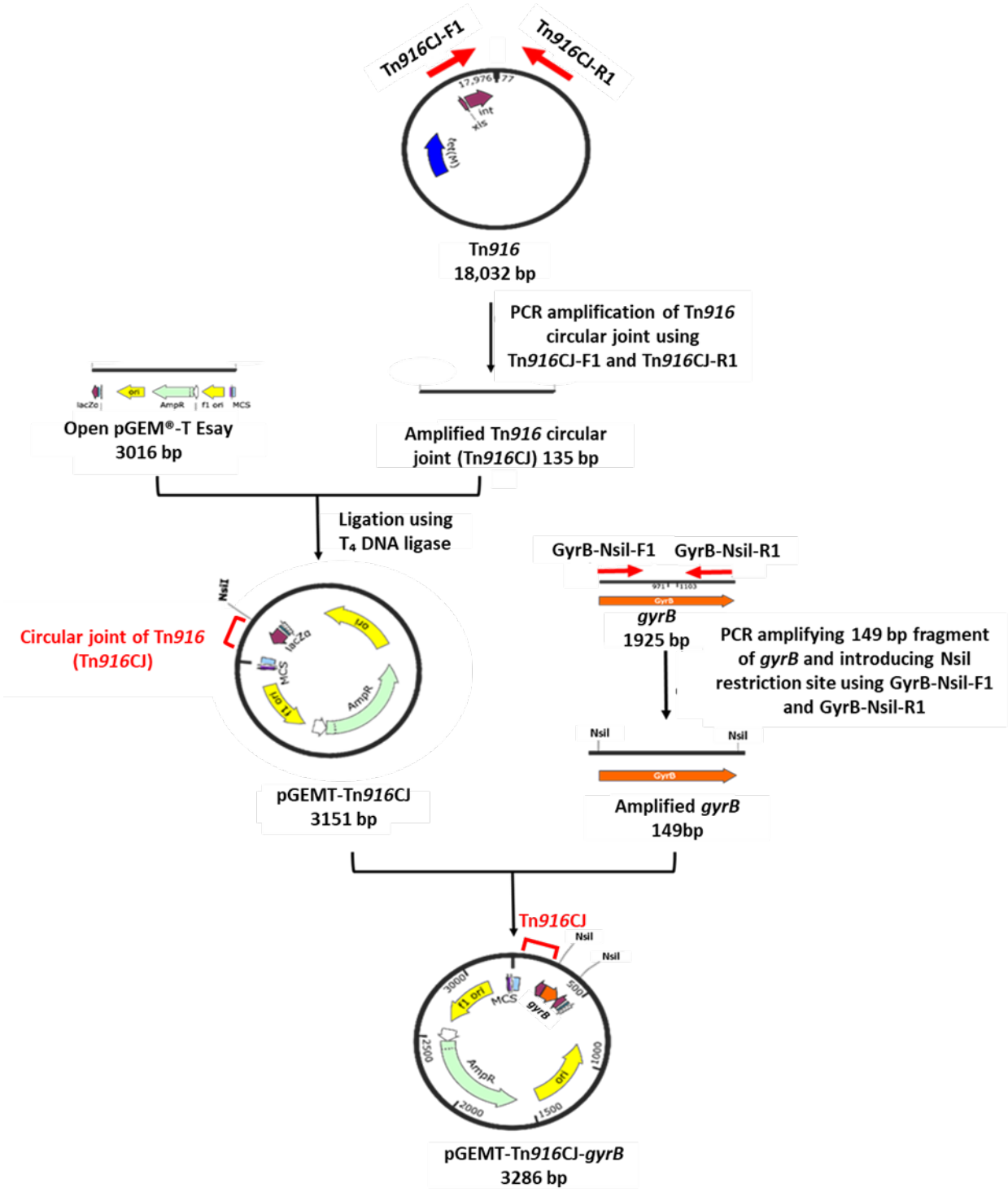

Figure 4.2: The construction of pGEMT-Tn*916*CJ-*gyrB* derivatives

The circular joint of Tn*916* was amplified fromTn*916* using outward primers (Tn*916*CJ-F1 and Tn*916*CJ-R1). The Tn*916* circular joint sequence was inserted into the open pGEM®-T Easy in lacZα site and formed pGEMT-Tn*916*CJ. The *gyr*B was amplified from *B. subtilis* DNA using a primer with NsiI restriction enzyme recognition sites at the 5' end (GyrB-NsiI-F1 and GyrB-NsiI-R1) and cloned into pGEMT-Tn*916*CJ in NsiI site, forming pGEMT-Tn*916*CJ-*gyr*B.The construct was drawn using SnapGene version 3.2.1 and modified on PowerPoint.

4.2.4 Quantitative polymerase chain reaction (qPCR) for quantifying excision using SYBR Green

All of the DNA samples (BS34A total DNA and pGEMT-Tn*916*CJ-*gyr*B DNA were 10-fold serial diluted. QPCR amplification and analysis was performed using a Rotor-Gene 6000 instrument with software version 1.7 (Corbett Research). The qPCR was carried out in triplicate with a 50 µl final reaction mixture using the SensiMix SYBR No-ROX Kit (Bioline Reagents, UK). The qPCR reaction contained 1.25 µl of each primer (final concentration of 250 nM), 25 µl of 2x SensiMix SYBR No-ROX solution, 5 µl of template DNA (pGEMT-Tn*916*CJ-*gyrB* or BS34A DNA), and PCR-grade water up to 50 µl. The thermal cycling protocol was as follows: initial denaturation for 10 min at 95 °C followed by 40 cycles for 15 s at 95 °C, 30 s at 62 °C and 15 s at 72 °C. The fluorescence signal was measured at the end of each extension step at 72 °C.

4.2.5 Calculation of the circularised Tn*916* copy number

After the qPCR run was completed, the threshold cycle (Hughes and Datta, 1983) value was determined by automatic adjustment of the baseline and manual adjustment of the fluorescent threshold on the lower amplification curve. Ct values were created automatically and exported to Excel, and the average and the standard deviation were calculated (Bismuth et al., 1990). Samples with a Ct value, SD greater than 0.3, were excluded from standard curve generation and copy number calculation.

The Ct values were plotted against the log concentration of the pGEMT-Tn*916*CJ-*gyr*B DNA to establish the standard curves. The absolute copy number of circular Tn*916* from the total DNA of BS34A was determined by comparing the Ct values of the samples with the Ct value of the standard using the standard curves (Yu et al., 2005). The average of Tn*916* copy numbers (triplicates) was calculated (Appendix 9 and 10) and illustrated in a graph. The copy number was calculated using the following (Whelan et al., 2003):

Equation 4.1: $$\frac{\text{DNA (copy)}=\left(6.02 \times 10^{23}\left(\frac{\text{copy}}{\text{mole}}\right) \times \text{DNA amount (ng)}\right)}{\left(\text{DNA length (bp)} \times 1 \times 10^{9}\left(\frac{\text{ng}}{\text{g}}\right) \times 660\left(\frac{\text{g}}{\text{mole}}\right)\right)}$$

6.02×10^{23} (copy/mole) = Avogadro's number

1×10^{9} (ng/g) = convert from gram to nanogram

660 (g/mole) = average mass of 1 bp dsDNA

4.3 Results

4.3.1 Verification of the pGEMT-Tn*916*CJ- *gyrs*B sequence

The pGEMT-Tn*916*CJ- *gyrsB* construct containing a single copy of the circular joint of Tn*916* and *gyr*B was analysed and this confirmed the presence of the expected sequence (**Figure 4.3**). The quantification of the copy number of this construct represented a control compared with the amount of Tn*916* circular forms from the total DNA of BS34A cell, which was detected when Tn*916* was excised.

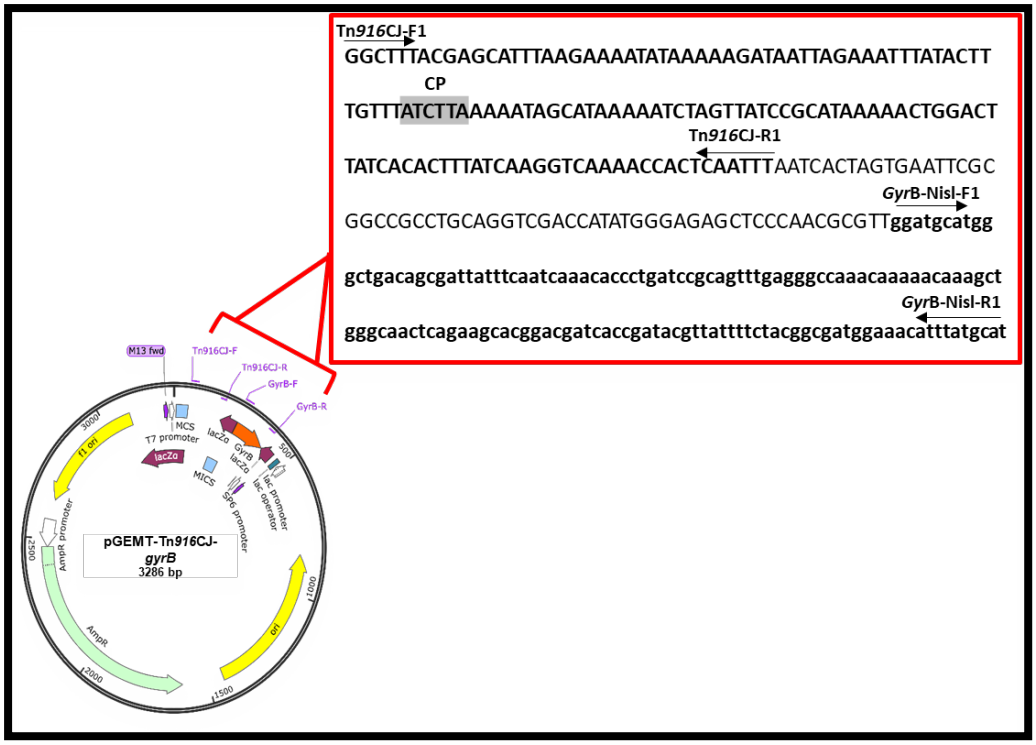

Figure 4.3: Sequence and locations of the inserted DNA in pGEMT-T-Tn*916*CJ-*gyrB* construct

The bold upper and lower case indicates Tn*916* circular joint and the *gyr*B sequence, respectively. The grey highlights indicate the coupling sequence.

4.3.2 QPCR analysis

4.3.2.1 QPCR specificity and sensitivity

During the PCR amplification process, the amount of the PCR product is doubled per cycle resulting in an increase in the intensity of fluorescence after each cycle by binding strongly to the double strand DNA. The serial dilution of the triplicate samples showed that the fluorescence curves closely overlapped (**Figure 4.4**).

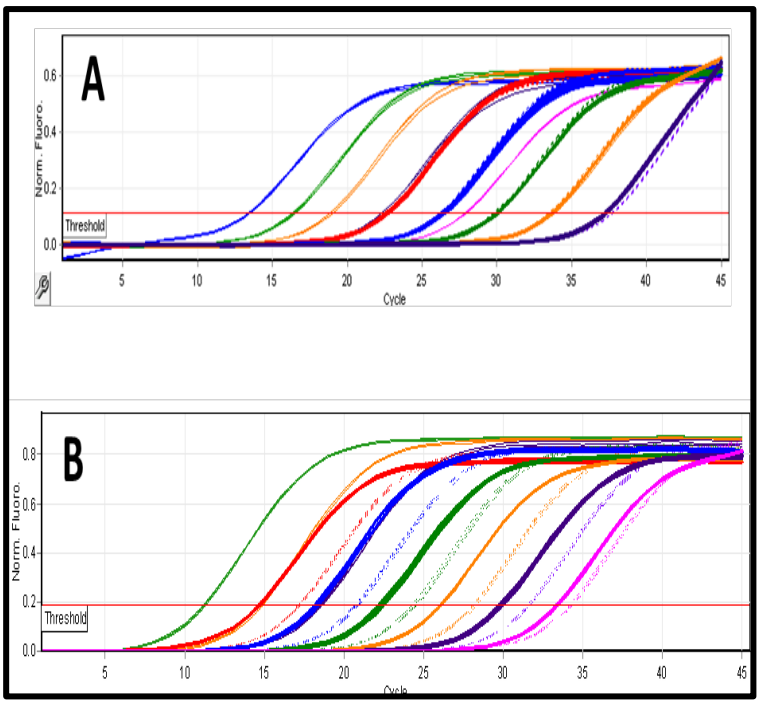

Figure 4.4: The SYBR green fluorescence chart produced in qPCR targeted the joint ends of Tn*916*

The normalized fluorescence signal (y-axis) is plotted against the PCR cycle number (x-axis) for the triplicates of the DNA templates. The fluorescence intensity increases during every cycle, as the amplicon copy number increases. The threshold cycle (Hughes and Datta, 1983) is the fractional cycle number at which a significant increase in fluorescence signals above a baseline signal. **A)** The total DNA template for BS34A incubated aerobically and anaerobically**. B)** The total DNA template for BS34A mixed with *E. faecalis* JH2-2. The dashed lines indicated DNA extracts from aerobic conditions, and the thick lines indicate from anaerobic conditions. The amplification curve with different colour represents different concentration of DNA samples.

The primer pair of Tn*916*CJ-F1/ Tn*916*CJ-R1 targeting the circular joint of Tn*916* were used. The data obtained from the melting curve of each reaction indicated that the specific PCR products were amplified with the primer set, which showed a single sharp peak at the melting temperature of 77 ºC for both the standard sample (pGEMT-Tn*916*CJ-*Gyr*B) and total DNA sample of BS34A (**Figure 4.5**). These results indicated that no non-specific PCR products were detected in the temperature range used (Ririe et al., 1997).

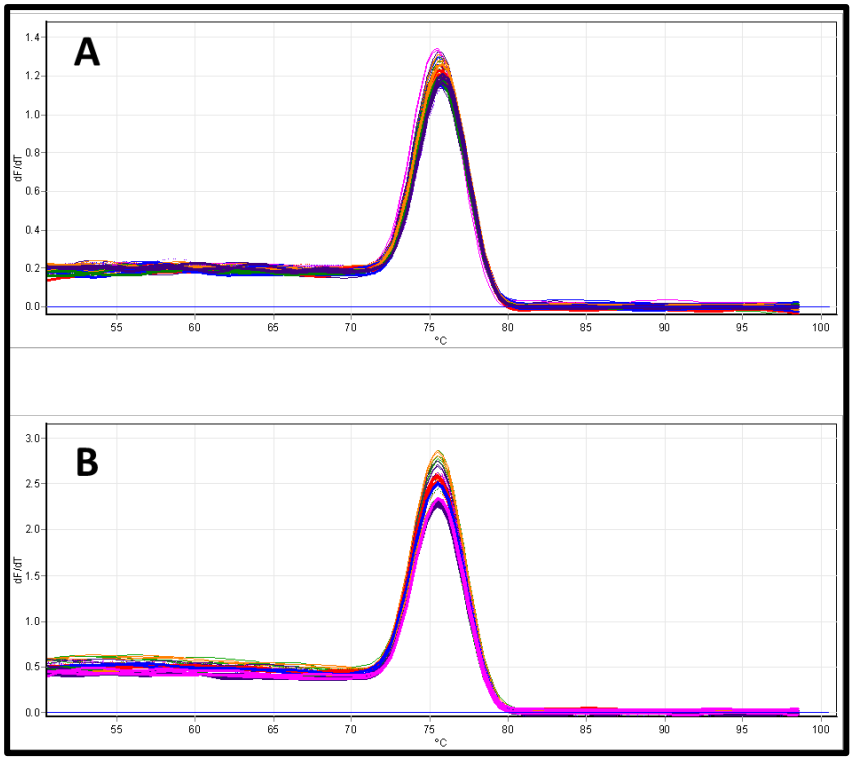

Figure 4.5: The melting curve plot in qPCR targeted the joint ends of Tn*916*

A) total DNA of BS34A cultured anaerobically and aerobically, and **B)** total DNA of BS34A + *E. faecalis* JH2-2 incubated aerobically and anaerobically. The *x* axis represents the temperature (°C), and the *y* axis represents the derivate of the fluorescence. The peaks indicate the melting temperature (77 ºC) for the PCR amplicons. The peak with different colour represents amplification product for different concentration of DNA samples.

4.3.2.2 Standard curves and amplification efficiencies

Standard curves were established by a 10-fold serial dilution in triplicate for the pGEMT-Tn*916*CJ-*Gyr*B (standard) in both experiments. The curves showed a plot of the Ct versus the log copy number, which ranged from 1 × 10^2 to 1 × 10^{10} copies/µl. A linear regression passing through the points was generated (R2 = 0.991 and 0.999), and the slopes were −3.6 in both experiments. This indicated that the PCR amplification results with an efficiency 90% (**Figure 4.6**) in both reactions were reliable and represented a

biological property and not misprocessing samples. The slope was automatically calculated with the following (Rasmussen, 2001):

Equation 4.2: $E = -1+10^{(-1/slope)}$

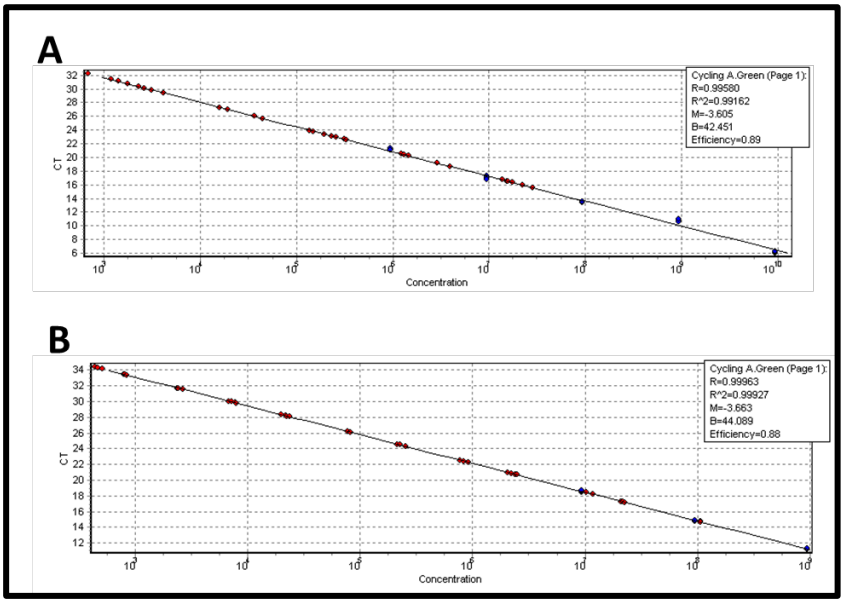

Figure 4.6: The standard curve of qPCR

A) The total DNA for BS34A incubated aerobically and anaerobically**. B)** The total DNA of BS34A mixed with *E. faecalis* JH2-2. The Ct on the x-axis plotted against the log copy number on the y-axis. The black dots represent the data for the standard samples (pGEMT-Tn*916*CJ-*Gyr*B), and the red dots represent the data for the unknown total DNA samples. The standard curve shows a linear regression: R^2 = 0.991 and 0.999.

4.3.2.3 Determination and the analysis of the copy number of the circularised Tn*916*

Using the standard curve of pGEMT-Tn*916*CJ-*Gyr*B, absolute quantification was performed for the circular molecule with the same concentration as the genomic DNA. As shown in **Figure 4.7**, the copy number of the excised Tn*916* showed no difference when extracted from aerobically or anaerobically grown cultures (**Figure 4.7 A**) (Appendix 9). However, when

the recipient *E. faecalis* was present, a 10-fold significant ($p<0.0001$) increase of excised Tn*916* was observed in anaerobic conditions (**Figure 4.7 B**) (Appendix 10). The statistical significance was determined by two-way ANOVA followed by Bonferroni's post hoc test.

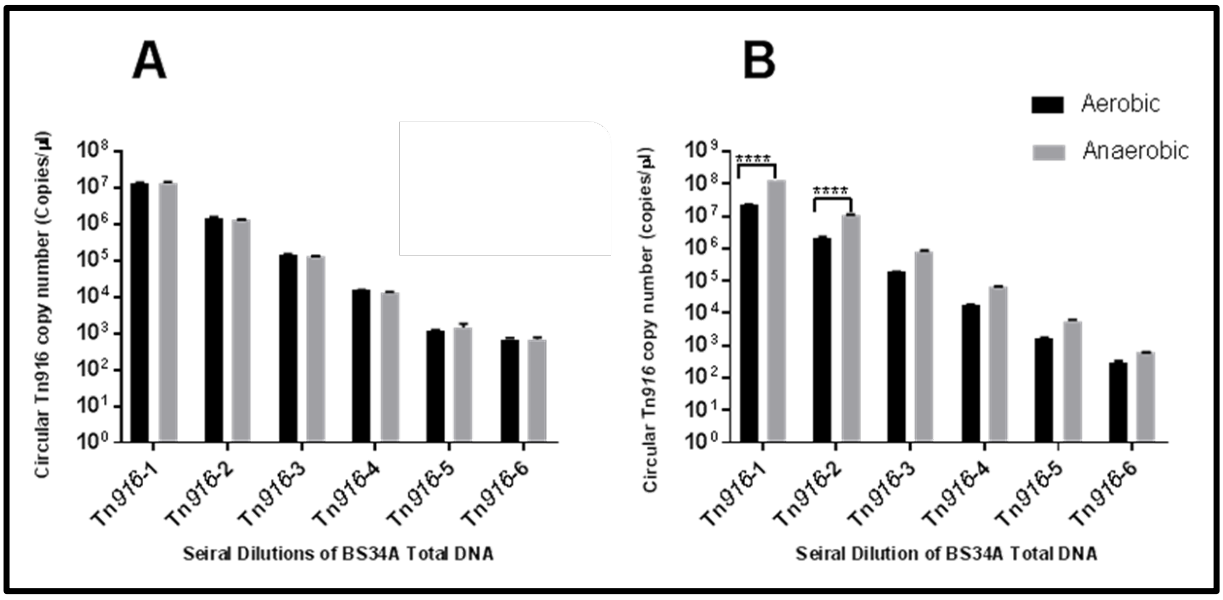

Figure 4.7: The influence of culture conditions on the copy number of circular Tn*916* in BS34A, as determined by qPCR

A) The total DNA for BS34A**. B)** The total DNA of BS34A mixed with *E. faecalis* JH2-2. The copy numbers in aerobic conditions are marked as black bars, and in anaerobic conditions, they are marked as grey bars. The error bars represent the standard errors for the three replicates. The asterisks (*) represent the statistically significantly difference between the copy numbers of the circular Tn*916* in aerobic and anaerobic conditions with a *p*-value <0.0001 (****), which was determined by using a two-way ANOVA followed by Bonferroni's post hoc test.

4.4 Discussion

Conjugative transposition of MGEs increases the spread of antibiotic resistance genes and the establishment of multidrug resistance strains in various bacterial pathogens. The first step of the transfer of the conjugative element Tn*916* is the excision from the host chromosome (Gawron-Burke

and Clewell, 1982), which was verified by a mutant Tn*916* (Δ*xis*), that lost the ability to excise and transfer from the donor DNA molecule (Storrs et al., 1991). Moreover, it was proposed that an excised Tn*916* in a Gram-positive donor did not accumulate in the cell, as it transferred rapidly into the recipient genome (Scott et al., 1988). In addition, it was reported that the number of circular forms of Tn*916* was proportional to the conjugation frequency (Jaworski and Clewell, 1994, Manganelli et al., 1995). Therefore, it was thought that excision was the rate-limiting step for the conjugation process to occur (Gawron-Burke and Clewell, 1982, Caparon and Scott, 1989). However, Marra et al. (1999) changed this view after observing that there was no difference in copy number of Tn*916* circular form between high and low frequency donors. In addition, overexpression of Int and Xis in *E. faecalis* DM103, *B. subtilis* CK101 and CK102 donor cells caused an increase in the circular form of Tn*916*, but it did not affect the frequency of transfer, which was the same before and after the expression (Marra et al., 1999).

Tn*916* in circular form has been detected by several methods, such as the Southern blot (Scott et al., 1988, Marra and Scott, 1999), PCR amplification and measuring the band density (Jaworski and Clewell, 1994) and droplet digital PCR (ddPCR) (Lunde et al., 2019). Moreover, circular Tn*916* has been detected in *E. faecalis* (GP423.5, GP422.1 and GP423.1) strains with and without tetracycline by using a two-step PCR. The copy number of circular Tn*916* and the conjugation frequency has been shown to increase with tetracycline (Manganelli et al., 1995). These methods (except ddPCR) are time consuming and not as accurate as qPCR therefore, to investigate the effect of environmental conditions, on Tn*916* excision, an assay

developed to determine Tn*916* copy number in circular form using qPCR. This technique was easy, fast and accurate (Lee et al., 2006, Al-Shanti et al., 2009).

Following technique described by Lee et al. (2006) pGEMT-Tn*916*CJ-*gyr*B (**Figure 4.2**) designed to determine the copy numbers using qPCR for either absolute or relative methods. A small segment of Tn*916* DNA bearing joint end sequences was cloned into the construct to allow quantification of the absolute copy number of circular Tn*916* present in BS34A total DNA, which was based on the Ct values from the corresponding standard curve (**Figure 4.6**) (Yu et al., 2005). In addition, the *gyrB* is a highly conserved gene that encodes the ATPase domain (β subunit) of DNA gyrase (topoisomerase II). Amplification of *gyrB* is known for its accuracy and precision in distinguishing the phenotype of a closely related species belonging to the *Bacillus* genus (Yamada et al., 1999, La Duc et al., 2004). A small fragment sized 128bp of *gyrB* was cloned into pGEMT-Tn*916*CJ as a housekeeping gene for the relative method. In the relative method, the copy number could then be determined by the ratio of the construct, which was used as the calibrator (reference gene), and the total DNA using two primer sets Tn*916*CJ-F1/ Tn*916*CJ-R1 and GyrB-Nsil-F1/ GyrB-Nsil-R1. However, due to the limited number of samples to be applied in just one run using Rotor-Gene 6000 and insufficient time, the construct was used to run only the absolute method to determine the excised copy number of Tn*916.*

Concentration of all the starting DNA molecules (circular and genomic) was normalised to 100 ng/µl to establish the same amount of DNA molecules in experiments. QPCR amplifications were performed in the same concentration, for the standard samples (pGEMT-Tn*916*CJ-*Gyr*B) (circular DNA) and for the total DNA samples (genomic DNA) of BS34A, which harboured Tn*916*. Incubation conditions were either aerobic or anaerobic with and without the recipient (*E. faecalis* JH2-2). Absolute quantification was performed from the corresponding standard curve using the Ct value (**Figure 4.6**) (Yu et al., 2005). The amplification efficiency of the target sequence was 90%. This means that the efficiency between the reactions were the same.

The copy number of Tn*916* was calculated from qPCR data. As shown on the graph (**Figure 4.7**), when the donor (*B. subtilis* BS34A) was incubated in either aerobic or anaerobic conditions, the copy number of the excised Tn*916* was equal and ranged between 6 $X10^2$ - 1.3 $X10^7$ copies/µl. Since the level of Tn*916* circular form in both conditions was the same, it was assumed that conditions alone had no effect on the excision of Tn*916* and that the detected excised copies of Tn*916* were due to the presence of tetracycline (Showsh and Andrews, 1992).

Furthermore, it has been assumed that the step prior to filter mating could have had an effect, as was proposed in a review by Scott (1992) and in the current work was observed that in different conditions (aerobic and anaerobic), the circular copy number of Tn*916* was the same. Therefore, the copy number of excised Tn*916* in both conditions in the presence of the recipient (*E. faecalis* JH2-2) the original host of Tn*916* was investigated and observed (Franke and Clewell, 1981).

Using nested PCR, Manganelli et al. (1995) demonstrated that the copy number of circular Tn*916* in *E. faecalis* did not change with cell contact. However, the condition of growth was not mentioned. In the current work, it was detected that in the presence of the recipient, a significant increase in the copy number of circular Tn*916* occurred only in anaerobic conditions. As *E. faecalis* is an intestinal organism and the conjugation process requires cell-to-cell contact (Tatum and Lederberg, 1947), it was assumed that the excision and/or transposition of Tn*916* was stimulated by an anaerobic regulatory factor encoded by the donor in the presence of the recipient e.g. FNR (fumarate and nitrate reductase). A further investigation will be required to determine the effect of FNR on Tn*916* transfer in anaerobic conditions.

In conclusion, the influence of conditions and the recipients on the excision of Tn*916* was determined. QPCR based absolute methods were applied to quantify the Tn*916* copy number within BS34A cells, which were incubated aerobically or anaerobically. The calculated copy number of Tn*916* was similar in both conditions (aerobic and anaerobic), which indicated that the gaseous requirement did not have an effect on excision alone. Furthermore, the results showed that Tn*916* excision in different growth conditions was enhanced in the presence of recipient. In anaerobic conditions, the excised Tn*916* circular copy numbers increased significantly (10-fold) compared to the circular copy number in aerobic conditions in the presence of the recipient.

Chapter 5

Determination of the role of the Fumarate and Nitrate Reductase (FNR) binding site on Tn*916* transcription

Chapter 5 Determination of the role of the Fumarate and Nitrate reductase (FNR) binding site on Tn*916* transcription

5.1 Introduction

Bacteria face environmental challenges which they must be able to respond in order to survive. Bacteria can be stressed by a variety of conditions, such as changes in physiochemical and chemical parameters (e.g. temperature (Benson and Haldenwang, 1993, Maul et al., 1995, Voelker et al., 1996, Brigulla et al., 2003, Holtmann et al., 2004, Mendez et al., 2004, Budde et al., 2006), radiation, pressure, pH, oxygen (Volker et al., 1994), osmolyte salt concentration and redox state (Boylan et al., 1993, Maul et al., 1995, Zhang and Haldenwang, 2005)), nutritional deprivation (e.g. glucose, phosphate, amino acids, carbon and nitrogen) (Volker et al., 1994, Maul et al., 1995, Voelker et al., 1995, Bernhardt et al., 2003, Zhang and Haldenwang, 2005) and the presence of toxic substances (e.g. antibiotics (Bandow et al., 2002, Mascher et al., 2003), toxins and heavy metals (Voelker et al., 1996, Moore et al., 2004)). Some types of bacteria have a wide range of regulatory genes (e.g. global regulatory systems) that encode a regulatory protein, which controls the expression of other genes in response to environmental changes to allow bacteria to survive (Boylan et al., 1993, Antelmann et al., 1997). Signals surrounding the bacteria initiate the transcription of a specific gene from the promoter, which is recognised by sigma factor and directed by RNA polymerase (RNAP) (Burgess et al., 1969). Sigma factors are proteins that specify transcription by recognising a specific DNA sequence on the

promoter. It allows RNAP to bind and initiate transcription, which is terminated by a short sequence complementary to the sequence before it (Khesin et al., 1969, Burgess et al., 1969, Gusarov and Nudler, 1999, Buck et al., 2000, Cramer, 2002, Browning and Busby, 2004). Bacteria have a primary (housekeeping) sigma factor, such as σ^{70} in *Escherichia coli* and σ^{A} in *B. subtilis,* and alternative sigma factors with an extracytoplasmic function (regulate a wide range of genes in response to conditions) (Khesin et al., 1969, Lonetto et al., 1994).

Primary sigma factors promote the transcription of most genes that are required in most growth conditions, whereas alternative sigma factors initiate the transcription of specific genes required to overcome environmental stress or developmental states. In *B. subtilis*, one alternative sigma factor is σ^{B}, which is induced in response to environmental and energy stress (Völker et al., 1999, Price, 2000, Price et al., 2001, van Schaik et al., 2004).

Changes in growth conditions from an aerobic to an anaerobic environment activates fumarate and nitrate reductase (FNR) (Spiro and Guest, 1990, Cruz Ramos et al., 2000). This transcriptional regulator contains a dimer structure $[4Fe\text{-}4S]^{+2}$ that changes the DNA binding affinity in the absence of oxygen, allowing it to regulate a range of different genes essential for anaerobic metabolism, and repress the genes involved in aerobic metabolism (**Figure 5.1** and **Figure 5.2**) (Spiro and Guest, 1990, Iuchi and Lin, 1993, Cruz Ramos et al., 1995, Khoroshilova et al., 1997, Jordan et al., 1997). However, in the presence of oxygen, the protein-binding affinity is altered (structure changed) via immediate degradation of the $[4Fe\text{-}4S]^{+2}$ clusters to $[2Fe\text{-}2S]^{+2}$

after oxygen binds to the iron, causing dissociation of FNR into its subunits (weak DNA binding) (**Figure 5.2**). FNR is a member of the catabolite activator protein (CAP) family (Kolb et al., 1993) and contains a C-terminal DNA binding domain recognising TGTGA-N_6-TCACA (**Figure 5.4**) (Cruz Ramos et al., 1995, Reents et al., 2006b) and an N-terminal sensory domain (Reents et al., 2006a).

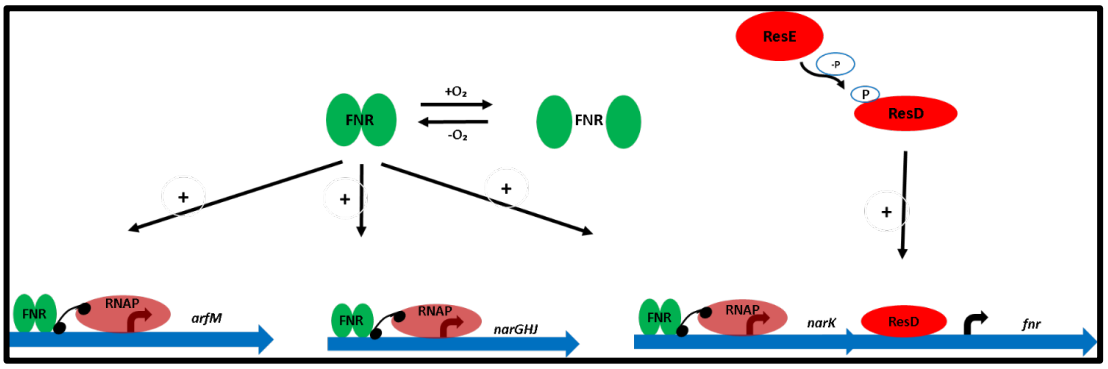

Figure 5.1: Regulation of gene expression by redox sensitive Res and FNR pathways

Under anaerobic conditions, phosphorylated ResD positively regulates the transcription of FNR. FNR is inactive in the presence of oxygen and active in the absence of oxygen (**Figure 5.2**). Active FNR binds to and induces transcription from *arfM* (an anaerobic respiration and fermentation modulator), *nar*GHJI (nitrate reductase) and *nark* (nitrite efflux).

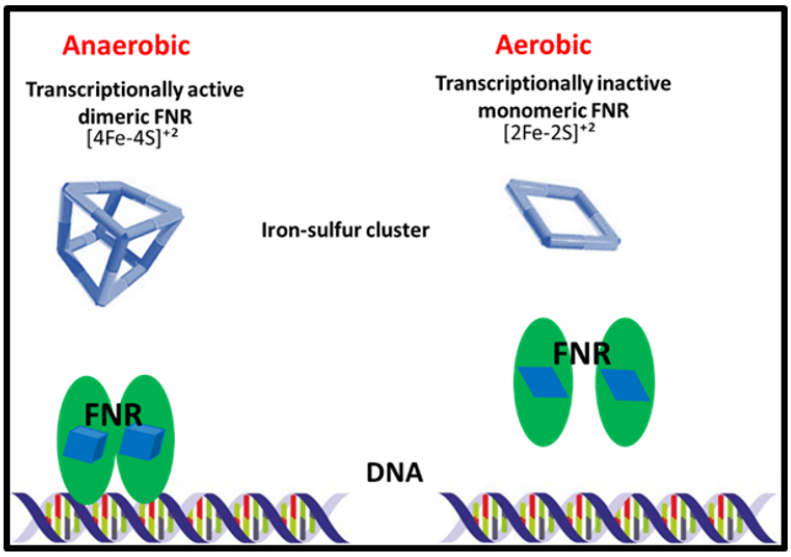

Figure 5.2: Model of FNR-DNA interaction in response to the oxygen presence

Right: Under aerobic conditions, FNR (green oval shape) is inactive with a monomeric structure containing a $[2Fe\text{-}2S]^{+2}$ cluster (blue square shape). **Left**: Under anaerobic conditions, FNRs dimerized containing $[4Fe\text{-}4S]^{+2}$ (blue cube shape) and binds to a specific sequence on the DNA.

The work presented in Chapter 4 showed that the copy numbers of circular Tn*916* in *B. subtilis* with *E. faecalis* present was significantly greater in an anaerobic environment. *B. subtilis* responds to anaerobic growth via the FNR protein to upregulate genes required for anaerobic respiration. Genes regulated by FNR have a specific binding site (TGTGA-N_6-TCACA) upstream of their start codon (**Figure 5.1**) (Reents et al., 2006b, Cruz Ramos et al., 1995). It was hypothesised that Tn*916* transfer in anaerobic conditions is partly regulated by the FNR regulatory system in addition to the recipient effect. This chapter aimed to investigate the effect of FNR on Tn*916* transcriptional regulation. This was tested by screening Tn*916* for a putative FNR-binding site (TGTGA-N_6-TCACA) and measuring the activity of a cloned FNR-binding site upstream of *gusA* in a reporter system aerobically

and anaerobically. Different *gusA* reporter constructs (with and without FNR binding sequence or replaced the FNR binding sequence) were developed. If this site showed a higher activity when conditions changed (from aerobic to anaerobic) in the presence of the FNR binding sequence and showed the opposite in the absence or the substituted sequence, it would suggest that FNR binds and enhances the transcription within and excision of Tn*916*.

5.2 Materials and Methods

5.2.1 In silico analysis to determine the FNR-binding site on Tn*916*

The prokaryotic database of gene regulation (PRODORIC) an online software was used to identify the FNR palindrome motifs of *B. subtilis* (http://www.prodoric2.de/) and create the position weight matrix model (Münch et al., 2003). The Tn*916* sequence was screened in silico to determine the potential FNR-binding site using the pattern TGTGA-N_6-TCACA (Reents et al., 2006b) with some variability in the numbers of N_6. This search was not limited to the upstream promoter sites because the entire Tn*916 orfs* and terminators sequences wanted to be screened.

5.2.2 Bacterial strains and plasmid culture conditions

All the bacterial strains and plasmids used in this chapter were listed in Table 2.1. *B. subtilis* BS34A harboring Tn*916* was used as a host for all the *gusA* reporter plasmids to measure the expression of the *gus*A under different conditions (aerobic, anaerobic or both conditions). *B. subtilis* BS34A

harboring the *gusA* reporter was grown in 10 ml of BHI broth supplemented with 10 µg/ml of chloramphenicol. The control *B. subtilis* BS34A without a plasmid was cultured without antibiotic (negative control). For aerobic growth, the bacterial cultures were incubated at 37 ºC in a shaker incubator (200 rpm) for 18 h. For anaerobic growth, conditions containing 80% N_2, 10% H_2 and 10% CO_2, the bacterial cultures were incubated using a tissue culture flask T25 (Sarstedt Ltd, UK) at 37 ºC with shaking (50 rpm) for 18 h. Another culture of *B. subtilis* BS34A harboring the *gusA* reporter was incubated aerobically at 37 ºC in a shaker incubator (200 rpm) for 17 h; and for the last hour of incubation, the culture was transferred to the anaerobic chamber. *E. coli* α-select silver (Bioline, UK) was used as a host for pHCMC05-p*tet*(M)-Δ*orf12-gus*A (*gusA* reporter system) (Seier-Petersen et al., 2014). *E. coli* α-select silver transformants bearing *gusA* reporter were cultured on LB agar or LB broth supplemented with 10 µg/ ml of chloramphenicol (Sigma, UK); it was then incubated aerobically at 37 °C on a rotary shaker (200 rpm) for 16 h. Moreover, for RNA extraction, two tubes containing 10 ml of BHI broth were inoculated with *B. subtilis* BS34A and incubated at 37 °C, aerobically and anaerobically.

5.2.3 Investigating *Bacillus subtilis* FNR expression

5.2.3.1 RNA extraction

Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to determine expression of *fnr*. RNA was extracted from an overnight culture of *B. subtilis*, incubated aerobically or anaerobically. This involved harvesting

cell pellets at 12000 *X g* (Eppendorf centrifuge 5804 R) for 5 min at 4 ºC and then discarding the supernatant. Cell pellets were lysed with 800 µl of TRIzol (lysozyme solution), and then incubated for 5 min at room temperature. Two-hundred microliters of chloroform was added with mixing for 15 sec, and then incubated at room temperature for 15 min. After being centrifuged at 12000 *x g* for 15 min at 4 ºC, the top colourless aqueous layer was transferred into a 1.5 ml microcentrifuge tube, mixed with 5 µl of DNase I (Life Technologies, UK) and incubated for 30 min in a 37 ºC water bath. Five-hundred microliters of isopropanol were mixed with the samples and centrifuged at 12000 *x g* for 8 min at 4 ºC. The supernatant was discarded and the RNA pellets were washed with 75% ethanol. The samples were centrifuged at 7500 *x g* for 5 min at 4 ºC. Then the pellets were air dried for 10 min. The pellets were dissolved in 100 µl molecular grade H_2O (Sigma, UK) and incubated for 10 min in a 60 ºC water bath. Before conducting RT-PCR, the RNA samples were tested for DNA contamination using the extracted RNA as the template for standard PCR and visualised on gel (not shown).

A TURBO DNA-free™ Kit (Thermo Scientific, UK) was used to remove DNA contamination from the RNA samples following the manufacturer's instructions. The DNA-free reaction was prepared in a 1.5 ml microcentrifuge tube as follows: 3.5 µl of 10X TURBO DNase buffer, 1 µl of TURBO DNase (2 units/ µl) and 35 µl of the RNA samples. The preparation was gently mixed and incubated at 37 ºC for 30 min. DNase activity was inactivated via mixing the samples with 3.5 µl of DNase inactivation reagent, and then incubating them for 5 min at room temperature while mixing occasionally. The samples

were centrifuged at 10000 *x g* for 1.5 min, and then the RNA supernatant was transferred to a new tube. Decontaminated samples were used and visualised by standard PCR and gel electrophoresis to check for sample purity and absence of DNA.

5.2.3.2 Reverse-transcriptase polymerase chain reaction (RT-PCR)

First strand complementary DNA (cDNA) was synthesised at 55 ºC using the SuperScript™ III reverse transcriptase kit (Termfisher, UK). The cDNA synthesis reaction was prepared following the manufacturer's protocol and consisted of: 2 µl of gene-specific reverse primer (FNR-R or Veg-R) (2 µM), 1 µl of dNTP (10 mM), 2 µl of template RNA (1 µg) and water up to 13 µl. The reaction was mixed well, heated at 65 ºC for 5 min and then transferred to ice for 1 min. The contents of the tubes were collected by centrifugation and used to prepare the reverse transcriptase reaction via the addition of 4 µl of 5X first strand buffer, 1 µl of 0.1 M DTT, 1 µl of RNaseOUT (40 units /µl) recombinant RNase inhibitor and 2 µl of superscript TMIII RT (200 units/µl). The reaction was mixed gently, and incubated at 55 ºC for 1 h. Samples were heated for 15 min at 70 ºC to inactive the reaction. The generated cDNA for *fnr* and *veg* were used as a template for PCR amplification. *Veg* was used as a positive control for RT-PCR, because in *B. subtilis* it expresses consistent in different conditions (Le Grice et al., 1986, Gilman and Chamberlin, 1983, Ollington and Losick, 1981). The PCR and cycle conditions were carried out as described in Section 2.2.2.

5.2.4 Investigation of the role of the putative FNR-binding sequence on *orf12* of Tn*916*

5.2.4.1 Mutation on the cloned *orf12* terminator sequence

To determine the role of the putative FNR-binding site on the *orf12* terminator, four site-directed mutagenesis reactions were carried out using the Q5® Site-Directed Mutagenesis Kit (NEB, UK) and four oligonucleotides pairs (Table 2.2). The pHCMC05-*ptet*M-*orf12-gusA* plasmid, previously described by (Seier-Petersen et al., 2014), was used as a template amplified via four different primers, resulting in the deletion of *orf12* (Δ*orf12*), the deletion of *orf12* terminator T_2 (Δ*orf12*(T_2)), the partial deletion of *orf12* terminator T_1 (Δ*orf12*(T_1)) and the substitution *orf12* terminator (T_1) sequence (s*orf12*(T_1)) (**Figure 5.3**). The amplified, PCR products containing the mutations were ligated in a 10 µl ligation reaction using the Kinase-Ligase-DpnI (KLD) enzyme mix (NEB, UK), incubated at room temperature for 10 min. Five µl of the ligation reaction were transferred to *E. coli* α-select silver and cultured on LB agar supplemented with 10 µg/ml of chloramphenicol for sequencing. After verifying the sequences, the constructs were transformed to *B. subtilis* BS34A and isolated on BHI agar supplemented with 10 µg/ml of chloramphenicol after 24–48 h incubation aerobically at 37 ºC. All the reaction mixtures, cycling conditions of the site-directed mutagenesis and transformation methods were performed as previously described in Chapter 2.

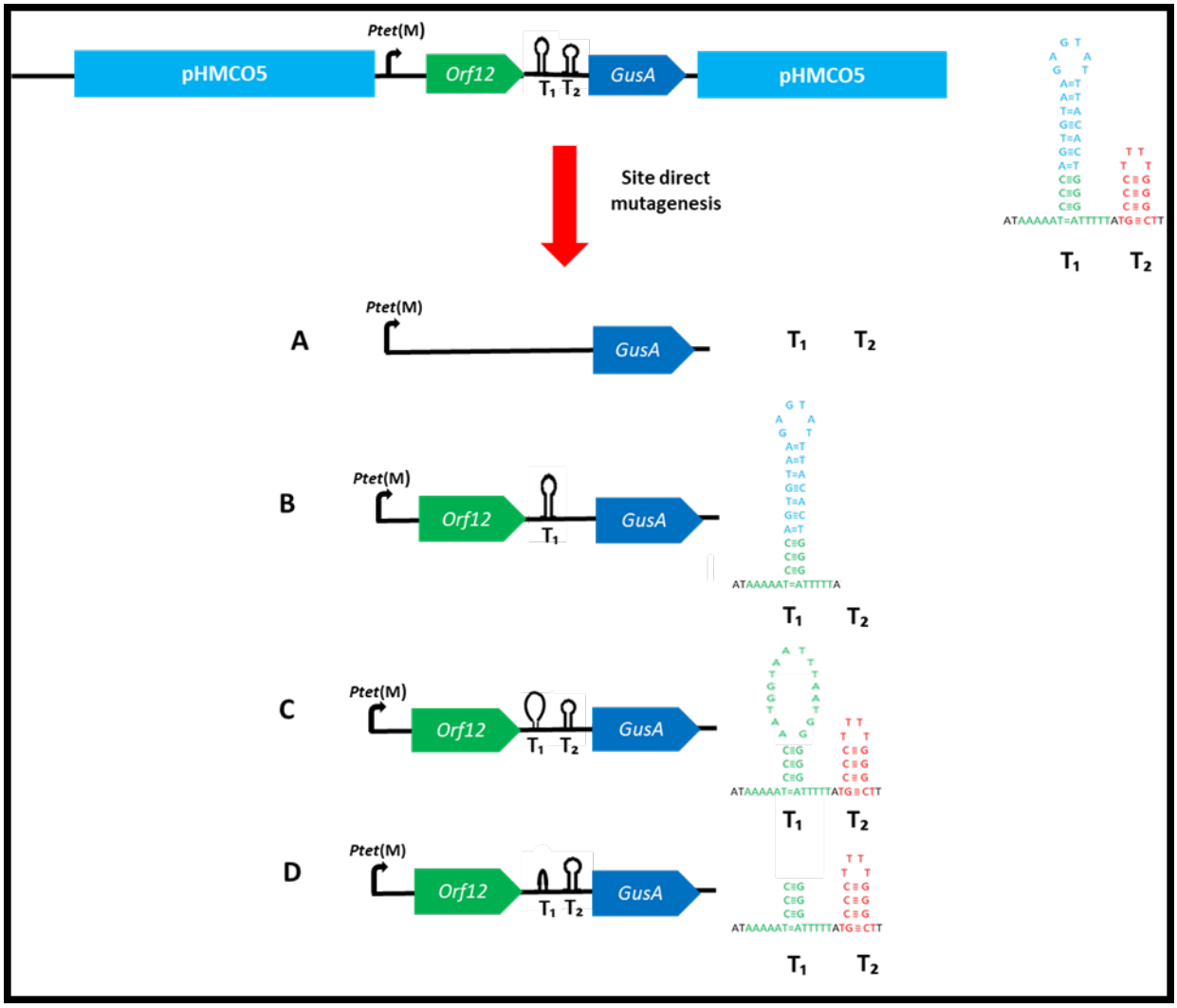

Figure 5.3: Diagrammatic representation of *orf12* mutagenesis

The top image represents the p*tet*(M)-*orf12-gusA* construct. The green arrows represent *orf12* on Tn*916,* the dark blue arrows represent the *gusA* and the light blue boxes represent the pHCMC05 backbone. The large terminator sequence (T_1) represented in green, the small terminator sequence (T_2) represented in red and the sequence of FNR binding site on *orf12* represented in blue. pHCMC05-p*tet*(M)-*orf12-gusA* was used as a template to generate: **A)** the *orf12* deletion construct (p*tet*(M)-Δ*orf12-gusA*), **B)** the *orf12* terminator T_2 deletion construct (p*tet*(M)-Δ*orf12*(T_2)-*gusA*), **C)** the sequence substitution of the *orf12* terminator T_1 construct (p*tet*(M)-s*orf12*(T_1)-*gusA*) and **D)** the partial deletion of the *orf12* terminator T_1 construct (p*tet*(M)-Δ*orf12*(T_1)-*gusA).*

5.2.4.2 Measurement of reporter gene expression

The ß-glucuronidase enzymatic assay was performed to measure the potential FNR- binding site activity based on the expression of *gusA*, following the protocol previously described by Belitsky et al. (1995), with some modifications. The optical density of all the *B. subtilis* samples containing the reporter construct and cultured overnight in 10 ml of BHI broth supplemented with 10 µg/ml of chloramphenicol was measured at OD_{600} nm. All the cultures were centrifuged for 10 min at 3000 x *g*, and the cell pellets were stored at -70 ºC for 1h to inhibit growth and promote lysis. The cell pellets were thawed at room temperature and re-suspended in 800 µl of Z-buffer (pH 7, appendix 1) and 8 µl of toluene. The mixture was transferred to a 2 ml cryotube with unwashed glass beads (150–212 µm in diameter) (Sigma, UK). The tubes were vortexed twice for 5 min each, with 1 min of incubation on ice in between. The cryotubes were centrifuged at 3000 *x g* for 3 min at 4 °C to remove the glass beads. An appropriate volume of cell lysate was mixed with Z buffer to make a total volume 800 µl, and the sample was then incubated at 37 °C for 5 min. One-hundred-sixty µl of 6mM p-nitrophenyl-ß-D-glucuronide (PNPG) was added to the reaction and incubated at 37 °C for 5 min to start the enzyme reaction. The reaction was stopped by the addition of 400 µl of 1 M Na_2CO_3, and then the samples were centrifuged at 3000 x g at 25 ºC for 10 min to remove the cell debris. The absorbance of the supernatant was measured using a spectrophotometer at a wavelength of 405 nm. Three biological replicates of the ß-glucuronidase enzymatic assay were performed. The ß-glucuronidase Miller units were calculated using Equation 5.1 (Miller, 2018):

Equation 5.1: $\text{ß} - \text{glucuronidase Miller units} = \frac{A^{405}\text{X}1000}{OD^{600}\text{X time in min} \quad \text{X } 1.25 \text{ X volume (ml)}}$

pHCMC05-P*tet*(M)-*gusA* was used as a positive control in the experiment because it had been previously shown expression of the *gusA* (Seier-Petersen et al., 2014). Statistical analysis was performed using the GraphPad Prism 7 (GraphPad Software, Inc., USA).

5.3 Results

5.3.1 Identification of the FNR-binding sequences on *orf12* terminator of Tn*916*

The position weight matrix model of the FNR-binding site in *B. subtilis* was created online by PRODORIC software (Münch et al., 2003) and shown as a sequence logo (**Figure 5.4 A**). Putative FNR binding motifs were found present along the Tn*916* sequence (**Figure 5.4 B**). One of the putative FNR consensus binding site was identified on the *orf12* terminator (large terminator (T_1)) upstream the *tet*(M*)* with a 12 bp space in between the conserved sequence (**Figure 5.5**).

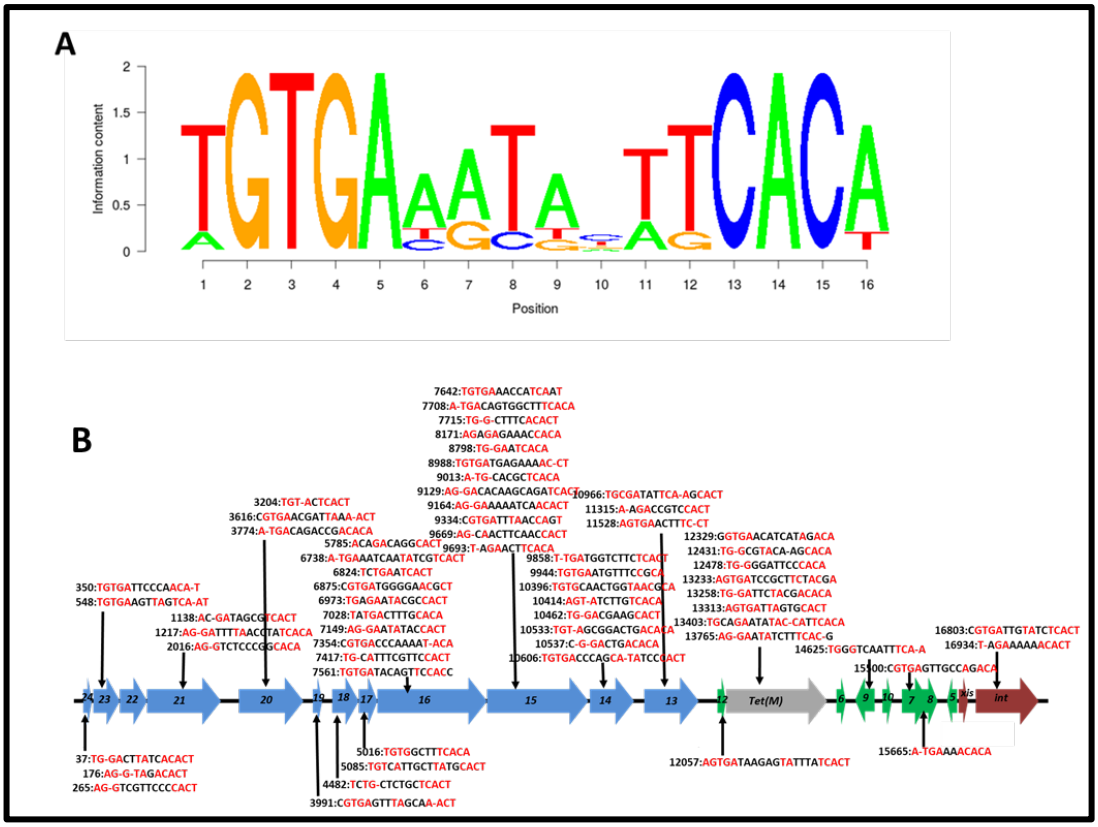

Figure 5.4: *B. subtilis* FNR-binding site

A) The sequence logo of the *B. subtilis* FNR-binding consensus based on the position weight matrix model. The height of each letter represents the frequency of the sequence. **B)** Sequences and locations of the putative FNR binding sites identified on Tn*916*. The black nucleotides represent Tn*916* sequence and the red nucleotides represent putative FNR binding motifs on Tn*916*. The numbers before each sequence indicates the location of the sequence on Tn*916*.

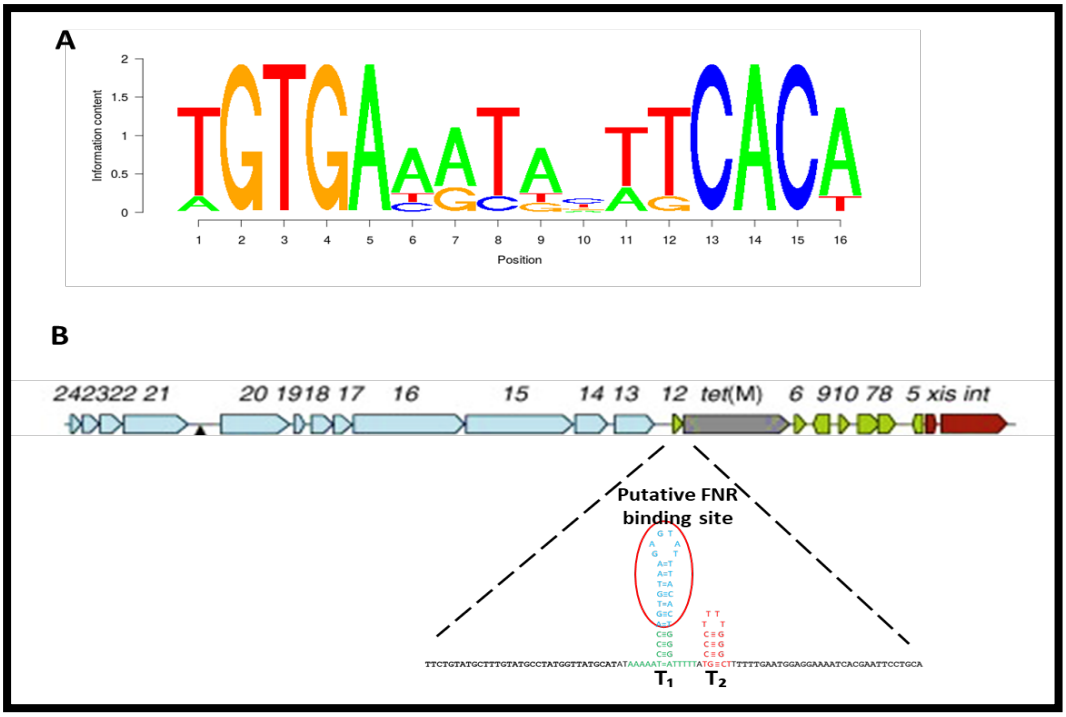

Figure 5.5: Location of the potential FNR-binding sequence on the *orf12* terminator (T_1) of Tn*916*

The sequence in blue represent the putative FNR-binding site which is contained within the *orf12* large terminator (T_1) (green) and upstream the small terminator (T_2) (red).

5.3.2 The *fnr* gene is expressed aerobically and anaerobically

To confirm that *fnr* was transcribed aerobically and anaerobically, RT-PCR was performed on the RNA extracted from BS34A grown aerobically and anaerobically. As shown in **Figure 5.6 B**, the RNA of *fnr* is present in both conditions aerobically and anaerobically.

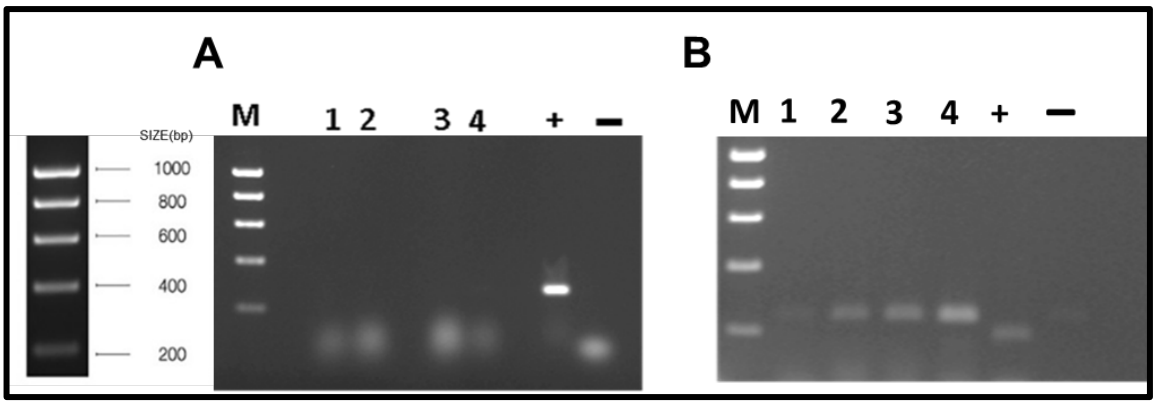

Figure 5.6: Gel electrophoresis of RT-PCR for *fnr* RNA

A) A strandard PCR on the RNA samples to detect DNA contamination showing no bands and **B)** RT-PCR results of cDNA showing faint bands. **M**: 1kb hyper ladder Marker, **lane 1 and 2**: RNA extracted from the BS34A-incubated anaerobically, **lane 3 and 4:** RNA extracted from the BS34A-incubated aerobically. **+** in **A)** represents the *fnr* amplified from BS34A DNA using FNR-F/FNR-R primers (positive control for PCR) and **+** in **B)** represents *veg* amplified from the cDNA using Veg-F/Veg-R primers (positive control for RT-PCR).

5.3.3 Generation of the P*tet*(M)-*gusA* reporter mutants

Four of the P*tet*(M)-*orf12*-*gusA* reporter constructs containing either deletion of the entire *orf12* (*Δorf12*), partial deletion of the *orf12* large terminator sequence (*Δorf12*(T_1)), substitution of *orf12* large terminator sequence (s*orf12*(T_1)) or deletion of *orf12* small terminator (Δ*orf12*(T_2)) were successfully generated and sequenced as indicated in **Figure 5.7**.

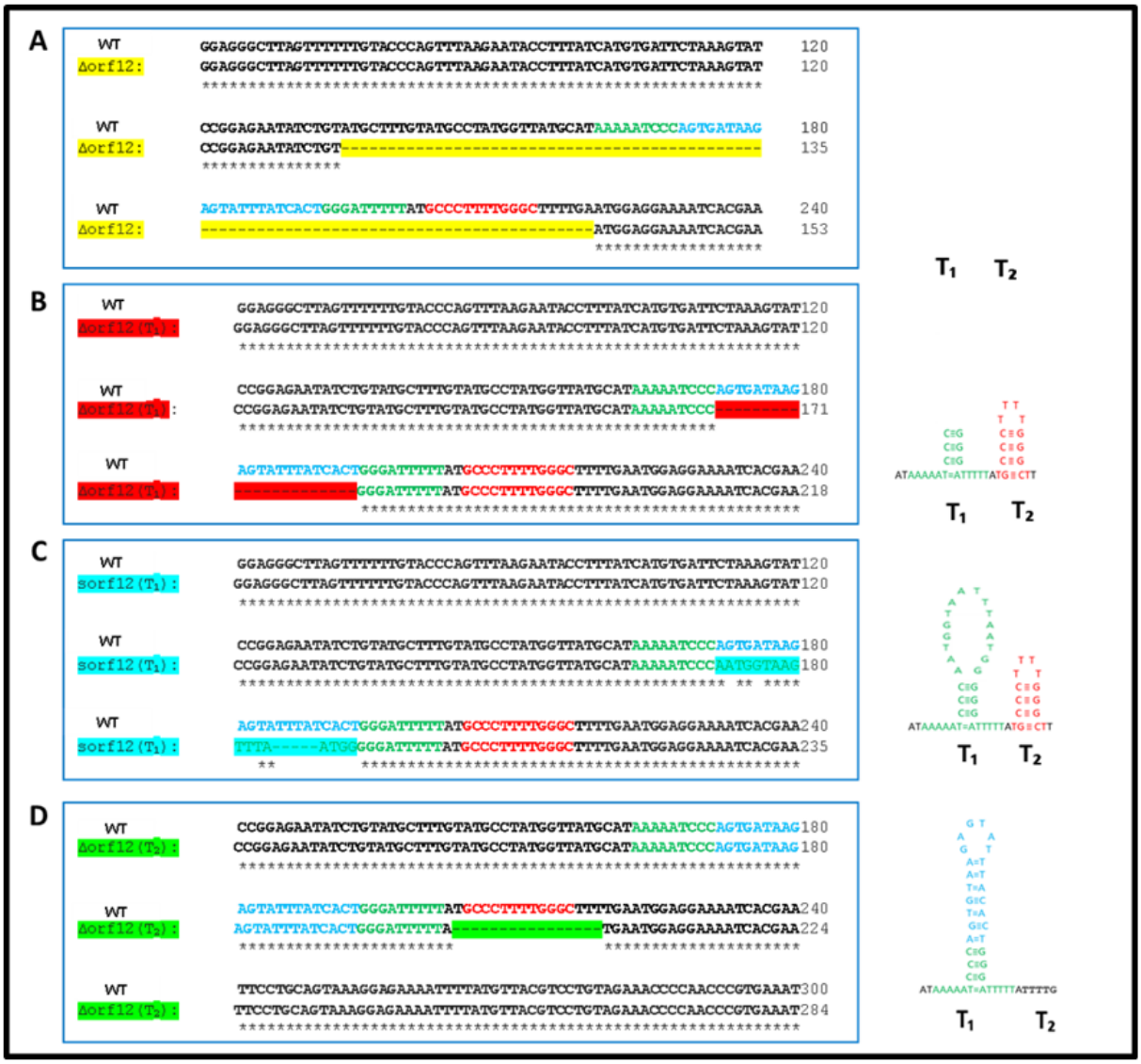

Figure 5.7: Sequence alignment of the mutagenesis of the P*tet*(M)-*orf12-gusA* reporter constructs

The sequence of T_1, T_2 terminators and FNR putative binding site are represented in green, red and blue respectively. **A)** The sequence of the deleted *orf12* (*Δorf12*) constructs are highlighted in yellow. **B)** The large terminator mutation of *orf12* (*Δorf12* (T_1)) is highlighted in red. **C)** The substitution of the *orf12* large terminator (s*orf12*(T_1)) is highlighted in blue. **D)** The sequence of the deletion of the *orf12* small terminator (Δ*orf12*(T_2)) is highlighted in green.

5.3.4 Effect of oxygen limitation on the transcriptional read-through of *orf12* and its mutants

The effect of oxygen availability on the transcriptional read-through of *orf12* and its mutants was investigated on the cells grown under aerobic, anaerobic or both conditions (aerobic first, and then transferred to anaerobic conditions) (**Figure 5.8**) (Appendix 11). The results showed that the highest β-glucuronidase activity occurred when *B. subtilis* BS34A sample harboring P*tet*(M)-*orf12*-*gusA* contained different mutations in the *orf12* terminator (Δ*orf12,* Δ*orf12*(T_1), Δ*orf12*(T_2) and s*orf12*(T_1)). Comparing all of these mutant constructs in anaerobic culture and with culture transferred from aerobic to anaerobic conditions, revealed a significant increase (P=0.02) in the enzyme activity when the culture was transferred.

As illustrated in **Figure 5.8**, constant enzyme activity was found in *B. subtilis* BS34A with the P*tet*(M)-*orf12* and BS34A (without the construct), which were used as the positive and negative control respectively, despite the incubation condition.

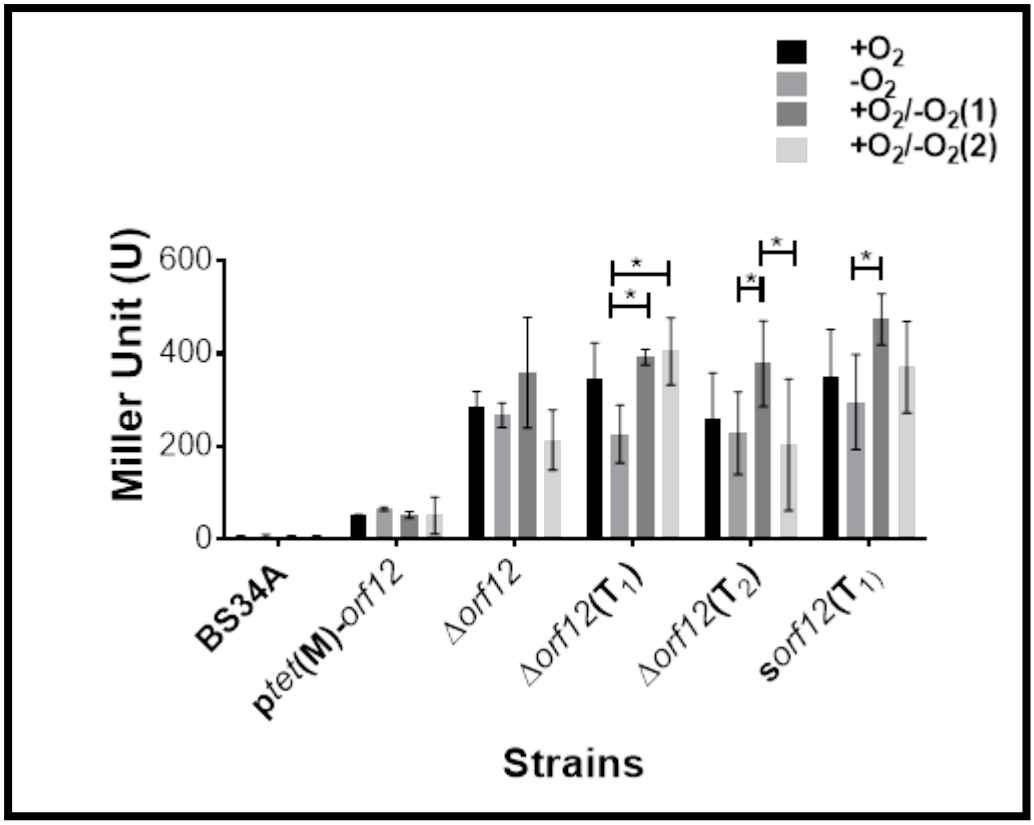

Figure 5.8: β-glucuronidase activity from the transcriptional read-through of *orf12* and its mutants in response to aerobic and anaerobic growth

The activity of *gusA* in strains harboring p*tet(M)-orf12* was low in comparison to the strains that harbored the *gusA* reporter with different mutations in the *orf12* terminator sequence (*Δorf12, Δorf12*(T_1), *Δorf12*(T_2) and *Δsorf12*(T_1)). The black bars represents growth with oxygen ($+O_2$), the gray bars represents growth without oxygen ($-O_2$), the dark gray bars represents growth with oxygen transferred to anaerobic conditions ($+O_2/-O_2(1)$) and the light gray bars represents growth with oxygen transferred to anaerobic and processed in anaerobic condition never exposed to oxygen($+O_2/-O_2(2)$). Error bars indicate the standard deviation of three biological triplicates. The data analysis was performed using two-way ANOVA followed by Tukey test. The (*) indicates a statistically significance of P=0.01 to 0.05.

5.4 Discussion

B. subtilis is a soil organism in which the oxygen level in its environment fluctuates (Priest, 1993). Therefore, a regulatory mechanism for metabolic adaptation to this change using an alternative electron acceptor is required. *B. subtilis* is capable of anaerobic respiration in the presence of nitrate as an

electron acceptor or via fermentation in the presence of pyruvate (Nakano et al., 1997, Cruz Ramos et al., 2000). FNR is a regulatory protein that induces expression of the genes required for anaerobic metabolic adaption in the presence of nitrate in an environment with low oxygen levels (Glaser et al., 1995, Hoffmann et al., 1995, Cruz Ramos et al., 1995, Kiley and Beinert, 1998). Expression of *fnr* is regulated anaerobically by a two-component signal transduction system, ResDE (**Figure 5.1**) (Nakano et al., 1996, Sun et al., 1996, Ye et al., 2000, Nakano and Zhu, 2001, Nakano, 2002, Geng et al., 2007). The FNR-binding region carries a highly conserved sequence, TGTGA-N_6-TCACA, which is centred -41.5 bp and -40.5 bp upstream of the transcriptional start point (Cruz Ramos et al., 1995). In Chapter 4, the copy numbers of circular Tn*916* in *B. subtilis* in the presence of *E. faecalis* was significantly 10-fold greater in an anaerobic environment. It was hypothesised that Tn*916* transfer in anaerobic conditions is partly regulated by the FNR regulatory system in addition to the recipient effect. Due to insufficient time, this chapter has investigated only the effect of FNR (no recipient) on Tn*916* transcriptional regulation in different conditions.

In silico screening of Tn*916* identified several putative FNR-binding motifs present on Tn*916* (**Figure 5.4 B**). Since the proposed Tn*916* regulatory network starts from the P*tet*(M) promoter in the presence of tetracycline (Celli and Trieu-Cuot, 1998, Su et al., 1992), it was assumed that the anaerobic regulatory protein (FNR) could play a role in the transcriptional regulation of Tn*916.* Therefore, the putative FNR-binding motif on the *orf12* large terminator (T_1) of Tn*916* investigated.

The transcription of *fnr* in *B. subtilis* BS34A under different atmospheric conditions was detected via RT-PCR. The extracted RNA samples were reverse transcribed, creating the cDNA, which was then amplified with standard PCR and visualised by gel electrophoresis, resulting in a 240 bp product (**Figure 5.6 B**). The vegetative gene (*veg)* encodes a small conserved protein, which is transcribed at the same level under different growth conditions and has been used widely as a control in transcriptional investigation mechanism in *Bacillus* (Ollington and Losick, 1981, Gilman and Chamberlin, 1983, Le Grice et al., 1986, Lei et al., 2013). Therefore, *veg* have been used as a positive control in the RT-PCR showing a product size 180bp on the gel (**Figure 5.6 B**). FNR was expressed under both tested conditions (aerobic and anaerobic). This result can be explained by the fact that *fnr* is expressed in both conditions, but the activity is based on the availability of oxygen (Cruz Ramos et al., 1995). The oxygen level is sensed by a centre iron (Fe) surrounded by three cysteine residues in the C-terminal (DNA binding site) (Reents et al., 2006a). In the presence of oxygen, the protein-binding affinity is altered (structure changed) via immediate degradation of the [4Fe-4S]$^{+2}$ clusters to [2Fe-2S]$^{+2}$ after oxygen binds to the iron, causing dissociation of FNR into its subunit (weak DNA binding) (**Figure 5.2**). In the absence of oxygen, [4Fe-4S]$^{+2}$ binds to DNA to induce the expression of genes for the proteins that are essential for anaerobic metabolism, and it represses the genes involved in aerobic metabolism (**Figure 5.2**) (Khoroshilova et al., 1997).

The role of transcriptional activity of the putative FNR-binding site present on the *orf12* terminator of Tn*916* was determined under aerobic and anaerobic conditions. As shown in **Figure 5.8**, the enzyme activity was low for the

bacterial strains with p*tet*(M)-*orf12* grown in either conditions. This outcome can be explained by the fact that transcription was terminated at the terminator of *orf12* (Su et al., 1992). The expression level in all the strains with a mutation on the *orf12* terminators (Δ*orf12,* Δ*orf12*(T_1), Δ*orf12*(T_2) and s*orf12*(T_1)) were high compared to the control p*tet*(M)-*orf12.* The *gusA* expression was high because the terminator sequence was disturbed thus, the transcription was not terminated (**Figure 5.8**) (Su et al., 1992).

In addition, comparing the activity level of all the mutants of the *orf12* terminator (Δ*orf12,* Δ*orf12*(T_1), Δ*orf12*(T_2) and s*orf12*(T_1)) under aerobic or anaerobic conditions to the activity of the mutants that transferred from one condition to another (aerobic to anaerobic($+O_2/-O_2$)), the activity of *gusA* was low in both conditions (aerobic and anaerobic). This is explained by the low amount of charged tRNA, as a result of the high protein synthesis in the aerobic condition. In addition, in the anaerobic condition, *B. subtilis* growth would be low due to the limited amount of oxygen and the low levels of ATP generated, would lead to slow protein synthesis (Volker et al., 1994). However, when the *Δorf12, Δorf12*(T_1), *Δorf12*(T_2) and *Δsorf12*(T_1) strains transferred from the aerobic condition to the anaerobic condition, a significant increase in the transcriptional activity of *gusA* was observed. This suggests, that the promoter (p*tet*(M)) was condition-dependent, as induced by the alternative sigma factor σ^B, which redirect RNAP to transcribe the regulatory genes of Tn*916* such as *orf12* and *tet*(M) from p*tet*(M) in response to environmental stress and/or indirectly by FNR binding (Hecker and Volker, 1998, Hecker and Volker, 2001). The σ^B structural genes is possess an 8-gene cluster that are transcribe from an upstream σ^A dependent promoter

(housekeeping) (**Figure 5.9**) (Kalman et al., 1990). It is postulated that, during growth and when transferring from one condition to another (aerobic to anaerobic(+O_2/-O_2)), the protein synthesis increased rapidly using the accumulated tRNA (present from the previous aerobic culture) in response to the new growth condition (limited levels of oxygen). It has been proposed that Tn*916* transcription is increased by any event that increases the level of charged tRNA, such as exposure to biocides (Seier-Petersen et al., 2014) and tetracycline (Showsh and Andrews, 1992, Roberts and Mullany, 2009). This suggests that the element senses the stresses on the host, and causes its excision and transfer to a new host.

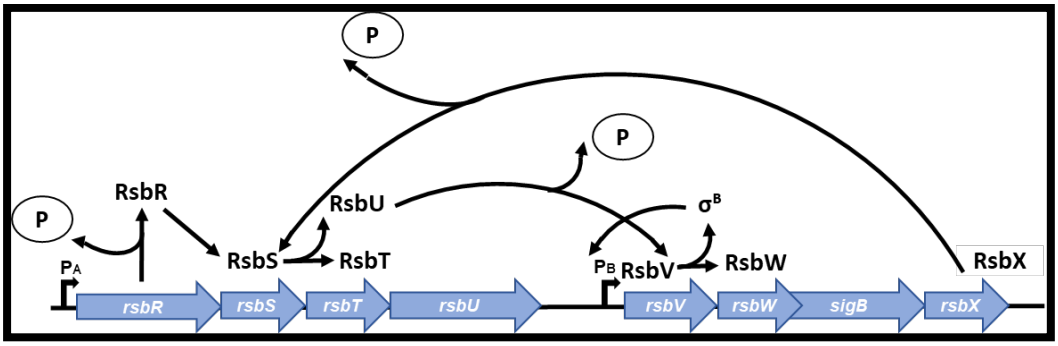

Figure 5.9: Schematic model for the alternative σ^B regulation in response to environmental stress

The σ^B regulon consists of an eight-gene cluster that includes the rsbV, rsbW, sigB and rsbX operon, which is regulated by a σ^B dependent promoter, and an upstream rsbR, rsbS, rsbT and rsbU operon, which is transcribed from a σ^A dependent promoter. In the presence of environmental or energy stress, RsbX dephosphorylates RsbS or RsbR respectively allowing RsbS to interact with RsbT and/or RsbR resulting in a release of RsbU. Free RsbU dephosphorylate RsbV, which interact with RsbW resulting in a release of σ^B. Subsequently released σ^B start to autoregulate from σ^B dependent promoter.

FNR protein degrades immediately after exposure to oxygen (Bauer et al., 2009) , and, because samples were prepared aerobically prior to measuring the cell growth (OD_{600}), it was unclear whether the enzyme activity in (+O_2/-O_2)(1)) was due to FNR binding or due to changes in the conditions. Therefore, another enzymatic assay was conducted. After changing the incubation condition (aerobic to anaerobic ((+O_2/-O_2)(2)), the samples were prepared for measuring the OD_{600} and covered in the anaerobic chamber until the freezing step in which the cells lysed. The enzyme activity of *gusA* for all the strains p*tet*(M)-*orf12, Δorf12, Δorf12*(T_1), *Δorf12*(T_2) and *Δsorf12*(T_1) was lower in (+O_2/-O_2)(2) than in (+O_2/-O_2)(1). This could be due to the fact that the samples were kept for a longer period in anaerobic conditions after being transferred from air, causing a reduction in protein synthesis similar to that observed in the anaerobic condition (-O_2). It is also possible that the observed low activity was because of cell death and degradation of the GusA protein. A further investigation is necessary to observe the effect of these mutations on *orf12* terminator on Tn*916* transfer in anaerobic conditions (e.g. doing a mutation on the *orf12* large terminator sequence of Tn*916* in the host genome).

In summary, an analysis of the transcriptional response of a putative FNR-binding site present on the *orf12* terminator of Tn*916* in *B. subtilis* showed that the exposure of cells to either an aerobic condition or an anaerobic condition had no effect on transcription of Tn*916* (low expression of β-glucuronidase). Also observed that, the transcription level was higher in strains with terminator mutations, suggesting that transcription was not

terminated and continued through, reaching the downstream genes. Moreover, it was found that environmental stress affected the expression from the promoter P*tet*(M) of Tn*916*. This evolution may benefit the element in sensing the stresses on the host and lead to its transfer to a new host e.g. *E. faecalis* which will be investigated in the future work.

Chapter 6

Investigation of the transfer of Tn*916* in different conditions after introducing a mutation on the large terminator

Chapter 6 Investigation of the transfer of Tn*916* in different conditions after introducing a mutation on the large terminator

6.1 Introduction

The horizontal transfer of MGEs and their associated resistance genes is a major concern in spreading antibiotic resistance (Canchaya et al., 2004, Brüssow et al., 2004, Jasni et al., 2010).The conjugative element Tn*916* confers resistance to tetracycline by a *tet*(M) determinant that encodes a ribosomal protection protein (Su et al., 1992, Flannagan et al., 1994). Usually, Tn*916* is stable when integrated in the host genome, but under certain stimulating conditions, it can excise, forming a covalently closed circle, and transfers to a new host (Scott et al., 1988, Caparon and Scott, 1989, Rice and Carias, 1994, Manganelli et al., 1997). Enhancement of the conjugal transfer of Tn*916* has been demonstrated in the presence of tetracycline and biocides (Showsh and Andrews, 1992, Seier-Petersen et al., 2014). In addition, a recent investigation has reported the transfer of Tn*916* after treatment with macrolides, lincosamides and streptogramin (MLS) groups, although Tn*916* does not encode resistance to these antibiotics (Scornec et al., 2017). Moreover, the conditions of Tn*916* transfer in soil, such as humidity, pH, nutrients and temperature, have been determined between environmental bacteria *B. subtilis* and *B. thuringiensis* subsp. *israelensis* and between *B. subtilis* and *E. faecalis* at frequencies of 2.1 x 10^{-5} and 2.3 x 10^{-6} transconjugants per donor, respectively (Haack et al., 1996).

The regulatory mechanism of Tn*916* involves transcriptional attenuation, which is controlled by an inverted repeat sequence within *orf12* (Su et al., 1992, Celli and Trieu-Cuot, 1998). The regulatory region is conserved in almost all Tn*916*-like elements, which indicates it is essential for the function and maintenance of the elements. In the absence of tetracycline, transcription initiating at p*tet*(M) is blocked by the terminator structure present on the *orf12* RNA upstream of *tet*(M). However, in the presence of tetracycline, the few ribosomes that are protected by the basal level of Tet(M), translate *orf12* RNA rapidly, by catching up with RNAP, and inhibiting the formation of a stem loop, allowing the transcription to pass to the downstream genes (**Figure 1.17**). This transcription from P*tet*(M) stops the translation of *orf9* by antisense *orf9* and thus lowers the Orf9 repression activity on P*orf7*. This results in a high transcription of *orf7*, *orf8* and downstream genes *xis* and *int*, and the transfer genes if Tn9*16* is in a circular form (Su et al., 1992, Celli and Trieu-Cuot, 1998). However, the regulation and behaviour of Tn*916* when exposed to a limited oxygen environment has not yet been investigated.

In the previous chapter 5, a significant increase was observed in the expression level of *gusA* in strains containing a mutation in the *orf12* terminator fused to the reporter, when transferred from aerobic to anaerobic conditions. This chapter aimed to investigate the effect of this mutation on Tn*916* transfer aerobically and anaerobically. In a previous study on the *orf12* terminator, a mutation had been made by an allelic replacement, but this mutational methodology might interrupt Tn*916* transcriptional regulation via introduction of a new resistance gene (Jasni, 2013). Therefore, this chapter, aimed to investigate the transfer of Tn*916* under different conditions (aerobic vs

anaerobic) after introducing a deletion mutation in the *orf12* large terminator of Tn*916* in the *B. subtilis* genome using a single-plasmid CRISPR-Cas9 system.

Clustered regularly interspaced short palindromic repeats (CRISPRs) and their CRISPR-associated (Cas) proteins represent a high-efficiency approach for genome editing and has been applied to *B. subtilis* (Altenbuchner, 2016, Westbrook et al., 2016, Burby and Simmons, 2017a, Burby and Simmons, 2017b, So et al., 2017). CRISPR is an adaptive immune response of prokaryotes which uses short sequences copied from invading mobile elements to direct Cas nucleases to the target DNA upstream of a protospacer adjacent motif (PAM) (Deveau et al., 2008, Mojica et al., 2009), leading to a double-strand break (DSB). This system can be used to select for mutants by using editing templates to replace the sequence targeted by the guide RNA (gRNA) allowing mutants to survive (**Figure 6.1**).

Recently, a simple CRISPR-Cas9 system for targeting specific sequences via a synthetic guide RNA (sgRNA), consisting of a protospacer, Cas9-binding hairpin (CBH) and transcriptional terminator, has been developed (Jinek et al., 2012) (**Figure 6.1**). The CRISPR-Cas9 system has been used to produce a marker-less deletion in *yvmC* (encodes cyclodipeptide synthesis) and *amyE* (encodes α-amylase synthesis) and point mutations in *trpC* (encodes enzyme catalyse tryptophan synthesis) in *B. subtilis* (Altenbuchner, 2016, Burby and Simmons, 2017a)

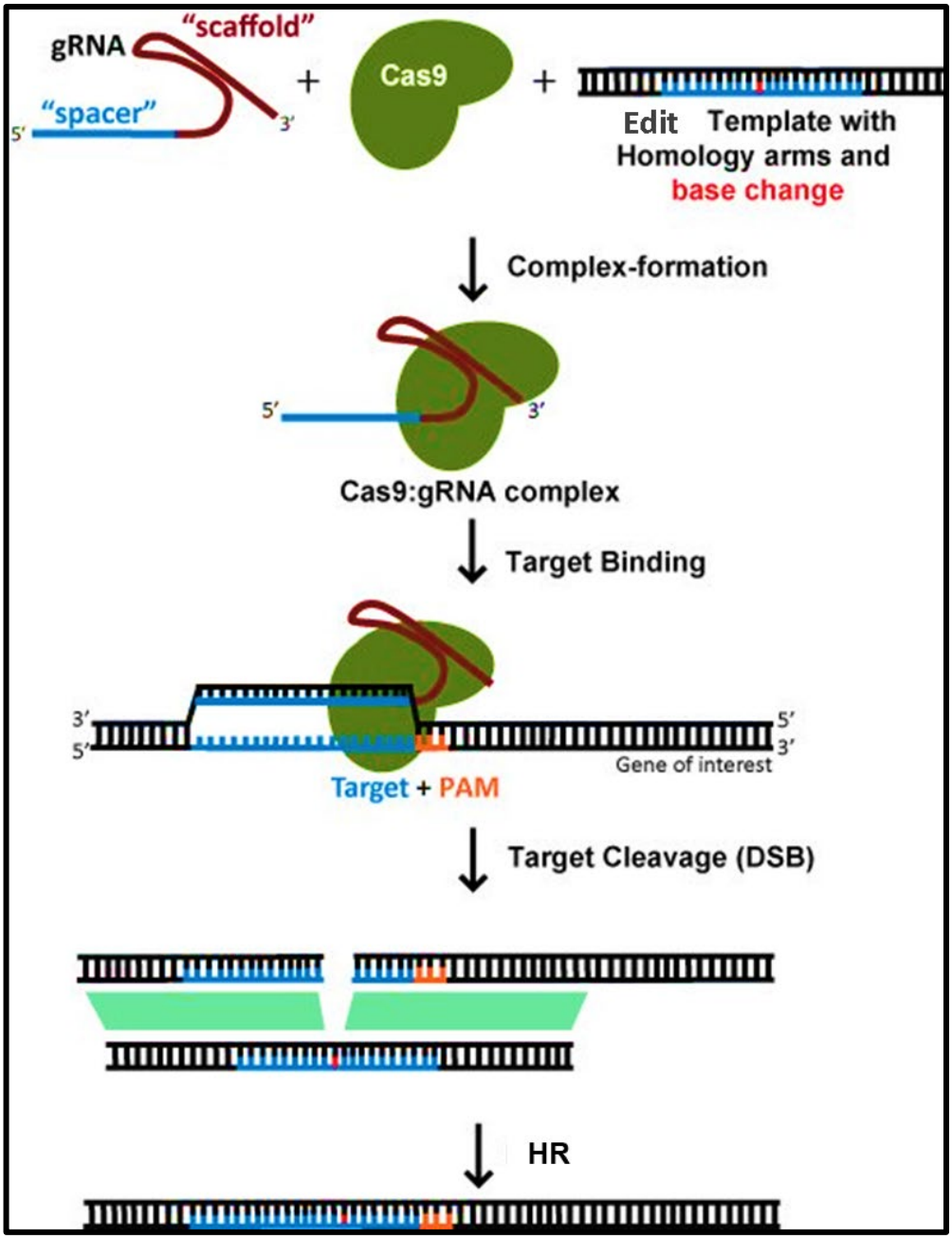

Figure 6.1: Crispr-Cas9 system

The guide RNA (gRNA), consists of a spacer sequence (20 bp) specific to the DNA target site and a scaffold sequence (tracrRNA) that interacts with Cas9. The Cas9:gRNA complex is directed via gRNA to the target site, and it generates a double-strand breakage (DSB) via Cas9. The cleavage site is repaired by introducing exogenous DNA (editing template) with the desired mutation by homologous recombination (HR). The PAM sequence (orange) indicates the protospacer-adjacent motif sequence. The target site with the desired mutation is represented in blue and red, respectively. Adapted from "How to design your gRNA for CRISPR Genome editinf," by J, Doench, 2017 (https://blog.addgene.org/how-to-design-your-grna-for-crispr-genome-editing).

6.2 Material and methods

6.2.1 Bacterial strains, plasmid and culture conditions

All bacterial strains and plasmids used were listed in Table 2.1. *E. coli* α-select silver was used as a host for DNA cloning and plasmid preparation. The pJOE8999 (7794bp) vector, given by Altenbuchner (2016) from the University of Stuttgart (Germany), was used for CRISPR-Cas9 gene editing. The *E. coli* α-select silver-bearing pJOE8999 was cultured on LB agar or LB broth containing 30 µg/ml of kanamycin (Sigma, UK), aerobically at 37 °C on a rotary shaker (200 rpm) for 16–18 h.

B. subtilis BS34A was the host for genome editing using pJOE8999 Ω sgRNA-HA. Transformants were isolated on LB agar containing 5 µg/ml kanamycin and incubated aerobically overnight at 37 °C.

In the filter mating experiment, *B. subtilis* BS34A and *B. subtilis* BS34AΔ*orf12*(T_1) (mutants with deletion in the *orf12* large terminator(T_1)) were cultured in 10 ml of BHI broth supplemented with 10 µg/ml tetracycline and used as donors. In addition, *S. oralis, S. pyogenes and E. faecalis* were cultured in 10 ml BHI broth and used as recipients.

6.2.2 In-silico analysis for PAM and gRNA on Tn*916* as a target sequence

The possible proto-spacer-adjacent motif (PAM = NGG) sequences on Tn*916* were identified using the online tool chopchop, (http://chopchop.cbu.uib.no/), including the possible gRNA (guide RNA) sequences (20bp) upstream of the PAM (Table 6.1). One of these sequences was chosen for cloning (highlighted in Table 6.1) with four overhanging nucleotides at the 5' end of each. The cloning strategy is illustrated in **Figure 6.2**.

Table 6.1: List of potential PAM and gRNA sequences located on Tn*916*.

The blue highlighted sequence indicates the region used for in pJOE8999 targeting the orf12 large terminator (T_1) located in Tn*916* and harboured by *B. subtilis* BS34A.

Target sequence	Genomic location	Exon	Strand	GC (%)	Self-complementarity	Off-targets				Efficiency
						0	1	2	3	
AAAAGGAGTTAATAAATATGCGG	seq:519	1	+	20	N/A	0	0	0	0	0.62
ACTTTACCGAATCTGAACAATGG	seq:1285	1	+	35	N/A	0	0	0	0	0.59
TTCATGCACTTAGGAAAATGGGG	seq:1120	1	+	35	N/A	0	0	0	0	0.58
AGAGGAATTACAATTCAGACAGG	seq:936	1	+	35	N/A	0	0	0	0	0.58
CGAAATTGTAATCAAACAGAAGG	seq:1232	1	+	30	N/A	0	0	0	0	0.57
TGATAAGAGTATTTATCACTGGG	seq:719	1	+	25	N/A	0	0	0	0	0.56
AAATCCTATTCACAATCGTAAGG	seq:262	1	+	30	N/A	0	0	0	0	0.56
AAATGGAATTGATTTATCAACGG	seq:1178	1	+	20	N/A	0	0	0	0	0.56
TTCATTATCAGTTTTAGATGGGG	seq:1043	1	+	25	N/A	0	0	0	0	0.56
CTTTCCTACTATGTGAATGACGG	seq:194	1	+	35	N/A	0	0	0	0	0.56
CTTTTAGAGAAATATATGTCCGG	seq:1335	1	+	25	N/A	0	0	0	0	0.55

6.2.3 Introducing gRNA and an editing template to the pJOE8999 plasmid

The construction of a CRISPR/Cas9 genome-editing plasmid using pJEO8999 was done in two cloning steps. The primers sgRNA-F and sgRNA-R were reverse complementary to each other with four overhanging nucleotides at the 5' ends of each (**Figure 6.2**). The protruding four nucleotides were complementary to the backbone of pJEO8999 when digested with BsaI. The sgRNA-F and sgRNA-R primers were annealed to each other by mixing 10 µl of each primer (10 µM) in one tube and incubating at 95 °C for 5 min and cooling slowly by leaving on the bench until it reaches room temperature for 25 min. The annealed, double-stranded primers were phosphorylated using the T4 polynucleotide kinase kit (NEB, UK), prepared as follows: 1 µM of the annealed primers,1 µl T4 PNK (10 units/µl), 5 µl of T4 PNK reaction buffer, 5 µl ATP (10 mM) and H_2O up to 50 µl. Then, the reaction was incubated at 37 ºC. T4 PNK was inactivated by heat at 65 ºC for 20 min (**Figure 6.2**). Phosphorylated double-strand primers were cloned into pJEO8999 at site BsaI, replacing *lacZ* α fragment, and resulting in the pJOE8999 Ω sgRNA vector (**Figure 6.3**). Plasmids were transformed into *E. coli* α-select silver, cultured on LB agar supplemented with 30 µg/ml kanamycin, purified and sequenced. Sequencing was performed by Genewize (Takeley, UK) using TS-F1, TS-F2 and TS-F3 primers (Table 2.2).

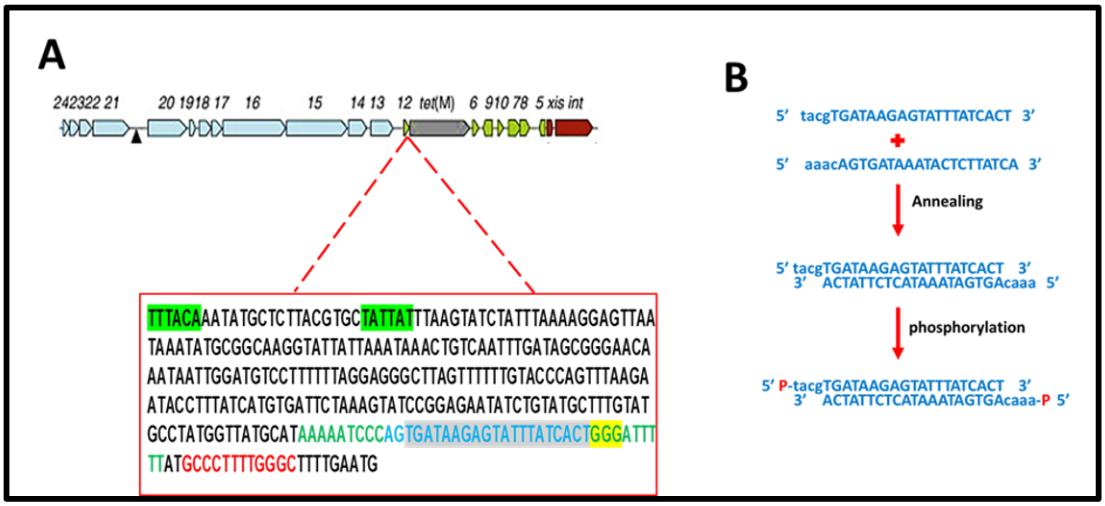

Figure 6.2: Proto-spacer location and preparation

A) The proto-spacer adjacent motif (PAM) is highlighted yellow and the proto-spacer is highlighted grey on the Tn*916* sequence. The *tet*(M) promoter is highlighted green. The green and red nucleotides represent the large and small terminators on Tn*916*, respectively. **B)** Oligonucleotides with four overhang nucleotides were annealed and phosphorylated to generate the dsDNA proto-spacer to ligate into pJEO8999.

The editing region was prepared through the amplification of *orf12* containing a deletion of the target sequence using the HA-xbal-F and HA-xbal-R primers (**Figure 6.3**). The PCR product was cloned into pJOE8999 Ω sgRNA after digested with Xbal, forming pJOE8999 Ω sgRNA-HA (**Figure 6.3**). The plasmid was transformed into *E. coli* α-select silver, isolated on LB supplemented with 30 μg/ml kanamycin, purified and sequenced using T7 primer.

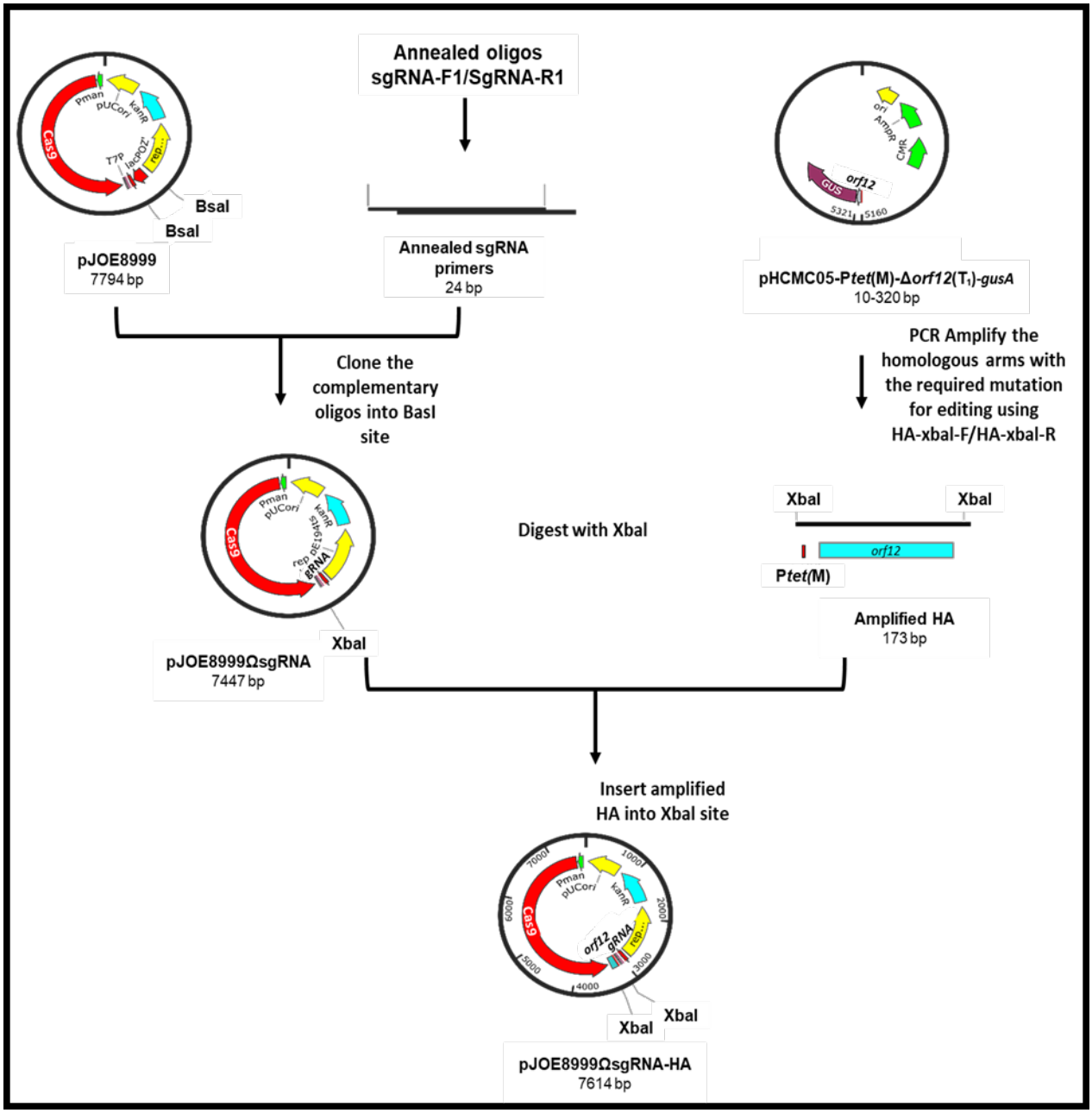

Figure 6.3: Construction of the pJOE8999 Ω sgRNA-HA constructs

The 20-bp dsDNA proto-spacer replaced *lacZ* α in pJOE8999 in the BsaI site, creating pJOE8999 Ω sgRNA. The editing region was amplified from pHCMC05-*ptet*(M)-Δ*orf12*(T_1)-*gusA* using primers with an XbaI restriction enzyme recognition site (HA-xbaI-F and HA-xbaI-R) and cloned into pJOE8999 Ω sgRNA after digested with XbaI, forming pJOE8999 Ω sgRNA-HA. The construct was drawn using SnapGene version 3.2.1 and modified on PowerPoint.

6.2.4 Transformation of the pJOE8999 Ω sgRNA-HA vector into BS34A

To introduce a mutation on the Tn*916 orf12* large terminator, pJOE8999 Ω sgRNA-HA was transformed into *B. subtilis* BS34A and isolated on LB with 5 µg/ml kanamycin. The protocol for transformation of *B. subtilis* was as described in section 2.2.14.2.

6.2.5 Inducing and removing pJOE8999 Ω sgRNA-HA from the cell

Colonies of *B. subtilis* BS34A transformants were re-inoculated onto LB agar supplemented with 5 µg/ml kanamycin and 0.2% mannose (Oxoid, UK) for Cas9 induction. After 2 days of incubation at 30 °C, colonies were inoculated on LB agar without antibiotics and incubated for 14 h at 50 °C for plasmid loss. The next day, colonies were streaked on LB agar and incubated at 42 °C for 16 h. Isolates were screened for plasmid loss by re-streaking on LB agar with and without kanamycin. Kanamycin-sensitive isolates were screened by PCR followed by Sanger sequencing to verify that the deletion mutation was present.

6.2.6 Conjugation experiments (filter mating) and calculating the frequency of conjugal transfer

Two conjugation experiments were carried out in parallel. One experiment was incubated aerobically and the other anaerobically using *B. subtilis* BS34A::Δo*rf12*(T_1) as the donor and *S. oralis*, *S. pyogenes*. and *E. faecalis* as

recipients (Table 2.1). In addition, wild-type *B. subtilis* BS34A (donor) was used as a positive control anaerobically and as a negative control aerobically. The filter mating protocol and the measurement of the frequency of transfer were performed as described in sections 3.2.

Data were statistically analysed using the GraphPad Prism 7 (GraphPad Software, Inc., USA).

6.2.7 Identifying and screening transconjugants

All the recipients, the donors and the transconjugants were identified by amplifying and sequencing the 16S rRNA gene using universal primers (27F/1492R) and by screening for *orf12* of Tn*916* by PCR using two pairs of primers, one that binds to the deleted region (*orf12*-F/M12-R) and another flanking the deleted region (*orf12*-F/*orf12*-R) (**Figure 6.4**). All the primers used are listed in Table 2.2. Sequence analysis was performed as described previously in section 3.2.4

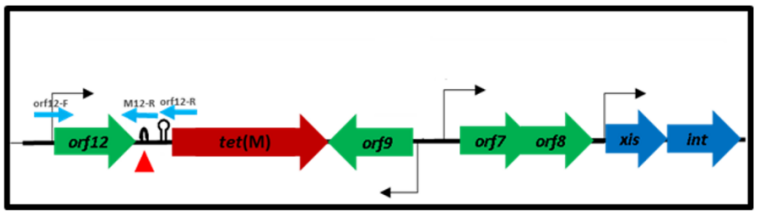

Figure 6.4: A schematic representation of primers binding sites on Tn*916*

The small blue arrows show the binding site of each primer. *orf12*-F with *orf12*-R design to amplify *orf12*. Primer M12-R designed to bind to the deleted region (*orf12* large terminator pointed with red triangle). ORFs represented as arrows: green arrows represent the transcriptional regulatory region, the red arrow represents the accessory gene (*tet*(M)) and blue arrows represent the site-specific recombinase. The black thin small arrows represent the promoters.

6.3 Results

6.3.1 Verification of *B. subtilis* BS34A::Δo*rf12* (T_1) isolates

Sequences of isolates, unable to grow on LB agar with 5 µg/ml kanamycin (which had lost the plasmid), showed the desired deletion compared with the wild-type (**Figure 6.5**).

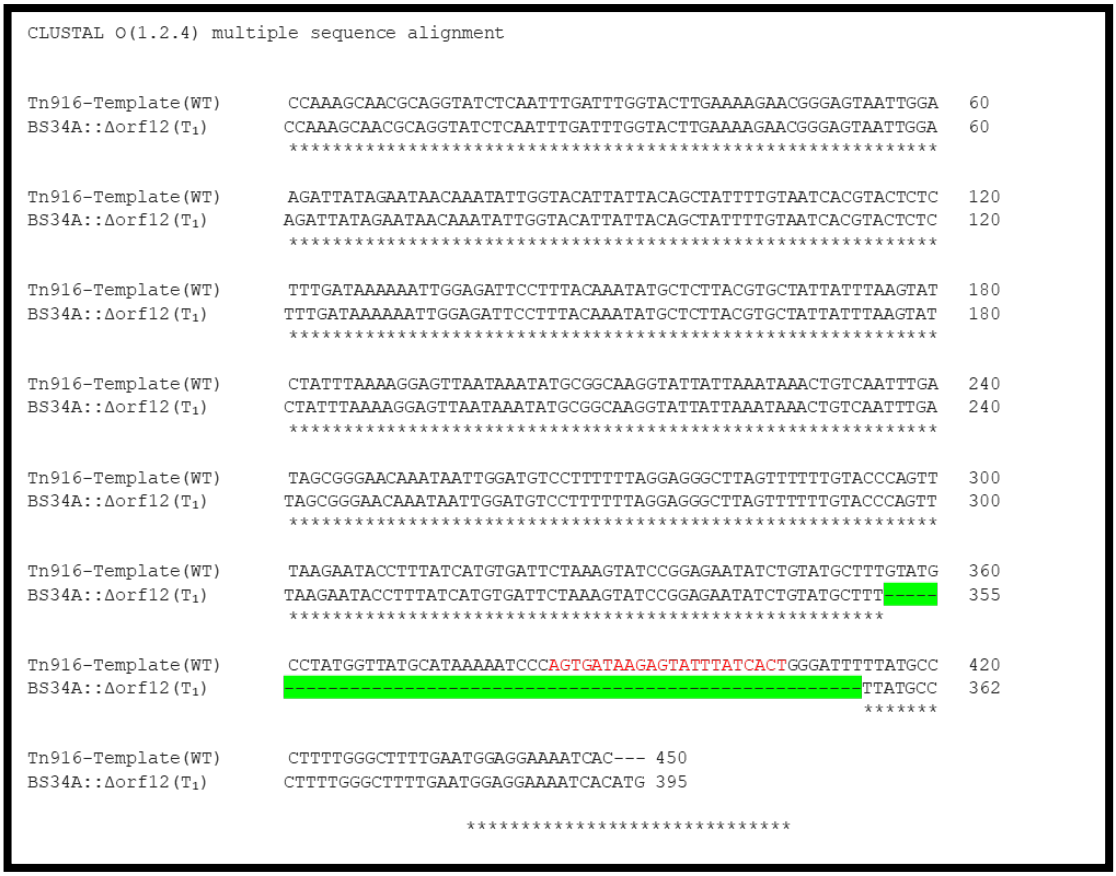

Figure 6.5: Sequence alignments of *orf12* in *B. subtilis* BS34A::Δ*orf12* (T_1) with Tn*916* wild-type

The red nucleotides represent the target site located on *orf12* terminator (T_1) in wild-type Tn*916*. The deletion region is highlighted in green in BS34A::Δ*orf12* (T_1) Tn*916*.

6.3.2 Tn*916* transfer

Mating of BS34A::Δo*rf12* (T_1) (donor) with recipients (*S. oralis, S. pyogenes and E. faecalis*) in aerobic conditions resulted in transconjugants with a frequency ranging from 1.8 x 10^{-9} to 1.5 x 10^{-8} (**Figure 6.6**) (Appendix 13). However, the wild-type Tn*916* did not transfer in this condition (aerobic).

In contrast, no transconjugants were obtained when *S. oralis* and *E. faecalis* were mated with BS34A::Δo*rf12* (T_1) (donor) anaerobically. However, when mated with *S. pyogenes,* transconjugants were isolated with a frequency 6.1 x 10^{-10} (**Figure 6.6**) (Appendix 13). In addition, the wild-type *B. subtilis* BS34A showed a transfer of Tn*916* in the anaerobic condition to all the recipients.

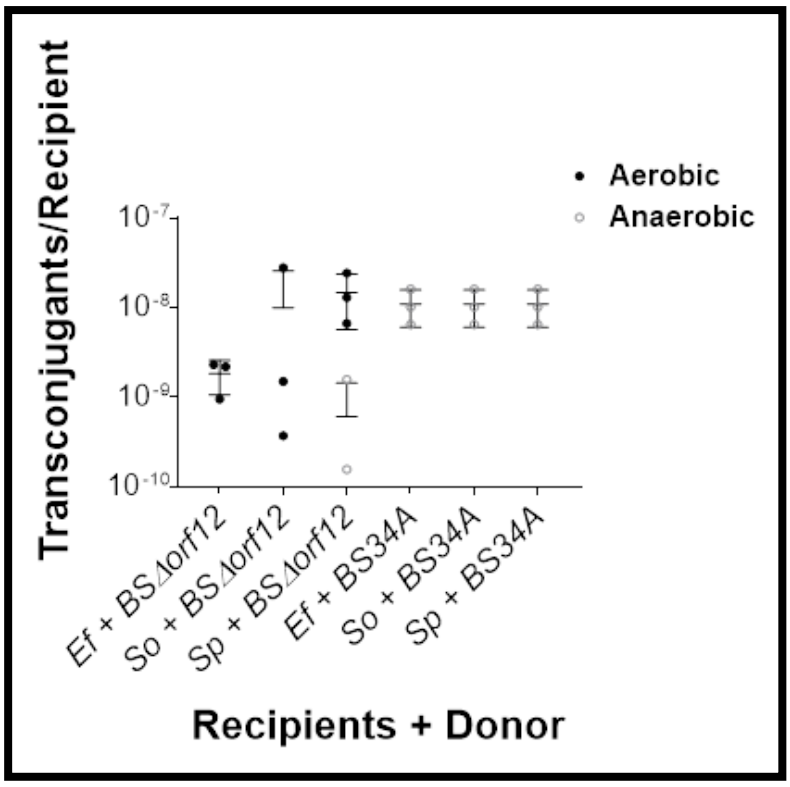

Figure 6.6: Effect of deletion *orf12* terminator on the Tn*916* conjugative transposition frequency between the recipient and the donor in different conditions compared with the wild-type

Mating between *B. subtilis* BS34A::Δo*rf12* (T_1) or BS34A with *E. faecalis* (Ef)*, S. oralis* (So) and *S. pyogenes* (Sp) plotted against the frequency of Tn*916* transfer. The conjugal transfer in aerobic condition is marked as black dots and in anaerobic condition as white dots. The data was analysed by two-way ANOVA followed by Tukey test and the error bars correspond to the standard deviation for three biological repeats. Most of the data clustered within the same group except a single reading with *S. pyogenes* (in anaerobic) and S. *oralis* (in aerobic) were outlined from the rest of the data in the same group (Appendix 13).

The conjugation frequency of *S. pyogenes* with BS34A::Δo*rf12*(T_1) observed anaerobically was non significantly (p=0.57) decreased 20-fold compared with the conjugation frequency with the wild-type BS34A (**Figure 6.6**) (Appendix 13).

6.3.3 All transconjugants and BS34A::Δo*rf12* (T_1) contain Tn*916* Δo*rf12* (T_1)

PCR analysis showed products sized 396bp for the mutant *orf12* terminators of Tn*916* in all the transconjugants (*S. oralis*, *S. pyogenes* and *E. faecalis*), which was the same as in the donor BS34A::Δo*rf12* (T_1) compared to 440bp in the wild type BS34A donor (**Figure 6.7**).

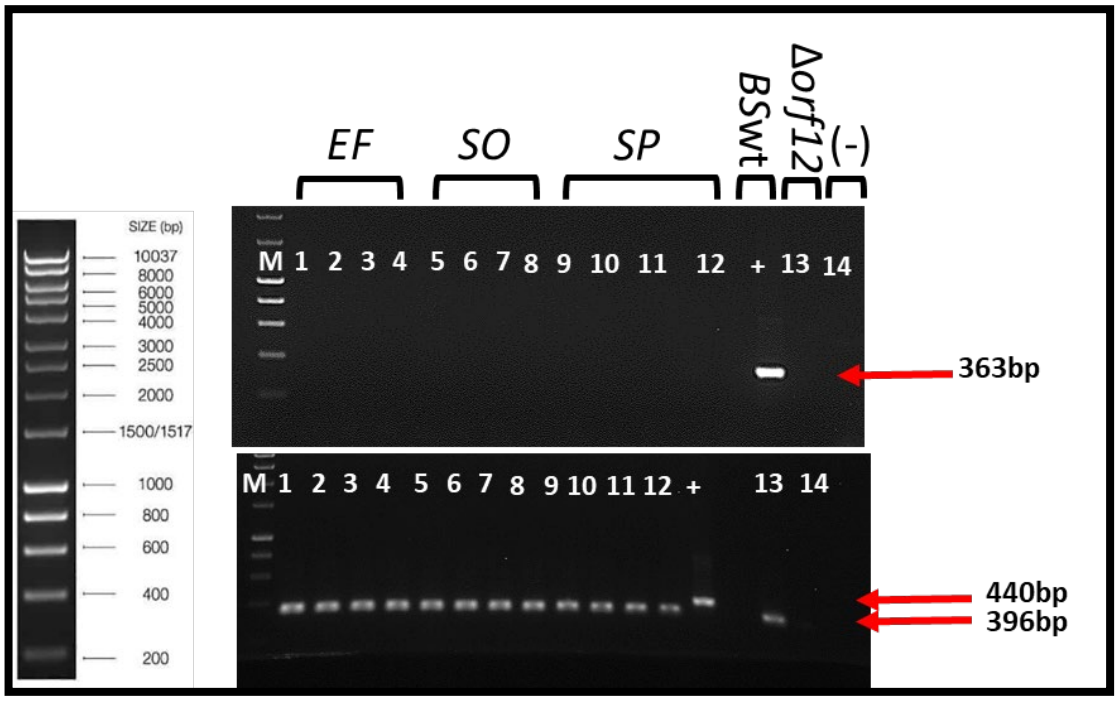

Figure 6.7: Gel electrophoresis image of the *orf12* PCR products

Lane M: 1 kb HyperLader marker; **Lanes 1–4**: transconjugant of *E. faecalis*; **Lanes 5–8**: transconjugant of *S. oralis*; **Lanes 9–12**: transconjugant of *S. pyogenes;* **Lane 13**: BS34A::Δo*rf12* (T_1) (donor); **Lane +**: *B. subtilis* BS34A (wild-type donor) and **Lane 14**: negative control. The top image showed no PCR products for all transconjugants and BS34A::Δo*rf12* (T_1) when one of the primers was homologous to the sequence that had been deleted, except for wild-type BS34A, in which a 363-bp product was detected. The bottom image illustrates amplified products sized 396 bp for all transconjugants and BS34A::Δo*rf12* (T_1) and 440 bp for wild-type BS34A when amplified with primers flanking the deleted region.

Sequence analysis for the amplified *orf12* of all the transconjugants and the *B. subtilis* BS34A::Δo*rf12* (T_1) donor aligned and demonstrated that the deletion exists in all transconjugants (**Figure 6.8**). In addition, the sequences of the 16S rRNA gene for all the transconjugants were identical to the recipient's 16S rRNA sequence, confirming that Tn*916* had transferred to the recipients.

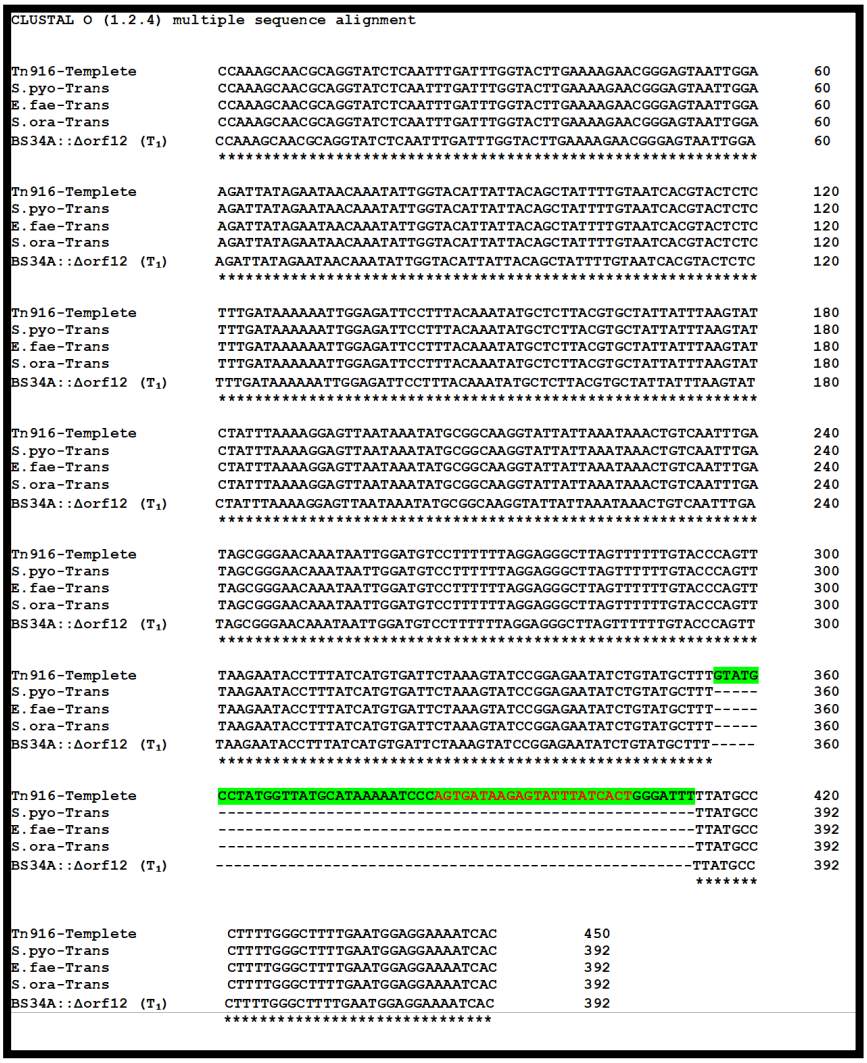

Figure 6.8: Sequence alignments of *orf12* in transconjugants (*S. oralis*, *S. pyogenes* and *E. faecalis*) and *B. subtilis* BS34A::Δ*orf12* (T_1) with Tn*916* wild-type

The target site located on *orf12* terminator (T_1) represented in red nucleotides and the deletion site is highlighted green.

6.4 Discussion

It had been thought that Tn*916* transfer was only induced by tetracycline (Su et al., 1992, Showsh and Andrews, 1992). However, it has also been found to be induced by subinhibitory concentrations of biocides, such as ethanol, hydrogen peroxide, chlorhexidine digluconate and sodium hypochlorite. Another investigation demonstrated that Tn*916* transposition occurred in the presence of other classes of antibiotics belonging to the MLS groups (macrolides, lincosamides and streptogramin) (Scornec et al., 2017). It has been proposed that any cellular condition that inhibits the translation process and increases the charged tRNA pool, stimulates the Tn*916* transcription mechanism (Roberts and Mullany, 2009).

In Chapter 5, the expression activity of *orf12* large terminator in different conditions have been measured using the *gusA* reporter system. Here, investigated the effect of deleting the large terminator on Tn*916* transfer in different conditions using an effective plasmid CRISPR-Cas9 system (pJOE8999) for editing *B. subtilis* BS34A genome. This system required two recombination stimulation events, which were integration gRNA and a cloning editing template. The gRNA was transcribed under a semisynthetic improved promoter, P*vanABK* of *Corynebacterium glutamicum* (Morabbi Heravi et al., 2015, Altenbuchner, 2016). The second step was cloning the editing template with the required mutation, after being amplified using pHCMC05-*ptet*(M)-Δ*orf12*(T_1) as a template.

Subsequently, pJOE8999 Ω sgRNA-HA was transferred into *B. subtilis* BS34A and induced via 0.2% mannose present in the LB agar. ManR (a regulatory gene) is present in *B. subtilis* which in the presence of mannose, activates the promoter P^{manp} (Sun and Altenbuchner, 2010), leading to Cas9 induction. The expressed Cas9 binds to the gRNA to guide to the target site present on the chromosome (Mojica et al., 2009), causing a double-strand cleavage, which is repaired by integrating the editing template with the required mutation. For a marker-less mutation, a temperature-sensitive replicon from $pE194^{ts}$ present on the plasmid allowed for successful removal by re-inoculating colonies on LB agar without antibiotics and incubation at 50 °C (Arnaud et al., 2004).

Filter mating for wild-type BS34A or BS34A::Δo*rf12* (T_1) with *S. oralis, S. pyogenes* and *E. faecalis* was conducted aerobically and anaerobically. In aerobic conditions, the wild-type Tn*916* did not transfer to any recipients, which was the same as observed previously in Chapter 3. However, the transfer of Tn*916* Δo*rf12* (T_1) occurred under aerobic conditions with frequencies of 9.9×10^{-9}, 1.5×10^{-8} and 1.8×10^{-9} transconjugants per recipient to *S. oralis, S. pyogenes* and *E. faecalis*, respectively. To clarify, it was hypothesised that due to the deletion of the o*rf12* large terminator Δo*rf12* (T_1) transcription read-through, reaching the downstream genes and inducing Tn*916* transposition (**Figure 6.9**).

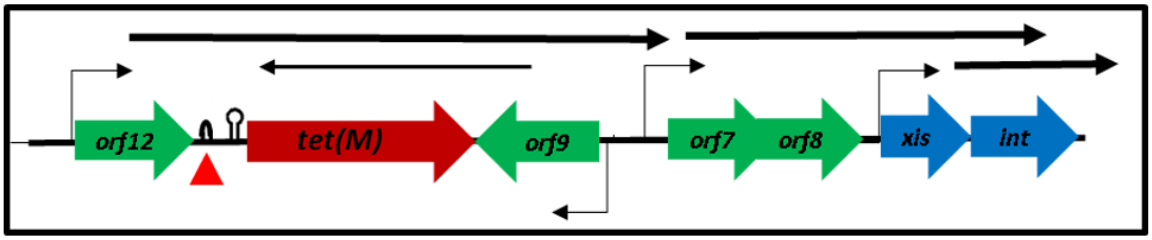

Figure 6.9: Regulatory transcription of *Δorf12* (T_1) on Tn*916* transposition

ORFs represented by arrows: green arrows represent the transcriptional regulatory region, the red arrow represents the accessory gene (*tet*(M)) and blue arrows represent the site-specific recombinase, *int* and the excisionasse *xis*. Thin small arrows symbolise promoters. Thick black arrows indicate transcript direction. Transcripts started from the p*tet*(M) and with deletion of *orf12* terminator (pointed with red tringle) transcription from *orf12* read-through, reaching *orf7* and *orf8*. Transcription from p*tet*(M) repress the activity of Orf9 antisense. Thus, p*orf7* activity is de-repressed and expressed by the overexpressed Orf7 and Orf8, leading to increased transcription of the downstream genes and P*xis* causing transposition.

In anaerobic conditions, *B. subtilis* containing wild-type Tn*916*, transconjugants were isolated from all (as observed in chapter 3). In contrast, mating with *B. subtilis* containing Tn*916*Δo*rf12* (T_1), resulted in transconjugants in *S. pyogenes* at frequency 20-fold lower than wild-type, and no transconjugants were obtained when *E. faecalis* and *S. oralis* were recipients. This maybe because the transposon was unstable due to absence of the terminator, leading to unregulated expressed of integrase (*int*) and excisionase (*xis*) which stops the transposon stably integrating into the genome.

The sequencing result of BS34A::Δo*rf12* (T_1) verified the existence of the deletion in the target sequence (*orf12* terminator) in the *B. subtilis* BS34A chromosome after removal of the plasmid. Moreover, the transconjugant sequencing results showed that the deletion sequence on the *orf12* large

terminator was present in all transconjugants after Tn*916*Δo*rf12* (T_1) transferred into the recipient.

In conclusion, the investigation of the Tn*916* transfer in different conditions after introducing a mutation in the *orf12* terminator in the *B. subtilis* genome using a single-plasmid CRISPR-Cas9 system has shown for the first time in this work, a transfer of Tn*916* from *B. subtilis* into *Streptococcus* and *Enterococcus* under aerobic conditions. In contrast, under anaerobic conditions, no transfer was observed, except for *S. pyogenes* with a 20-fold lower transfer frequency compared with the wild-type.

Chapter 7

General conclusions and scope for future work

Chapter 7 General conclusions and scope for future work

The current study was performed to investigate the transfer of the conjugative transposon Tn*916* in response to different environmental conditions. As the human oral cavity contains a multispecies community and as it is always exposed to multiple stressors, such as food, drink and oxygen availability, it is important to investigate the spread of antibiotic resistance genes (ARGs) in the oral cavity.

In the first experimental chapter, the effect of different possible environmental conditions in the oral cavity, such as the presence or absence of oxygen, on Tn*916* transfer was evaluated between an environmental bacteria *B. subtilis* as a donor and six oral *Streptococcus* spp. (*S. oralis, S. pyogenes, S. salivarius, S. mutans, S. sanguinis* and *S. parasanguinis*) and a gastrointestinal bacteria, *E. faecalis* as recipients. Tn*916* had the ability to transfer into *S. oralis, S. pyogenes* and *E. faecalis* within 1 min at frequencies ranging from 10^{-9} to 10^{-7} transconjugants per recipient, but only anaerobically. In contrast, no transfer was observed in air. In addition, the research demonstrated the transfer of Tn*916* in broth within 1 min and 5 min. The transfer of Tn*916* in a short time prior to culturing on selective media may be the result of competition between the parents and transconjugants. Therefore, a competitive test is required in the future to determine the compatibility of both transconjugants and parent cells in broth within different incubation periods. This will be achieved by culturing four separate BHI broth tubes containing a recipient, transconjugants, a 1:1 mix of transconjugants with a recipient and a 1:1 mix of a donor with a recipient separately and incubating aerobically and

anaerobically for different periods (e.g. 1 min, 5 min, 10 min, 15 min, 20 min, 25 min, 30 min and 24 h). The dilutions of the cultures are spread on BHI agar with and without tetracycline and incubated aerobically and anaerobically. This would show if the parent cells out compete the transconjugants.

In Chapter 4, the copy number of circular Tn*916* was compared between *B. subtilis* grown aerobically and anaerobically. In both conditions, the copy numbers were the same. This was followed by a comparison of the copy numbers of circular Tn*916* after mating with the recipient (*E. faecalis* JH2-2), resulting in a significant 10-fold increase in the circular copy numbers anaerobically. It would be useful if more information about gene regulation in Tn*916* underpinning these observations could be obtained. This could be done by adding *E. faecalis* filtrate to *B. subtilis* containing Tn*916*, and examining the effect on the circular copy numbers of Tn*916* aerobically and anaerobically. This will be achieved by culturing three separate BHI broth tubes containing a recipient (*S. oralis, S. pyogenes* and *E. faecalis*) separately and incubating aerobically with 5% CO_2. The filtrate of the cultures are mixed with *B. subtilis* harbouring Tn*916* prior to incubation aerobically or anaerobically. After 16-18 h incubation, a qPCR run using *B. subtilis* DNA comparing the circular copy number of Tn*916* in the presence and absence of *E. faecalis* with the presence of the recipients filtrate only. This would show if a product produced by the recipient, induce Tn*916* excision and increase circular copy numbers.

In Chapter 5, the expression of *orf12* containing different mutations on the terminator was fused in the *gusA* reporter and was measured in aerobic and anaerobic conditions. There was no significant difference in the expression

level of *gusA* in both conditions. The activity of *gusA* was reduced or increased depending on the presence or absence of the *orf12* terminator, respectively. In contrast, highest expression was observed in strains harbouring a construct with a mutation on the *orf12* terminator when transferred from an aerobic to an anaerobic condition. It has been proposed that exposing the cell to any conditions that affect the level of charged t-RNA, e.g. tetracycline or biocides, induces Tn*916* expression and transfer. Therefore, as *orf12* translation is linked with the amount of charged t-RNA, it would be useful to determine the amount of accumulated charged t-RNA to understand the regulation of the Tn*916* expression in an anaerobic condition. A method for this has been recently described by Evans et al. (2017). This method allows to determine the charged tRNA by high-throughput sequencing after chemically treated the isolated RNA to remove the 3'A residue of uncharged tRNA.

Finally, in the last result chapter (Chapter 6), a mutation was produced in the large terminator of *orf12*, which is located immediately upstream of *tet*(M) on Tn*916* in *B. subtilis* genome. This mutant showed Tn*916*Δ*orf12*(T_1) transferred at a frequency of 10^{-9} to 10^{-8} transconjugants per recipient in aerobic conditions compared to the wild-type Tn*916*, which did not transfer. This is likely because of the absence of the terminator structures upstream of *tet*(M), as the translation of *orf12* continued, reaching the downstream genes and leading to the excision of Tn*916*. In contrast, in anaerobic conditions, the mutants (Tn*916*Δ*orf12*(T_1)) were not conjugationally transferred into *S. oralis* and *E. faecalis*. However, a conjugal transfer of (Tn*916*Δ*orf12*(T_1)) occurred into *S. pyogenes*. This indicates that the *orf12* terminator is essential in the

transcriptional regulation of Tn*916* in different conditions and in maintaining the Tn*916* within the host.

The aim of this study was to investigate the effect of different environmental conditions that mimic the oral cavity on Tn*916* transfer from an environmental bacterium *B. subtilis*, into oral and gut bacteria such as streptococci and enterococci. This aim focused on:

- **Investigating the effect of different environmental conditions (aerobic and anaerobic), media (liquid and solid) and time required for Tn*916* transfer**. This showed the ability of Tn*916* to transfer by conjugation into *S. oralis*, *S. pyogenes* and *E. faecalis* only in anaerobic condition.
- **Investigating the copy number of Tn*916* transferred in aerobic and anaerobic conditions.** It was discovered that Tn*916* copy number within BS34A cells in both conditions were the same, which indicated that the gaseous requirement alone did not have an effect on excision. Furthermore, the results showed that in the presence of the recipient, Tn*916* circular copy numbers increased significantly (10-fold) in anaerobic conditions compared to the circular copy numbers in aerobic conditions.
- **Determining the activity of a putative FNR binding sequence and the transcriptional activity on the *orf12* terminator in different conditions**. The transcriptional response of a putative FNR-binding site present on the *orf12* terminator of Tn*916* in *B. subtilis* showed that the exposure of cells to either condition (aerobic or anaerobic) had no

effect on transcription of Tn*916*. Results also showed that the transcription level was higher in strains with terminator mutations, suggesting that transcription did not terminate and continued through, reaching the downstream genes. Moreover, it was found that environmental stress affected the expression from the promoter P*tet*(M) of Tn*916*.

- **Investigating the transfer of Tn*916* aerobically and anaerobically after introducing a mutation on the *orf12* large terminator.** Mutants showed a transfer of Tn*916* from *B. subtilis* into *Streptococcus* and *Enterococcus* under aerobic conditions compared to the wild-type Tn*916*, which did not transfer. In contrast, under anaerobic conditions, mutants showed no transfer except for *S. pyogenes* with a 20-fold lower transfer frequency compared to the wild-typeTn*916*. This is the first demonstration of the effect of the *orf12* terminator on Tn*916* transcription regulation and transfer in different conditions.

The conjugative transposon Tn*916* is one of the transposons responsible for the spread of ARGs within the oral cavity. It is important to resume investigation into the molecular basis of the excision and transfer of this element in different conditions. This will clarify the view of the effect of environmental conditions on the dissemination of ARGs and lead to more knowledge on Tn*916* transfer events in different conditions to contain and control its spread.

Chapter 8 References

n.d. The IV Secreation Systems.

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Appendices

Appendix 1: Compositions of media and solutions

Media/Solutions	Compositions
Brain heart infusion (Gould and Bal) agar(g/l)	12.5g Brain infusion solids 5g Brain heart infusion solids 10g Proteose peptone 5g Sodium chloride 2g Glucose 2.5g Disodium phosphate 10g Agar pH 7.4 ± 2.
Brain heart infusion (Gould and Bal) broth(g/l)	12.5g Brain infusion solids 5g Brain heart infusion solids 10g Proteose peptone 5g Sodium chloride 2g Glucose 2.5g Disodium phosphate pH 7.4 ± 2.

Luria-Bertani (LB) broth (g/l)	10 gTryptone 5 gYeast Extract 5 g Sodium chloride (NaCl).
Luria-Bertani (LB) agar (g/l)	15 g Agar 10 g Tryptone 5 g Yeast Extract 5 g Sodium chloride (NaCl).
SOC	2% Tryptone 0.5% Yeast Extract 10 mM Sodium chloride (NaCl) 2.5 mM Potassium chloride (KCl) 10 mM Magnesium chloride (MgCl2) 10 mM Magnesium sulphate (MgSO4) 20 mM Glucose.

Buffer PB (Binding buffer)	Guanidine hydrochloride and Isopropanol.
Buffer PE (washing solution)	70% ethanol.
Buffer QC (Solubilizing and binding buffer)	Guanidine thiocyanate and pH indicator.
Buffer P1 (Lysing solution)	LyseBlue reagent, RNaseA and PH indicator.
P2 (Lysing solution)	Sodium hydroxide (NaOH).
N3 (Neutralizing and precipitating solution)	Guanidine hydrochloride and acetic acid.
Hydration solution	1 mM EDTA, 10 mM Tris·Cl ,pH 7.5
Z buffer (L)	8 g Disodium hydrogen phosphate heptahydrate ($Na_2HPO_4.7H_2O$) 2.75 g Sodium dihydrogen phosphate Monohydrate ($Na_2H_2PO_4.H_2O$) 0.375 g Potassium chloride (KCl) 0.125 g Magnesium sulphate heptahydrate ($Mg_4SO.\ 7H_2O$) dH_2O to 500 ml, pH7 add 0.14 ml 2-mercaptoethanol to 50 ml Z buffer before use (50 mM)

Stop solution in β-glucuronidase assay (1M Na_2CO_3)	5.3 g Na_2CO_3 dH_2O up to 50 ml
SP4X (g/l)	56.0 g dipotassium phosphate 24.0 g potassium phosphate 8.0 g ammonium sulphate 4.0 g trisodium citrate dihydrate 0.8 g magnesium sulphate heptahydrate 4.0 g casamino acids 4.0 g yeast extract (pH 7.2)
SPI (v/v)	25 ml SP4X 2.5 glucose (20%) 2.86 ml thymine (35 mg/ml) 5.0 ml amino acids solution (histidine, threonine and methionine (1 mg/ml))

	64.6 ml distilled water
SPII (ml/90 ml)	22.5 SP4X 2.25 glucose (20%) 2.6 ml thymine (35 mg/ml) 62.65 ml distilled water
Hybridisation buffer (w/v)	2.92 sodium chloride 5.0 milk powder
20X SSC (1L)	0.1M trisodium citrate (pH 7.0) 3 M sodium chloride
Primary wash buffer (1L)	6 M urea 0.4% SDS 0.20.5X SSC
Depurination solution (1L)	0.3250 mM hydrochloric acid
Denaturation solution (1L)	1.5 M sodium chloride 0.40.5 M sodium hydroxide
Neutralisation solution (1L)	1.5 M sodium chloride 0.50.5 M Tris-HCl pH 7.5

Appendix 2: Raw data of the starting viable cell counts

Aerobic Condition										
Bacterial Strains	*S. mutans* ATCC 700610	*S. mutans* NCTC 10449	*S. mutans* UA 159	*S. sanguinis*	*S. parasanguinis*	*S. oralis*	*S. salivarius*	*S. pyogenes*	*E. faecalis*	*B. subtilis*
VCC (cfu/ml)	4.1×10^9	3.85×10^9	4×10^9	3.23×10^9	3.19×10^9	3.14×10^9	3×10^9	4.13×10^9	4.1×10^9	1.6×10^9
Anaerobic Condition										
Bacterial Strains	*S. mutans* ATCC 700610	*S. mutans* NCTC 10449	*S. mutans* UA 159	*S. sanguinis*	*S. parasanguinis*	*S. oralis*	*S. salivarius*	*S. pyogenes*	*E. faecalis*	*B. subtilis*
VCC (cfu/ml)	3.1×10^9	3.9×10^9	2.9×10^9	2.2×10^9	3.19×10^9	3×10^9	3×10^9	2.4×10^9	2.8×10^9	2.1×10^9

Appendix 3: Raw data of mating in different incubation periods

Strains	0.05 h			1 h			2 h			24 h		
E.faecalis	$3.224 X10^{-9}$	$2.878 X10^{-8}$	$4.605 X10^{-9}$	$1.22 X10^{-9}$	$6.71 X10^{-9}$	$1.117 X10^{-8}$	$1.25 X10^{-9}$	$5.69 X10^{-9}$	$7.79 X10^{-9}$	$1.65 X10^{-8}$	$1.08 X10^{-8}$	$6.52 X10^{-9}$
S. oralis	$6.86 X10^{-9}$	$2.79 X10^{-9}$	$1.082 X10^{-8}$	$6.651 X10^{-9}$	$1.497 X10^{-8}$	$1.927 X10^{-8}$	$4.14 X10^{-9}$	$1.8 X10^{-8}$	$7.05 X10^{-9}$	$1.02 X10^{-8}$	$6.46 X10^{-9}$	$1.61 X10^{-8}$
S.pyogenes	$1.192 X10^{-8}$	$8.365 X10^{-7}$	$1.819 X10^{-8}$	$8.642 X10^{-9}$	$2.9 X10^{-9}$	$9.95 X10^{-9}$	$7.09 X10^{-9}$	$2.54 X10^{-9}$	$8.13 X10^{-9}$	$6.40 X10^{-9}$	$1.58X10^{-8}$	$1.01 X10^{-8}$

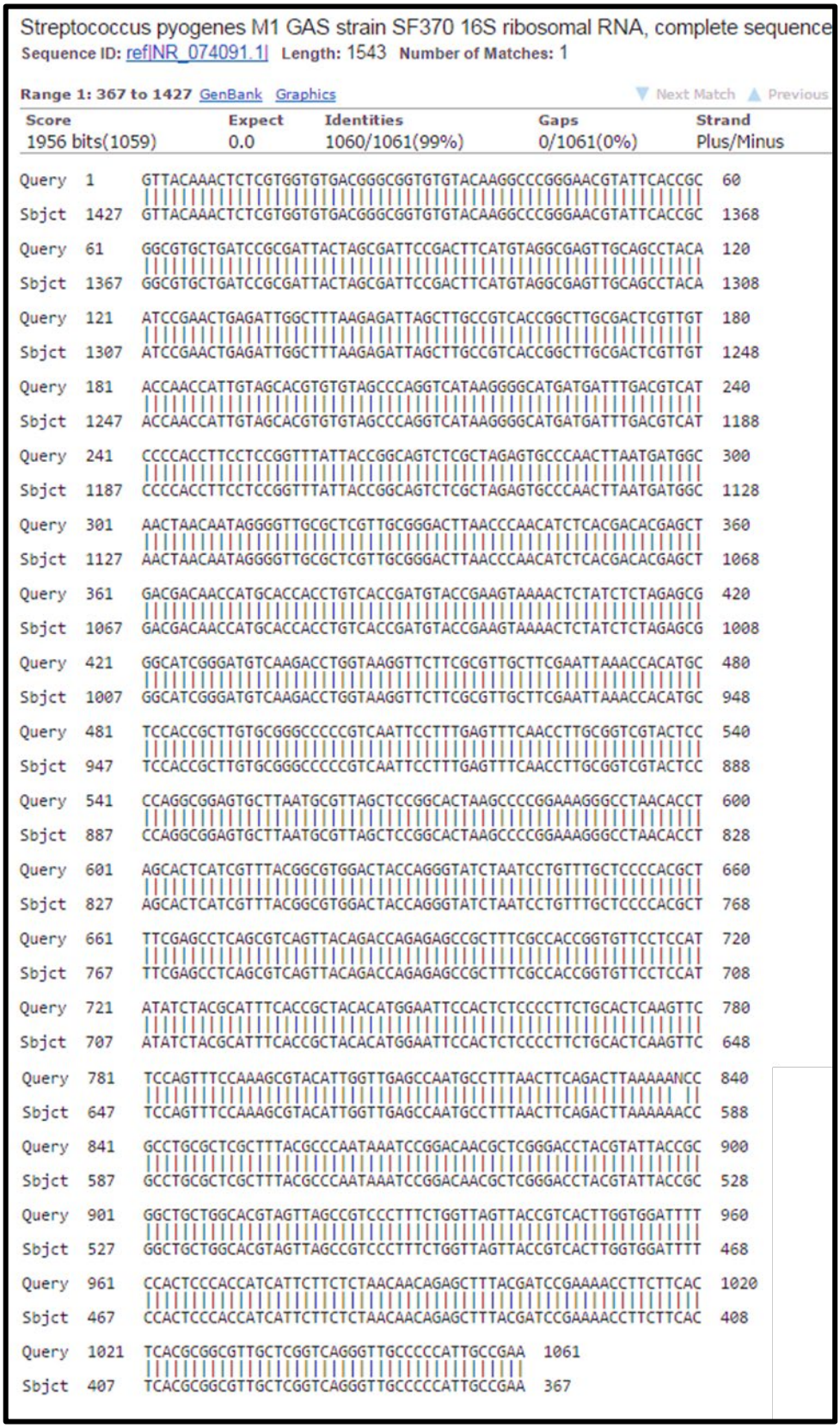
Appendix 4: Raw data of mating with and without DNaseI

Strains	+DNaseI 0.05h			-DNaseI 0.05h			+DNaseI 1h		
E. faecalis	$5.19X10^{-9}$	$1.5096 X10^{-7}$	$1.241 X10^{-7}$	$1.064 X10^{-8}$	$8.314 X10^{-9}$	$1.85 X10^{-8}$	$1.29 X10^{-8}$	$3.979X10^{-8}$	$2.78 X10^{-8}$
S. oralis	$8.94X10^{-9}$	$7.763 X10^{-8}$	$7.76 X10^{-8}$	$9.798 X10^{-9}$	$3.509 X10^{-8}$	$3.51 X10^{-8}$	$1.02 X10^{-9}$	$1.306X10^{-7}$	$1.311 X10^{-7}$
S. pyogenes	0	$2.0716 X10^{-8}$	$1.581 X10^{-7}$	$9.41 X10^{-8}$	0	$2.781 X10^{-7}$	$2.99 X10^{-9}$	$6.061X10^{-8}$	$1.0001 X10^{-7}$

Appendix 5: Raw data of mating in different media

Strains	Broth 1 min			Filter 1min			Broth 0.08h			Filter 0.08h		
E.faecalis	3.25 $X10^{-8}$	2.14 $X10^{-9}$	6.67 $X10^{-8}$	3.59 $X10^{-7}$	5.05 $X10^{-8}$	7.17 $X10^{-8}$	1.24 $X10^{-8}$	1.03 $X10^{-9}$	5.39 $X10^{-8}$	1.47 $X10^{-8}$	5.13 $X10^{-7}$	9.72 $X10^{-6}$
S. oralis	3.81 $X10^{-8}$	3.52 $X10^{-9}$	4.87e $X10^{-10}$	2.45 $X10^{-8}$	2.73 $X10^{-9}$	5.66 $X10^{-8}$	4.19 $X10^{-8}$	1.02 $X10^{-8}$	3.08 $X10^{-10}$	4.44 $X10^{-8}$	1.17 $X10^{-9}$	1.9 $X10^{-8}$
S.pyogenes	5.76 $X10^{-8}$	1.271 $X10^{-7}$	1.3 $X10^{-7}$	6.06 $X10^{-8}$	2.42 $X10^{-8}$	2.36 $X10^{-8}$	2.14 $X10^{-8}$	7.78 $X10^{-8}$	7.8 $X10^{-8}$	3.88 $X10^{-8}$	6.67 $X10^{-8}$	7 $X10^{-8}$

Appendix 6: 16S rRNA sequence of *S. pyogenes* transconjugant



Streptococcus pyogenes M1 GAS strain SF370 16S ribosomal RNA, complete sequence

Sequence ID: ref|NR_074091.1| Length: 1543 Number of Matches: 1

Range 1: 367 to 1427 GenBank Graphics ▼ Next Match ▲ Previous

Score	Expect	Identities	Gaps	Strand
1956 bits(1059)	0.0	1060/1061(99%)	0/1061(0%)	Plus/Minus

```
Query  1     GTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1427  GTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGC  1368

Query  61    GGCGTGCTGATCCGCGATTACTAGCGATTCCGACTTCATGTAGGCGAGTTGCAGCCTACA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1367  GGCGTGCTGATCCGCGATTACTAGCGATTCCGACTTCATGTAGGCGAGTTGCAGCCTACA  1308

Query  121   ATCCGAACTGAGATTGGCTTTAAGAGATTAGCTTGCCGTCACCGGCTTGCGACTCGTTGT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1307  ATCCGAACTGAGATTGGCTTTAAGAGATTAGCTTGCCGTCACCGGCTTGCGACTCGTTGT  1248

Query  181   ACCAACCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCAT  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1247  ACCAACCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCAT  1188

Query  241   CCCCACCTTCCTCCGGTTTATTACCGGCAGTCTCGCTAGAGTGCCCAACTTAATGATGGC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1187  CCCCACCTTCCTCCGGTTTATTACCGGCAGTCTCGCTAGAGTGCCCAACTTAATGATGGC  1128

Query  301   AACTAACAATAGGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1127  AACTAACAATAGGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCT  1068

Query  361   GACGACAACCATGCACCACCTGTCACCGATGTACCGAAGTAAAACTCTATCTCTAGAGCG  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1067  GACGACAACCATGCACCACCTGTCACCGATGTACCGAAGTAAAACTCTATCTCTAGAGCG  1008

Query  421   GGCATCGGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1007  GGCATCGGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGC  948

Query  481   TCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCC  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  947   TCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCC  888

Query  541   CCAGGCGGAGTGCTTAATGCGTTAGCTCCGGCACTAAGCCCCGGAAAGGGCCTAACACCT  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  887   CCAGGCGGAGTGCTTAATGCGTTAGCTCCGGCACTAAGCCCCGGAAAGGGCCTAACACCT  828

Query  601   AGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCT  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  827   AGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCT  768

Query  661   TTCGAGCCTCAGCGTCAGTTACAGACCAGAGAGCCGCTTTCGCCACCGGTGTTCCTCCAT  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  767   TTCGAGCCTCAGCGTCAGTTACAGACCAGAGAGCCGCTTTCGCCACCGGTGTTCCTCCAT  708

Query  721   ATATCTACGCATTTCACCGCTACACATGGAATTCCACTCTCCCCTTCTGCACTCAAGTTC  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  707   ATATCTACGCATTTCACCGCTACACATGGAATTCCACTCTCCCCTTCTGCACTCAAGTTC  648

Query  781   TCCAGTTTCCAAAGCGTACATTGGTTGAGCCAATGCCTTTAACTTCAGACTTAAAAANCC  840
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  647   TCCAGTTTCCAAAGCGTACATTGGTTGAGCCAATGCCTTTAACTTCAGACTTAAAAAACC  588

Query  841   GCCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTCGGGACCTACGTATTACCGC  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  587   GCCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTCGGGACCTACGTATTACCGC  528

Query  901   GGCTGCTGGCACGTAGTTAGCCGTCCCTTTCTGGTTAGTTACCGTCACTTGGTGGATTTT  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  527   GGCTGCTGGCACGTAGTTAGCCGTCCCTTTCTGGTTAGTTACCGTCACTTGGTGGATTTT  468

Query  961   CCACTCCCACCATCATTCTTCTCTAACAACAGAGCTTTACGATCCGAAAACCTTCTTCAC  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  467   CCACTCCCACCATCATTCTTCTCTAACAACAGAGCTTTACGATCCGAAAACCTTCTTCAC  408

Query  1021  TCACGCGGCGTTGCTCGGTCAGGGTTGCCCCCATTGCCGAA  1061
             |||||||||||||||||||||||||||||||||||||||||
Sbjct  407   TCACGCGGCGTTGCTCGGTCAGGGTTGCCCCCATTGCCGAA  367
```

Appendix 7: 16S rRNA sequence of *S. oralis* transconjugant

Streptococcus oralis strain CCUG 24891 16S ribosomal RNA gene, partial sequence

Sequence ID: ref|NR_115734.1| **Length:** 1472 **Number of Matches:** 1

Range 1: 446 to 1419 GenBank Graphics Next Match Previous M

Score	Expect	Identities	Gaps	Strand
1799 bits(974)	0.0	974/974(100%)	0/974(0%)	Plus/Minus

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Query  1     CCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1419  CCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAA  1360

Query  61    CGTATTCACCGCGGCGTGCTGATCCGCGATTACTAGCGATTCCGACTTCATGTAGGCGAG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1359  CGTATTCACCGCGGCGTGCTGATCCGCGATTACTAGCGATTCCGACTTCATGTAGGCGAG  1300

Query  121   TTGCAGCCTACAATCCGAACTGAGACTGGCTTTAAGAGATTAGCTTGCCGTCACCGGCTT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1299  TTGCAGCCTACAATCCGAACTGAGACTGGCTTTAAGAGATTAGCTTGCCGTCACCGGCTT  1240

Query  181   GCGACTCGTTGTACCAGCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1239  GCGACTCGTTGTACCAGCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATG  1180

Query  241   ATTTGACGTCATCCCCACCTTCCTCCGGTTTATTACCGGCAGTCTCGCTAGAGTGCCCAA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1179  ATTTGACGTCATCCCCACCTTCCTCCGGTTTATTACCGGCAGTCTCGCTAGAGTGCCCAA  1120

Query  301   CTGAATGATGGCAACTAACAATAGGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTC  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1119  CTGAATGATGGCAACTAACAATAGGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTC  1060

Query  361   ACGACACGAGCTGACGACAACCATGCACCACCTGTCACCTCTGTCCCGAAGGAAAACTCT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1059  ACGACACGAGCTGACGACAACCATGCACCACCTGTCACCTCTGTCCCGAAGGAAAACTCT  1000

Query  421   ATCTCTAGAGCGGTCAGAGGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAAT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  999   ATCTCTAGAGCGGTCAGAGGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAAT  940

Query  481   TAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTG  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  939   TAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTG  880

Query  541   CGGTCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCGGCACTAAACCCCGGAAAG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  879   CGGTCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCGGCACTAAACCCCGGAAAG  820

Query  601   GGTCTAACACCTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTT  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  819   GGTCTAACACCTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTT  760

Query  661   GCTCCCCACGCTTTCGAGCCTCAGCGTCAGTTACAAGCCAGAGAGCCGCTTTCGCCACCG  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  759   GCTCCCCACGCTTTCGAGCCTCAGCGTCAGTTACAAGCCAGAGAGCCGCTTTCGCCACCG  700

Query  721   GTGTTCCTCCATATATCTACGCATTTCACCGCTACACATGGAATTCCACTCTCCCCTCTT  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  699   GTGTTCCTCCATATATCTACGCATTTCACCGCTACACATGGAATTCCACTCTCCCCTCTT  640

Query  781   GCACTCAAGTTAAACAGTTTCCAAAGCGTACTATGGTTAAGCCACAGCCTTTAACTTCAG  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  639   GCACTCAAGTTAAACAGTTTCCAAAGCGTACTATGGTTAAGCCACAGCCTTTAACTTCAG  580

Query  841   ACTTATCTAACCGCCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTCGGGACCT  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  579   ACTTATCTAACCGCCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTCGGGACCT  520

Query  901   ACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTCCCTTTCTGGTAAGATACCGTCAC  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  519   ACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTCCCTTTCTGGTAAGATACCGTCAC  460

Query  961   AGTGTGAACTTTCC  974
             ||||||||||||||
Sbjct  459   AGTGTGAACTTTCC  446
```

Appendix 8: 16S rRNA Sequence of *E. faecalis trans*conjugant

Enterococcus faecalis strain ATCC 19433 16S ribosomal RNA gene, partial sequence

Sequence ID: NR_115765.1 **Length:** 1483 **Number of Matches:** 1

Range 1: 297 to 1418 GenBank Graphics ▼ Next Match ▲ Previous

Score	Expect	Identities	Gaps	Strand
2073 bits(1122)	0.0	1122/1122(100%)	0/1122(0%)	Plus/Minus

```
Query  1     GTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1418  GTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGC  1359

Query  61    GGCGTGCTGATCCGCGATTACTAGCGATTCCGGCTTCATGCAGGCGAGTTGCAGCCTGCA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1358  GGCGTGCTGATCCGCGATTACTAGCGATTCCGGCTTCATGCAGGCGAGTTGCAGCCTGCA  1299

Query  121   ATCCGAACTGAGAGAAGCTTTAAGAGATTTGCATGACCTCGCGGTCTAGCGACTCGTTGT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1298  ATCCGAACTGAGAGAAGCTTTAAGAGATTTGCATGACCTCGCGGTCTAGCGACTCGTTGT  1239

Query  181   ACTTCCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCAT  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1238  ACTTCCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCAT  1179

Query  241   CCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCGCTAGAGTGCCCAACTAAATGATGGC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1178  CCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCGCTAGAGTGCCCAACTAAATGATGGC  1119

Query  301   AACTAACAATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1118  AACTAACAATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCT  1059

Query  361   GACGACAACCATGCACCACCTGTCACTTTGTCCCCGAAGGGAAAGCTCTATCTCTAGAGT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1058  GACGACAACCATGCACCACCTGTCACTTTGTCCCCGAAGGGAAAGCTCTATCTCTAGAGT  999

Query  421   GGTCAAAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  998   GGTCAAAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGC  939

Query  481   TCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCC  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  938   TCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCC  879

Query  541   CCAGGCGGAGTGCTTAATGCGTTTGCTGCAGCACTGAAGGGCGGAAACCCTCCAACACTT  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  878   CCAGGCGGAGTGCTTAATGCGTTTGCTGCAGCACTGAAGGGCGGAAACCCTCCAACACTT  819

Query  601   AGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCT  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  818   AGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCT  759

Query  661   TTCGAGCCTCAGCGTCAGTTACAGACCAGAGAGCCGCCTTCGCCACTGGTGTTCCTCCAT  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  758   TTCGAGCCTCAGCGTCAGTTACAGACCAGAGAGCCGCCTTCGCCACTGGTGTTCCTCCAT  699

Query  721   ATATCTACGCATTTCACCGCTACACATGGAATTCCACTCTCCTCTTCTGCACTCAAGTCT  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  698   ATATCTACGCATTTCACCGCTACACATGGAATTCCACTCTCCTCTTCTGCACTCAAGTCT  639

Query  781   CCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAAC  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  638   CCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAAC  579

Query  841   CGCCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTTGCCACCTACGTATTACCG  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  578   CGCCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTTGCCACCTACGTATTACCG  519

Query  901   CGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGATACCGTCAGGGGACGTTCAG  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  518   CGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGATACCGTCAGGGGACGTTCAG  459

Query  961   TTACTAACGTCCTTGTTCTTCTCTAACAACAGAGTTTTACGATCCGAAAACCTTCTTCAC  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  458   TTACTAACGTCCTTGTTCTTCTCTAACAACAGAGTTTTACGATCCGAAAACCTTCTTCAC  399

Query  1081  CCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGAT  1122
             |||||||||||||||||||||||||||||||||||||||||
Sbjct  338   CCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGAT  297
```

Appendix 9: Raw data of circular Tn*916* copy numbers in aerobic and anaerobic condition

	Aerobic			Anaerobic		
Tn*916*-1	1.4333897e+007	1.3213585e+007	1.2324752e+007	1.180852e+007	1.4613179e+007	1.3773897e+007
Tn*916*-2	1577236	1213478	1455793	1176388	1343137	1319127
Tn*916*-3	157987	135103	130644	135500	134707	133358
Tn*916*-4	15568	14551	15634	13843	13704	12335
Tn*916*-5	1104	1226	1241	1242	1908	1251
Tn*916*-6	768	628	657	795	638	619

Appendix 10: Raw data of circular Tn*916* copy numbers in aerobic and anaerobic condition after mixed with *E. faecalis* (recipient)

	Aerobic			Anaerobic		
Tn*916*-1	2.3235409e+007	2.204995e+007	2.2802572e+007	1.31403501e+008	1.30887348e+008	1.30444509e+008
Tn*916*-2	2339006	2242354	2038682	1.176623e+007	1.0013939e+007	9789864
Tn*916*-3	193672	185608	183249	880646	797599	716955
Tn*916*-4	18979	17967	17774	69547	64156	66410
Tn*916*-5	1790	1765	1745	5421	6377	5661
Tn*916*-6	330	315	291	591	594	619

Appendix 11: Raw data of β-glucuronidase activity assay for a fused *orf12* (upstream) with a different mutation in response to oxygen.

Strains	$+O_2$			$-O_2$			$+O_2/-O_2(1)$			$+O_2/-O_2(2)$		
BS34A	7.06	8.244	6.592	6.0469	11.347	5.906	8.255	7.207	7.03	6.926	3.743	8.372
ptet(M)-orf12	53.756	52.419	47.725	69.346	66.867	62.491	61.37	49.241	48.937	52.013	11.569	90.974
Δorf12	250.689	298.233	312.195	237.172	283.559	282.873	494.059	312.825	269.755	254.711	139.994	247.964
Δorf12(T_1)	260.461	415.412	360.23	178.925	206.368	297.704	383.923	381.641	411.547	480.331	336.673	397.104
Δorf12(T_2)	149.573	313.69	320.451	134.288	241.557	310.741	460.292	278.389	397.769	361.498	161.394	88.337
sorf12(T_1)	325.701	462.171	260.586	182.149	327.316	379.205	532.251	465.168	423.493	477.006	353.87	282.265

Appendix 12: Raw data for the starting viable cell count of all the recipients and donors in aerobic and anaerobic condition.

Aerobic condition					
Bacterial Strains	*S. oralis*	*S. pyogenes*	*E. faecalis*	*B. subtilis*	*B. subtilisΔorf12(T_1)*
VCC (cfu/ml)	4.1×10^9	3.1×10^9	3×10^9	1.8×10^9	2.4×10^9
Anaerobic condition					
Bacterial Strains	*S. oralis*	*S. pyogenes*	*E. faecalis*	*B. subtilis*	*B. subtilisΔorf12(T_1)*
VCC (cfu/ml)	2.8×10^9	2×10^9	2.1×10^9	2.4×10^9	2.4×10^9

Appendix 13: Raw data of mating with *B. subtilies* wild type and *B. subtilis Δorf12* mutant in different condition (aerobic vs anaerobic)

Strains	Aerobic			Anaerobic		
Ef + BS*Δorf12*	9.5×10^{-10}	2.31×10^{-9}	2.2×10^{-9}	0	0	0
So + BS*Δorf12*	2.8×10^{-8}	1.5×10^{-9}	3.7×10^{-10}	0	0	0
Sp + BS*Δorf12*	6.66×10^{-9}	1.3×10^{-8}	2.44×10^{-8}	1.57×10^{-9}	1.56×10^{-10}	9.6×10^{-10}
Ef + BS34A	0	0	0	1.65×10^{-8}	1.08×10^{-8}	6.52×10^{-9}
So + BS34A	0	0	0	1.02×10^{-8}	6.46×10^{-9}	1.61×10^{-8}
Sp + BS34A	0	0	0	6.40×10^{-9}	1.58×10^{-8}	1.01×10^{-8}