Effect of the environment on gene transfer in *Clostridium difficile*

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Declaration

I, Ladan Khodadoost, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Clostridium difficile is a pathogenic bacterium that can colonise both humans and various animals. Toxin production leads to clinical symptoms ranging from mild to severe diarrhoea and can result in potentially fatal pseudomembranous colitis. These symptoms are caused by the disruption of the cytoskeleton and tight junctions of gut epithelial cells by the toxins. *C. difficile* responds to several biological compounds found in the human intestinal environment such as bile salts leading to spore germination and colonization. In this study we investigated the response of *C. difficile* to mammalian pancreatic α-amylase with production of a mucoid colony phenotype that results from increased secretion of extracellular proteins and carbohydrates. Furthermore, the effect of amylase on horizontal gene transfer in *C. difficile* was investigated using conjugative transposon Tn*5397* and non-conjugative mobilisable plasmid pMTL9301. A significant increase in the frequency of Tn*5397* transfer was observed when amylase was present. pMTL9301 transfer was not affected by amylase but significantly decreased when DNase was added to eliminate transformation. Further investigations into the molecular basis of the DNase-sensitive plasmid transfer into *C. difficile* showed that the *oriT* region of pMTL9301 (derived from RK2) is not required for transfer between *E. coli* and *C. difficile* strains 630Δ*erm* and CD37 and that this *oriT*-independent transfer is abolished in the presence of DNase when CD37 is the recipient. Transfer to the 630Δ*erm* strain is DNase resistant even without an obvious *oriT*, when *E. coli* CA434 is used as a donor and is sensitive to DNase when *E. coli* HB101 is the donor, suggesting that a 'novel cell-to-cell transformation-like mechanism' occurs in *C. difficile*.

Impact statement

A fundamental understanding of the evolutionary pressures which select for resistance is a prerequisite to design strategies to stop the spreading of antibiotic resistance genes (ARGs). The work in this study has shown that *C. difficile* has a remarkable ability to obtain new DNA. The unexpected observation that it can take up plasmid DNA encoding antibiotic resistance from an unrelated microorganism without a complete conjugation system or a *cis* acting *oriT* suggests that the organism has the potential to acquire almost any DNA sequence. Our study also has implications for the containment of genetically modified organisms, as we have shown that non-conjugative non-mobilisable plasmids can still be taken up by an organism that was previously thought not to be naturally competent, and it is important to determine how common this phenomenon is in nature.

The 'novel cell-to-cell transformation-like' mechanism shown here for the first time in *C. difficile*, may occur in environments outside the laboratory at non-negligible frequencies if several conditions are met. In this respect, further experiments using plasmid-free strains of *E. coli* and other *C. difficile* strains will be required. Furthermore, other results suggest that non-conjugative plasmids are more mobile than was previously believed (Zechner *et al.*, 2012). Our finding not only can provide insights into evolution of acquired resistance genes but also can offer a strategy for combatting the antibiotic-resistance crisis.

Acknowledgment

I would like to begin by thanking Professor Peter Mullany for providing me with the opportunity to conduct a PhD at UCL and for all his support with the lab work, the writing and all the helpful discussion. I would like to thank Dr. Sean Nair for his input and helpful comments on my thesis.

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List of abbreviations

µg	microgram
µl	microlitre
°C	degrees Celsius
AAD	antibiotic associated diarrhoea
ARG	antibiotic resistant gene
BHI	brain heart infusion
BHIB	brain heart infusion containing 5% defibrinated horse blood
BDM	basal defined medium
bp	basepair
CDI	*C. difficile* infection
CDS	coding sequence
CDT	*C. difficile* binary toxin
CPE	cytopathic effect
CTn	Conjugative transposon
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide-triphosphate
EBS	exon binding site
EPS	extracellular polymeric substance
g	gram
h	hours
Hfr	high frequency recombination
HGT	horizontal gene transfer
IBS	intron binding site

ICE	integrative and conjugative element
INDEL	insertion/ deletion
IPTG	Isopropyl-β-D-thio-galactoside
IS-element	insertion sequence element
kb	kilobase
l	litre
LCT	large clostridial toxin
M	molar concentration
mg	milligram
MGE	mobile genetic element
min	minutes
ml	millilitre
mM	milli molar
molH_2O	molecular biology grade water
mpf	mating pair formation
mRNA	messenger RNA
ng	nanogram
nm	nanometre
OD_{600}	optical density at 600 nm
ORF	open reading frame
oriT	origin of transfer
PaLoc	pathogenicity locus
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RAM	Retrotranspositional-activated marker

RNA	ribonucleic acid
RNA-seq	RNA sequencing
rpm	revolutions per minute
rRNA	ribosomal RNA
RT	room temperature
RT-PCR	reverse transcriptase PCR
S-layer	surface layer
sec	seconds
SD	standard deviation
skin	*sigK* intervening sequence
SNP	single nucleotide polymorphism
TcdA	*C. difficile* toxin A
TcdB	*C. difficile* toxin B
T4SS	type IV secretion system
w/v	weight per volume

Chapter 1 General Introduction

1.1. General characteristics of *Clostridium difficile*

Clostridium difficile (now also referred to as *Clostridioides difficile*) was originally isolated from the faeces of healthy human newborns and designated *Bacillus difficile* by Hall and O'Toole in 1935 (Hall & O'Toole, 1935). This report as well as a publication by Snyder (1937) characterized the organism as a Gram-positive rod-shaped bacterium (Figure 1.1). It was also revealed that the isolated Gram-positive bacterium is an obligate anaerobe, produces spores and secretes toxins. The toxin was shown to cause convulsions when administrated subcutaneously into animal models in a similar manner as tetanus toxin produced by *Clostridium tetani* (Snyder, 1937). The organism was later designated *Clostridium difficile* and it was shown that it usually infects human intestinal tract (Smith & King, 1962). The toxin of *C. difficile* was later found in patients suffering from antibiotic associated pseudomembranous colitis (Viscidi *et al.*, 1981). Furthermore, clindamycin-resistant toxin-producing *C. difficile* was found to be responsible for most incidents of clindamycin associated enterocolitis in hamsters (Bartlett *et al.*, 1977, Bartlett *et al.*, 1978).

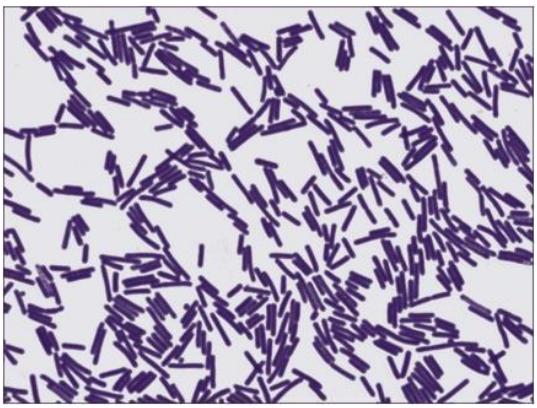


Figure 1.1 Gram staining of *C. difficile* strain 630.

C. difficile is Gram-positive, rod-shaped and spore-forming. This image was made using an Olympus BX51 microscope equipped with a Qlmaginig, MicroPublisher 5.0RTV camera (Retrieved from (Brouwer, 2013))

The first *C. difficile* strain to be fully sequenced was 630 which was first isolated from a patient with severe pseudomembranous colitis in a hospital outbreak in Switzerland (Wust *et al.*, 1982, Sebaihia *et al.*, 2006). *C. difficile* 630 genome consists of a 4.29 Mb chromosome and a 7.8 kb plasmid pCD630 (Sebaihia *et al.*, 2006). The average GC content of the chromosome is low (29%); however, a large number of mobile genetic elements (MGEs) (11%) were found within the chromosome with a relatively high GC content (up to 47%) (Sebaihia *et al.*, 2006). These include seven conjugative transposons (CTn*1*, CTn*2*, CTn*3* (Tn*5397*), CTn*4*, CTn*5*, CTn*6* and CTn*7*), one non-conjugative transposon (Tn*5398*), two prophages, a prophage-like *sigK* intervening sequence element and IStrons (Table 1.1) (Braun *et al.*, 2000, Farrow *et al.*, 2001, Haraldsen & Sonenshein, 2003, Sebaihia *et al.*, 2006).

Further analysis of the genomes of fifteen *C. difficile* strains showed that the organism has very low genome conservation (23-26%) and the core genome consists of 947 to 1033 coding sequences (CDS) (Scaria *et al.*, 2010). Other organisms with low genome conservations between their strains are *Streptococcus pneumoniae* (46.5%) and *Campylobacter jejuni* (59.2%) (Champion *et al.*, 2005, Hiller *et al.*, 2007). It is estimated that the shared core genome of *C. difficile* strains is as low as 16% which is lower than any bacterial species described so far (Knight *et al.*, 2015).

The pan genome is the entire gene set of all strains of a species and includes core genome (genes present in all strains) and variable genome (present only in some strains of a species) (Guimarães *et al.*, 2015). The pan genome of *Streptococcus agalactiae* and *S. pneumoniae* are 1806 CDS and 5100 CDS, respectively (Tettelin *et al.*, 2005, Hiller *et al.*, 2007), whereas the pan genome of *C. difficile* is predicted to be 9640 CDS (Scaria *et al.*, 2010).

The reason *C. difficile* has the largest predicted pan genome might be due to the presence of many MGEs in this organism that confers diversity among the species (Lewis *et al.*, 2017).

Feature	Chromosome	Plasmid
Size (bp)	4,290,252	7,881
G + C content (mole %)	29.06	27.9
Coding sequences	3,776	11
Coding density	0.087	1.39
Average gene size (bp)	943	563
Pseudogenes	32	0
rRNA operons	11	0
tRNA	87	0
Stable RNA	54	0

Table 1.1 General features of the genome of *C. difficile*.

(Retrieved from (Sebaihia *et al.*, 2006)). The table shows the features of the chromosome and plasmid pCD630 in strain 630

1.2. *C. difficile* infection, treatment and prevention

1.2.1. Disease and risk factors

Antibiotic associated diarrhea (AAD) is mediated by an unbalanced gut microbiome or dysbiosis resulting from numerous causes such as extensive antibiotic therapies, anticancer treatments and antiretroviral drugs for the treatment of HIV-infected patients (Dudek-Wicher *et al.*, 2018, Pinto-Cardoso *et al.*, 2018, Pouncey *et al.*, 2018). It is a mild and self-limiting condition affecting 5-39% of people who have a disrupted microbiota (Barbut & Meynard, 2002). *C. difficile* infection (CDI) is a form of AAD which causes gastrointestinal diseases ranging from mild diarrhea and fever to severe pseudomembranous colitis, toxic megacolon, multiorgan failure or even death (Antonara & Leber, 2016). One of the reasons that *C. difficile* causes serious problems within healthcare units is that spores produced by this organism survive for long

periods on abiotic objects and they are resistant to heat, acids and antibiotics (Barra-Carrasco & Paredes-Sabja, 2014). *C. difficile* spreads through the oral-faecal route and causes disease in humans by producing two protein exotoxins (toxin A and toxin B) with cytotoxic activities against intestinal epithelial cells (Rupnik *et al.*, 2009, Mullish & Williams, 2018). The host adaptive immune system plays an important part in determining the severity of the CDI in that high IgG production following exposure to *C. difficile* leads to a better protection (Rupnik *et al.*, 2009). The major risk factor for CDI is prolonged antibiotic consumption that disrupts diversity of the gut microbiota which in turn causes spore germination and vegetative growth of the organism in vulnerable people including elderly and immunosuppressed patients (Eze *et al.*, 2017). Risk factors for CDI are summarized in table 1.2.

Table 1.2 Major risk factors for *C. difficile* infection (CDI).
(Adapted from (Mullish & Williams, 2018)).

Risk factors	Details
Antibiotics	Almost all antibiotics (e.g., clindamycin and certain penicillin)
Acid suppressant medications	proton-pump inhibitors (PPI) and H_2-receptor antagonists
Age	CDI infection rate is 10-fold enhanced in people aged >65
Hospitalization	Recent hospitalisation, prolonged hospitalisation (>7 days), and/ or prolonged antibiotic courses, being admitted to a room where the previous patient had CDI
Immunosuppression	E.g., those receiving cancer chemotherapies (Cózar-Llistó *et al.*, 2016).

1.2.2. Treatment and prevention

Until recently, the antibiotics metronidazole and vancomycin were the only therapeutic options to treat CDI. Intravenous immunoglobulin (IVIg) and/ or surgical intervention (colectomy) have also been used in severe cases (Mullish & Williams, 2018). However, CDI has become more difficult to treat with conventional antibiotics for several reasons. First, the rate of CDI recurrence has increased (Nair *et al.*, 1998, Noren *et al.*, 2004). Second, the rate of CDI treatment failure with metronidazole has increased (>20%) (Musher *et al.*, 2005, Kelly & LaMont, 2008). Third, hypervirulent strains such as NAP1/ 027 with poor response to conventional antibiotics have emerged (Brazier *et al.*, 2008, Martin *et al.*, 2016). Novel approaches to treat CDI are now being taken including the use of new antibiotics such as fidaxomicin. It has been shown that fidaxomicin is an efficient therapeutic option to treat recurrent CDI (Lee *et al.*, 2016). However, its poor effectiveness against the epidemic NAP1/ 027 strain and severe colitis cases as well as the high cost of production have caused concerns regarding its use (Penziner *et al.*, 2015, Mullish & Williams, 2018).

Other therapeutic approaches to treat CDI are manipulation of the gut microbiota with probiotics and/ or faecal microbiota transplantation (FMT). There are very few studies regarding the effectiveness of probiotics in CDI treatment with all being uncertain about the type and dose of specific organism to be administrated. Therefore, probiotics are not recommended at present (Mullish & Williams, 2018). In contrast, it has been shown that FMT is more effective than vancomycin for treating recurrent CDI when delivered by colonoscopy (Cammarota *et al.*, 2015). FMT is now being administered in the UK (Mullish & Williams, 2015), but there is an interest to refine the route of delivery into a pill or drink to reduce the potential drawbacks (Kao *et al.*, 2017).

Another alternative therapeutic option is the development of vaccines to prevent the disease in adults at risk of CDI. Studies are being conducted to design a vaccine against the *C. difficile* TcdA toxin for which phase II trials are in progress (Foglia *et al.*, 2012).

At present, prevention of spread is one of the most important strategies to battle *C. difficile* in the hospital settings by isolation of infected patients, disinfection of the patient environment and good hand hygiene for health care workers to reduce the number of *C. difficile* outbreaks (Gerding *et al.*, 2008).

1.3. Molecular pathogenesis and virulence

1.3.1. Toxins

1.3.1.1. *C. difficile* TcdA and TcdB

The main virulence factors of *C. difficile* are cytotoxins TcdA and TcdB encoded on the pathogenicity locus (PaLoc ~ 19.6 kb). The PaLoc is a genetic locus that is only found in toxigenic strains of *C. difficile* and harbours five genes, *tcdA* encoding toxin A, *tcdB* encoding toxin B, two regulatory genes *tcdC* (anti-sigma factor), *tcdR* (sigma factor) and *tcdE* encoding a holin-like protein with lytic activity to facilitate the release of the TcdA and TcdB into the environment (Figure 1.2) (Rupnik *et al.*, 2005). TcdC is an acidic membrane associated protein acting as a negative regulator of toxin production. It has a unique mechanism of action and shares no homology with other regulatory proteins (Matamouros *et al.*, 2007). TcdR acts as a positive regulator of toxin expression and shares homologies with TetR and BotR regulators of tetanus and botulinum toxins, respectively (Rupnik *et al.*, 2005).

TcdA and TcdB are the main virulence factors responsible for symptomatic cases of CDI (Di Bella *et al.*, 2016). They belong to a family of large toxins present in different members of the genus *Clostridium*; including lethal toxin (TcsL) and haemorrhagic toxin (TcsH) from *Clostridium sordellii*, alpha toxin (TcnA) from *Clostridium novyi*, and TpeL from *Clostridium perfringens* (Busch *et al.*, 2000, Voth *et al.*, 2006, Amimoto *et al.*, 2007).

The entire PaLoc is absent from the majority of non-toxigenic strains and is replaced by a 115 bp sequence that is highly conserved among non-toxigenic *C. difficile* strains such as strain CD37 (Figure 1.2) (Braun *et al.*, 1996). Non-toxigenic *C. difficile*, however, can acquire the PaLoc from toxigenic organisms through horizontal gene transfer, resulting in the conversion of non-toxigenic strains to toxin producers (Brouwer *et al.*, 2013).

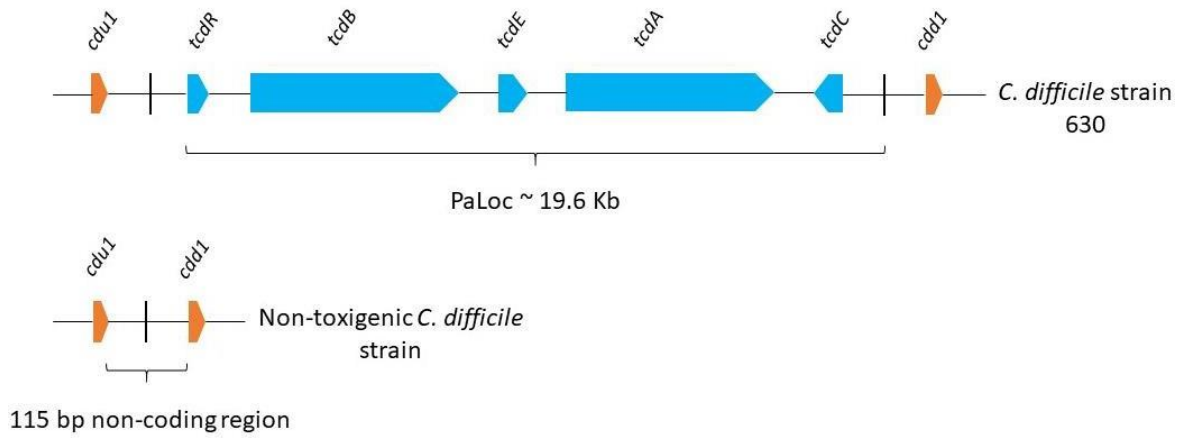


Figure 1.2 Schematic representation of PaLoc in toxigenic 630 and non-toxigenic *C. difficile* CD37.

The 5′ flanking gene *cdu1*, the 3′ flanking gene *cdd1*, positive regulator gene *tcdR*, negative regulator gene *tcdC*, holing-like pore-forming protein encoding gene *tcdE*, and toxin genes *tcdA* and *tcdB* are shown. In many non-toxigenic strains, PaLoc is replaced by a 115 bp non-coding region.

There are some exceptions regarding the PaLoc integration site in non-toxigenic strains (Elliott *et al.*, 2014). For example, a PCR ribotype (033) isolate contains only the *tcdC* gene and 2456 bp of the *tcdA* gene containing a 681 bp deletion, together representing 6521 bp of the PaLoc (Geric Stare & Rupnik, 2010). Immediately upstream of this region, a variant of conjugative transposon Tn*6218* is located (Figure 1.3) (Dingle *et al.*, 2014). Another exception is strain WA12 which was found to be responsible for a rare case of *C. difficile* bacteraemia in Australia. This isolate contains neither toxin A nor toxin B, but it has binary toxin genes (section 1.3.1.2). Besides, a 7.2 kb region unrelated to the PaLoc is present within the PaLoc integration site in this strain. The functions of the putative genes within this region have not yet been identified (Figure 1.3) (Elliott *et al.*, 2009).

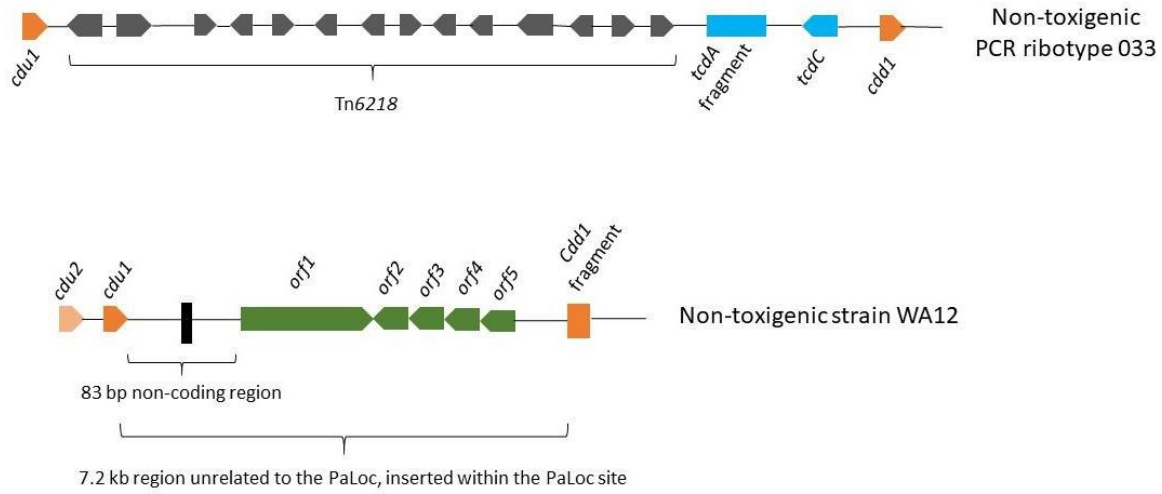


Figure 1.3 Schematic representation of the uncommon PaLoc integration sites in a small number of non-toxigenic *C. difficile* strains.

PaLoc is replaced by a 115 bp non-coding region in the majority of non-toxigenic *C. difficile* strains (Figure 1.2). However, there are strains that are exceptions to this including a PCR ribotype (033) isolate and *C. difficile* strain WA12.

The TcdA and TcdB toxins comprise different functional domains including a C-terminal binding domain for recognition of unknown receptors on the surface of intestinal epithelial cells, a translocation domain for entering the target cell and an N-terminal domain with catalytic activity to glycosylate the target molecule (Figure 1.4) (von Eichel-Streiber *et al.*, 1996, Rupnik *et al.*, 2009). Due to similarities between *C. difficile* toxins and other AB-type toxins such as diphtheria toxin, a structure- function relationship that consists of a biologically active domain and a binding domain was first proposed for the TcdA and TcdB (Collier, 2001). However, an ABCD domain structure model (multi-modular structure) was later suggested to better describe the structure-function relationship of these toxins (Figure 1.4) (Jank & Aktories, 2008).

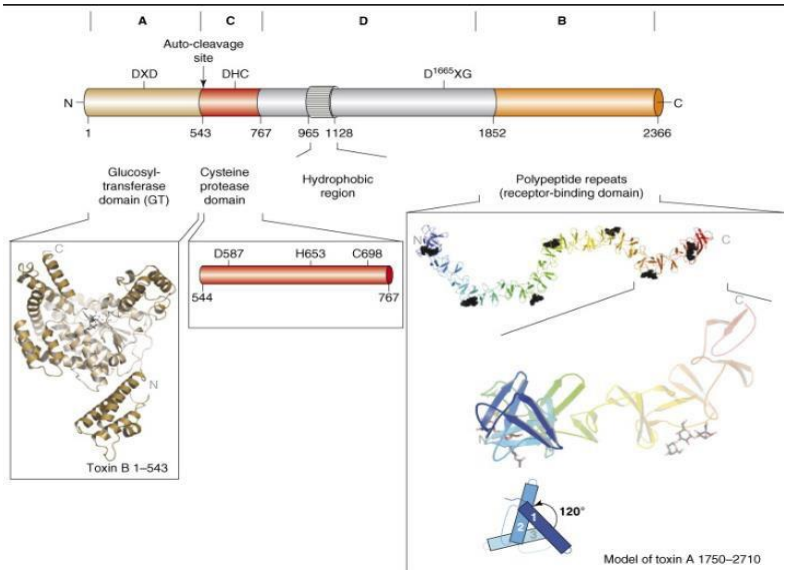


Figure 1.4 Proposed ABCD domain structure model of toxin B.

(Retrieved from (Jank & Aktories, 2008))**.** The biologically active glucosyltransferase A-domain is located at the N terminus and includes the DXD motif involved in Mn^{2+} coordination. The C terminus (domain B) consists of polypeptide repeats and is involved in receptor binding. It is hypothesized that the DXG motif is involved in processing the toxin and that the hydrophobic region may be involved in pore formation and delivery of the cysteine catalytic domain into the target cell.

Toxins enter the epithelial cells via endocytosis following C-terminal binding to a specific cell membrane receptor. In the endosome, the cysteine protease cleaves the catalytic domain by its proteolytic activity and mediates the entry of toxin into the cytoplasm. Both toxins can inactivate small regulatory proteins of the Rho and Ras superfamilies of GTPase via glycosylation (von Eichel-Streiber *et al.*, 1996, Di Bella *et al.*, 2016). This results in the disruption of actin cytoskeletons found in the tight junctions of epithelial cells and apoptosis (Figure 1.5) (Aktories & Just, 1995). The presence of the toxins in target cells is recognised by the innate immune system leading to cytokine IL-1β production and inflammation (Ng *et al.*, 2010). The destruction of the epithelial cells and the subsequent inflammatory response lead to pseudomembranous colitis and fluid accumulation in the gut (Rupnik *et al.*, 2009).

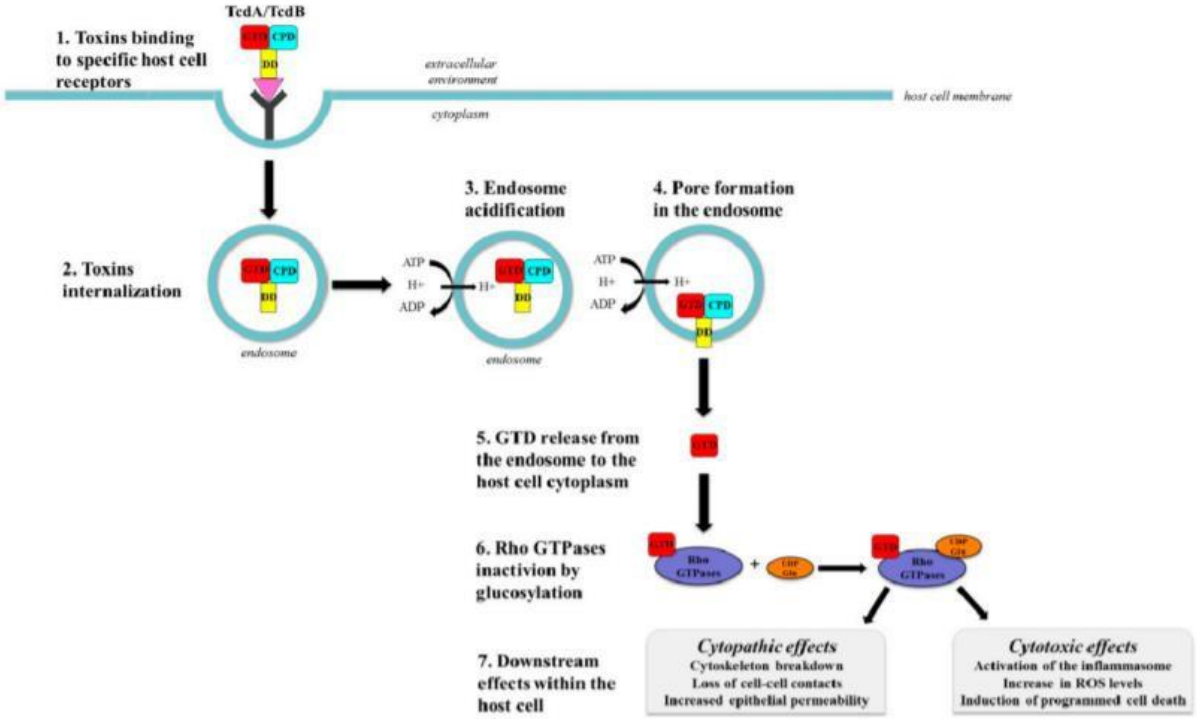


Figure 1.5 Mechanisms of action of TcdA and TcdB.

(Retrieved from (Di Bella *et al.*, 2016))**.** Seven main mechanisms of toxin delivery into epithelial cells. 1) toxin binding to the host cell surface receptor; 2) toxins internalization via receptor-mediated endocytosis; 3) endosome acidification via cysteine protease activity; 4) pore formation; 5) GTD release from the endosome to the host cell cytoplasm; 6) Rho GTPases inactivation by glycosylation; 7) downstream effects within the host cell such as toxins-induced cytokine IL-1β production. The colour codes are as follows: N-terminal glycosyltransferase domain (red), cysteine protease domain (blue), delivery domain (yellow).

1.3.1.2. *C. difficile* binary toxins

In addition to TcdA and TcdB, some *C. difficile* strains also produce a third toxin, binary toxin (Popoff *et al.*, 1988). This is a member of the family of clostridial binary toxins, which have ADP-ribosyltransferase activity, and include the *C. perfringens* iota toxin, *Clostridium spiroforme* toxin and *Clostridium botulinum* C2 toxins C and D (Popoff *et al.*, 1988, Perelle *et al.*, 1997). *C. difficile* binary toxin is encoded on the binary toxin (CDT) locus which consists of two toxin genes *cdtA* and *cdtB* as well as a positive transcriptional regulator *cdtR* (Figure 1.6) (Carter *et al.*, 2007). The toxin consists of two peptides, CdtB which binds an unknown surface receptor and translocates CdtA, the catalytic domain. CdtA ribosylates actin molecules and disrupts the cytoskeletons of the epithelial cells (Popoff *et al.*, 1988). PaLoc negative, CDT positive *C. difficile* strains can cause cytopathic effects (CPE) on cell lines *in vitro* and colonize hamster models *in vivo* (Geric *et al.*, 2006). It is possible that CDT increases *C. difficile* adherence to the epithelial cells by forming microtubule-based membrane structures (Schwan *et al.*, 2009).

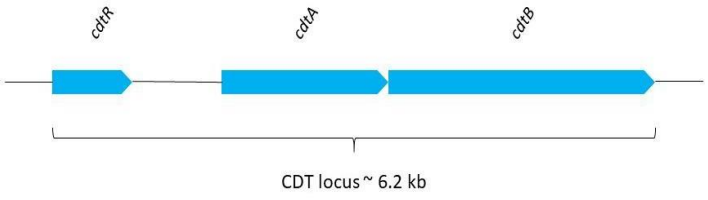


Figure 1.6 Schematic representation of CDT locus in *C. difficile*.

ORFs are represented by blue arrows. The complete locus is 6.2 kb long. The binary toxin consists of a binding component (CDTB) and an enzymatic component (CDTA).

1.3.2. Surface layer and cell wall proteins

The surface of many prokaryotic cells is covered by a proteinaceous layer, known as surface layer (S-layer) forming a two-dimensional structure which is visible by electron microscopy. S-layer is found on both Gram-positive and Gram-negative bacteria and is comprised of one or more types of S-layer proteins (SLP). SLPs play important roles in bacterial growth, survival, immune system and interactions with the host (Calabi *et al.*, 2001, Mori & Takahashi, 2018).

The crystalline or paracrystalline S-layer of *C. difficile* is composed of two distinct SLPs: high- molecular-weight SLP (HMW-SLP ~ 40 kDa) and low-molecular-weight SLP (LMW- SLP ~ 35 kDa) (Figure 1.7) (Sarker & Paredes-Sabja, 2012). A single gene, *slpA* that is conserved amongst all *C. difficile* strains, encodes a precursor protein SlpA which has three domains: (i) N-terminal signalling domain, (ii) highly variable LMW region, (iii) highly conserved HMW region (up to 97% sequence identity between the strains) (Fagan & Fairweather, 2011). The signalling domain guides the translocation of the SlpA across the cell membrane via an accessory Sec system (Fagan & Fairweather, 2011). The precursor protein is then cleaved by cysteine protease Cwp84 present in the cell wall (Kirby *et al.*, 2009) to generate two SLPs which later self-assemble to form the mature S-layer (Figure 1.7) (Kirk *et al.*, 2016).

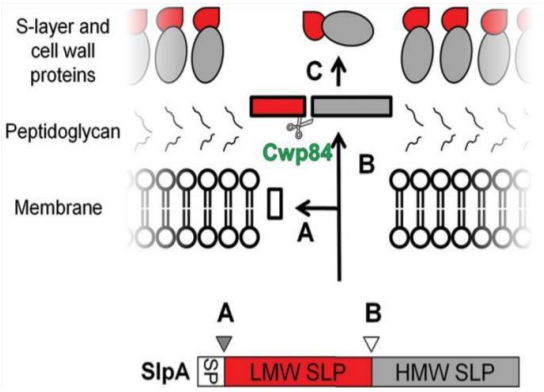


Figure 1.7 S-layer protein complex in *C. difficile.*

(Retrieved from (Awad *et al.*, 2014)**.** Figure shows the maturation steps of the SlpA protein. Three stages are shown: A) the removal of the signal peptide, B) cleavage of SLP by the protease Cwp84 to generate HMW SLP and LMW SLP, C) the formation of the S-layer matrix by re-association of the LMW and HMW SLPs.

The *slpA* locus (36.6 kb in strain 630) contains 11 *slpA* paralogs and there are 17 more paralogs throughout the genome (Sebaihia *et al.*, 2006, Fagan *et al.*, 2011, Monot *et al.*, 2011). In total, the *C. difficile* genome encodes 28 paralogues of SlpA that make up the clostridial cell wall protein (CWP) family (Kirk *et al.*, 2016). All these genes encode proteins with an N-terminal signal peptide and three putative cell wall binding domains with significant similarity to HMW SLP (Calabi *et al.*, 2001, Karjalainen *et al.*, 2001, Bradshaw *et al.*, 2018). Sequencing results of 57 *C. difficile* strains revealed that a 10 kb cassette within the S-layer locus has higher inter-strain variability compared to the rest of the locus (Figure 1.8) (Dingle *et al.*, 2013).

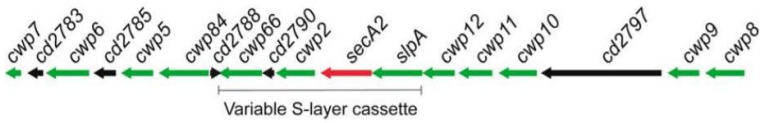


Figure 1.8 The S-layer locus.

(Retrieved from (Kirk *et al.*, 2016)**.** *C. difficile* strain 630 encodes 29 cell wall proteins. Twelve of these, including the S-layer precursor SlpA, are encoded within a single genomic locus (green arrows) that also encodes the S-layer secretion ATPase SecA2 (red arrow) and five unrelated proteins (black arrows). The variable S-layer cassette region is highlighted.

Cwp84, a cysteine protease involved in SlpA maturation, is so far the best characterized cell wall protein in *C. difficile* (Bradshaw *et al.*, 2015). This protein plays a significant role in SlpA processing since a *cwp84* insertional knockout strain was shown to be unable to cleave the SlpA precursor (Kirby *et al.*, 2009). Cwp84 is also able to degrade extracellular matrix proteins *in vitro*, indicating that it maybe involved in tissue degradation and bacterial dissemination during infection (de la Riva *et al.*, 2011). However, a *cwp84* mutant was shown to be fully virulent in a hamster model and complete protection was never achieved when Cwp84 was used as an immunization agent (Kirby *et al.*, 2009, Sandolo *et al.*, 2011). A second cysteine protease, Cwp13 is a paralogue of Cwp84, sharing 63% amino acid identity (de la Riva *et al.*, 2011). Cwp13 plays a role in the processing of Cwp84 but is not essential in Cwp84 activity or SlpA processing (de la Riva *et al.*, 2011). It is possible that Cwp13 is involved in the removal of damaged or misfolded proteins on the cell surface (de la Riva *et al.*, 2011).

Another cell wall protein involved in adherence is Cwp66 (66 kDa) containing a domain homologous to *Bacillus subtilis* autolysin, CwlB. Cwp66 was detected with antibodies raised against the surface proteins of heat-shocked bacteria, indicating that it plays a role as a surface-associated heat-shock protein (Waligora *et al.*, 2001).

The largest characterized member of the cell wall protein family that is encoded outside the S- layer cassette is CwpV (167 kDa). This protein has a phase variable expression, with only 5% of cells in a population expressing the protein *in vitro* (Emerson et al., 2009). CwpV plays a potential role in bacterial interaction and biofilm formation as well as protection against bacteriophage infection by preventing phage DNA replication (Emerson *et al.*, 2009, Reynolds *et al.*, 2011, Sekulovic *et al.*, 2015).

1.3.3. Polysaccharides

Three cell surface polysaccharides with immunogenic properties, PSI, PSII and PSIII are present in *C. difficile* strains, with ribotype 027 expressing the PSII most abundantly (Monteiro *et al.*, 2013). Investigations into vaccine production are mainly focused on these polysaccharides, particularly PSII. Glycan microarrays carrying synthetic PSI, PSII and PSIII detect IgG in the sera of CDI patients, and anti-PSII IgA has been detected in the faeces of patients (Martin *et al.*, 2013, Awad *et al.*, 2014). A vaccine candidate based on the PSII glycoprotein conjugated to recombinant TcdA and TcdB fragments has been shown to raise antibodies against PSII and *C. difficile* toxins in animal models. Therefore, it is suggested that vaccines containing a combination of polysaccharides and toxin antigens could be successfully manufactured in the future (Romano *et al.*, 2014).

1.3.4. Flagella

Flagella play an important role in host invasion, colonization and biofilm formation. The two best characterized *C. difficile* flagellar proteins are FliC, the major flagellin structural monomer, and FliD, the cap protein (Tasteyre *et al.*, 2000, Tasteyre *et al.*, 2001). The role of flagella in CDI is strain dependent. For example, flagella are important in the epidemic PCR ribotype 027 strain R20291 adhesion; however, the absence of flagella in strain 630Δ*erm* does not reduce the adherence (Dingle *et al.*, 2011, Baban *et al.*, 2013).

1.3.5. Fibronectin-binding proteins

Fibronectin is a large molecular weight glycoprotein involved in bacterial adhesion. *C. difficile* has a 68 kDa fibronectin-binding protein (Fbp68) which binds to extracellular matrix components such as fibronectin, fibrinogen and vitronectin (Cerquetti *et al.*, 2002). The precise role of Fbp68 in *C. difficile* pathogenesis is not completely understood. A *C. difficile* strain 630 *fbp68* mutant was unexpectedly shown to adhere more effectively *in vitro* than the wild-type strain. *In vivo* analysis demonstrated that an *fbpA* mutant was able to be shed in faeces at the same rate as wild-type strain in monoxenic mice but its colonization of caecal cells was reduced. The same result was observed when the experiment was performed on human-microbiota associated mice, suggesting that Fbp68 is primarily involved in *C. difficile* colonization (Barketi-Klai *et al.*, 2011).

Another Fibronectin-binding protein, ZmpI with sequence similarity to *Bacillus anthracis* lethal factor was shown to play a role in invasiveness and spread of *C. difficile* during infection (Cafardi *et al.*, 2013).

A collagen binding protein, CbpA was also found in *C. difficile* and shown to bind to collagens I and V, the most common collagen components present in many tissues including the gut (Péchiné *et al.*, 2018).

1.4. *C. difficile* epidemiology

1.4.1. Typing method

Restriction endonuclease analysis (REA), pulsed field gel electrophoresis (PFGE), PCR ribotyping, toxinotyping and multilocus sequence typing (MLST) are the most common typing methods to study *C. difficile*. REA and PFGE methods are based on the DNA patterns after restriction digestion (Clabots *et al.*, 1993, Gal *et al.*, 2005). PCR ribotyping is based on the fact that the intergenic spacer region between the 16S and 23S rRNA is different between the multiple pairs of alleles in an individual cell. The presence of multiple copies of these genes in most bacteria results in PCR products of various sizes when PCR is performed by using universal primers. Closely related isolates show identical patterns; however, distant isolates show variations which help to categorize the isolates (Cartwright *et al.*, 1995). Toxinotyping is a method in which PCR amplification of the PaLoc fragments restricted with one to four endonucleases is performed to identify restriction fragment length polymorphisms of the products (Rupnik *et al.*, 1998). In MLST, six to seven housekeeping genes are sequenced to reveal the genetic relationship between strains (Lemee *et al.*, 2004). Although PCR ribotyping is the preferred method for genotyping *C. difficile* (Janezic, 2016), whole genome sequencing (WGS) has been proved to be more efficient and accurate to distinguish between strains that have been categorized as indistinguishable by conventional typing methods (Yuan Kong *et al.*, 2016).

1.4.2. Epidemiology of CDI and hypervirulence

The occurrence, clinical presentation, severity and epidemiology of CDI have changed significantly over the last decade and *C. difficile* has become a major clinical problem (Legenza *et al.*, 2018). Healthcare associated infections (HCAIs) are a significant financial and social burden (Plowman *et al.*, 2001). Because of the rising incidence of CDI and associated mortality, a mandatory surveillance was announced in 2004 in the UK to facilitate epidemiological monitoring of *C. difficile* strains particularly those isolated from the healthcare units (Goldenberg *et al.*, 2011). *C. difficile* has historically been known as a nosocomial pathogen associated with prolonged antibiotic exposure. However, community-acquired *C. difficile* infections have been observed in populations previously considered low risk, such as healthy peripartum women, children, antibiotic naïve patients, and those with minimal or no recent healthcare exposure (Wilcox *et al.*, 2008, Depestel & Aronoff, 2013). As CDI initially emerged as a nosocomial infection in 1978 and became more prevalent in the community, creative strategies to reduce the risk factors are urgently required (Al-Jashaami & DuPont, 2016).

Reports of epidemic *C. difficile* outbreaks in the UK and other western countries have dramatically increased since 2000 (McDonald *et al.*, 2005, Warny *et al.*, 2005). The isolates from these outbreaks were all shown to be more virulent, secreting higher levels of toxins, more resistant to fluoroquinolones and more involved in CDI recurrence (Kuijper *et al.*, 2006). Most of these isolates were categorized as ribotype 027 and reported to be hypervirulent (Razavi *et al.*, 2007). As described earlier, the PaLoc region harbours genes such as *tcdC* and *tcdR* to regulate the expression of the *tcdA* and *tcdB* (Mani & Dupuy, 2001). Ribotype 027 strains have an 18 bp deletion in

the *tcdC* region which leads to the production of a truncated protein and disruption in the regulation of toxin genes (Warny *et al.*, 2005). In 2010 in the UK, hypervirulent strains isolated from patients all belonged to the ribotype 027 (Cartman *et al.*, 2010); however, in Netherlands, an emergence of isolates belonging to the PCR ribotype 078 was reported. Ribotype 078 contains a 39 bp deletion in the *tcdC* gene as well as an early stop codon (Goorhuis *et al.*, 2008). Although both ribotypes, 027 and 078 have deletions in the *tcdC* gene, the role of this gene in the emergence of hypervirulent strains is not clear since complementation of the *tcdC* mutation has given inconclusive results (Carter *et al.*, 2011, Cartman *et al.*, 2012). The emergence of other hypervirulent isolates was reported in China (ribotype 002), Germany (ribotype 001) and Italy (ribotype 018) (Borgmann *et al.*, 2008, Spigaglia *et al.*, 2010, Cheng *et al.*, 2011). Therefore, hypervirulence in *C. difficile* is no longer considered to be limited to ribotype 027. It has been revealed that some isolates which had previously been designated ribotype 027 were in fact ribotypes 176, 198 and 244. These ribotypes are highly similar to the ribotype 027; however, deletions in the *tcdC* regions of these ribotypes have never been reported (Valiente *et al.*, 2012).

The elevated production of the TcdA and TcdB toxins has been reported *in vitro* for ribotype 027 (Warny *et al.*, 2005). However, a study using a human gut model showed that the production of toxins by ribotype 027 strains is not higher per unit of time, but the duration of toxin production is prolonged, which may lead to the increased severity of symptoms for patients infected with a ribotype 027 isolate (Freeman *et al.*, 2007). It was also reported that ribotype 027 isolates have a higher level of sporulation (Merrigan *et al.*, 2010). However, another study showed that the rate of sporulation varies between strains within a ribotype, and increased or decreased sporulation has been observed for both epidemic and non-epidemic ribotypes.

Therefore, sporulation is no longer considered as a specific characteristic of hypervirulent isolates (Burns *et al.*, 2011). Although a significant difference in the epidemiology of CDI has been noticed over the past two decades, no solid criteria have yet described the hypervirulence. Therefore, most reports simply refer to them as epidemic strains (Robinson *et al.*, 2014).

Between 2008 and 2016 the number of *C. difficile* related deaths in England and Wales has decreased gradually in both men and women, most likely due to the improvements in hospital procedures and better management (Figure 1.9).

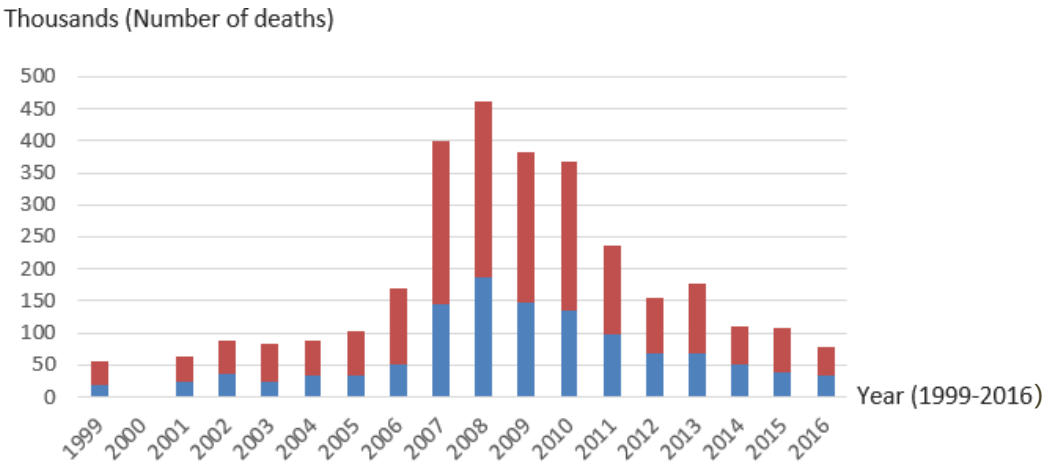


Figure 1.9 Number of death certificates with *C. difficile* mentioned, by sex, in England and Wales, deaths registered between 1999 and 2016.

Graph drawn based on the data provided by Office for National Statistics website. Release date: 30 October 2017. Colour codes: blue represents men and red represents women (Adapted from (Windsor-Shellard, 2017)).

1.5. Horizontal gene transfer via mobile genetic elements

Horizontal gene transfer (HGT) involves DNA transfer from donor cells to recipient cells, followed by replication (in the case of plasmids) and integration (in the case of transposons and bacteriophages). Therefore, it differs from vertical transmission of the genes from parent to offspring (Burmeister, 2015). It was first described in microorganisms in the late 1940s (Tatum & Lederberg, 1947), and has long been recognized as an important factor in the evolution of bacteria and archaea. Mobile genetic elements (MGEs), which include cytoplasmically located replicating plasmids and chromosomally located transposons and genomic islands, are important mediators of HGT. The three main mechanisms of HGT are transformation, transduction and conjugation.

1.5.1. Transformation

The first identified HGT mechanism was transformation, which is a direct uptake of exogenous DNA from the environment. It was first observed in *S. pneumoniae* that became virulent when exposed to heat-killed virulent cells (Griffith, 1928). This was confirmed later by demonstrating that nonvirulent strains acquired virulence when inoculated together with the extracted DNA from heat-killed virulent strains (Avery *et al.*, 1944). DNA uptake occurs during the competence state of bacteria, which can occur naturally or can be artificially induced. A total of 82 species are known to be naturally transformable, with an approximately equal number between Gram-negative and Gram-positive bacteria (Johnston *et al.*, 2014).

The competence stage of naturally transformable bacteria is usually triggered by specific conditions such as pH and nutrient availability or at a specific phase of bacterial growth (Wilson & Bott, 1968, Solomon & Grossman, 1996). The mechanisms of bacterial transformation are similar between transformable bacteria. Conserved proteins encoded by genes that form the *com* regulon are expressed during the competence state. Double-stranded DNA (dsDNA), as a substrate for transformation, is bound and captured by a transformation pilus (Johnston *et al.*, 2014). The binding sites on competent cells vary among bacteria (20- 50 sites in *B. subtilis*, 33-75 sites in *S. pneumoniae* and 4-8 sites in *Haemophilus influenzae*) (Singh, 1972, Deich & Smith, 1980, Dubnau, 1999). The captured dsDNA is then internalised as single-stranded DNA (ssDNA) through a transmembrane channel. As Gram-negative bacteria contain two layers of membrane, there are two transmembrane channels, one for each layer, while Gram-positive bacteria use only one transmembrane channel (Fig 1.10).

Once the DNA enters the cell, it could be degraded to nucleotides that can be used in other metabolic functions such as DNA replication. The internalised ssDNA could be recombined into the bacterial genome through homologous recombination (Johnston *et al.*, 2014). The internalised ssDNA is bound by DNA processing protein A (DprA), which then recruits RecA recombinase to the DNA and promotes recombination (Mortier-Barriere *et al.*, 2007). In the case of plasmids, if it contains an origin of replication that can be recognised by the host, recircularisation and second strand synthesis will occur and result in plasmid maintenance (Thomas & Nielsen, 2005).

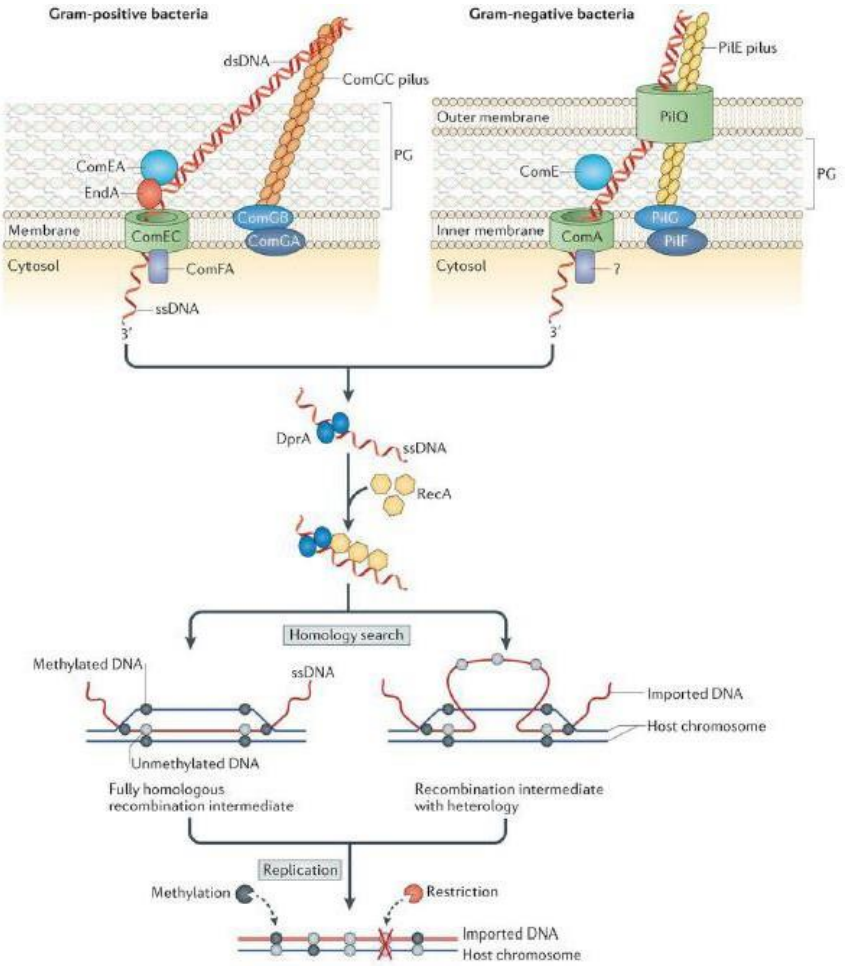


Figure 1.10 The processes of transformation in Gram-negative and Gram-positive bacteria.

(Retrieved from (Johnston *et al.*, 2014)**.** The exogenous double-stranded DNA (dsDNA) is captured by a transformation pilus (Tfp), which consists mainly of ComGC and PilE subunits in Gram-positive and Gram-negative bacteria, respectively. dsDNA is transported into cytosol via transmembrane channels (ComEC for Gram-positive bacteria, and PilQ and ComA for Gram-negative bacteria). The captured dsDNA is internalised as single-stranded DNA (ssDNA) into the cytosol, which is then bound by DNA processing protein A (DprA). The recombinase RecA multimers assemble on ssDNA and promotes homologous recombination.

1.5.2. Transduction

Transduction is a mechanism of HGT during which DNA from a phage-infected bacterium is transferred into a recipient cell via bacteriophage. It was discovered by Lederberg and Zinder (1952), who showed the transfer of chromosomal DNA from one strain of *Salmonella typhimurium* to another mediated by phage P22. By having phage as a vector, this mechanism does not require a cell-to-cell contact, and the donor DNA can be protected from physical and chemical agents (Calero-Caceres & Muniesa, 2016). There are two types of transduction: generalized and specialized. In generalized, bacteriophages can pick up any portion of the host DNA, whereas in specialized, they only pick up specific parts of the host DNA (Figure 1.11) (Wagner & Waldor, 2002). The transferable DNA sequences range from chromosomal DNA to MGEs such as plasmids, transposons and genomic islands (Brown-Jaque *et al.*, 2015).

The first report of the bacteriophage-mediated antibiotic resistance gene transfer between *C. difficile* strains was published in 2013. In this work, an erythromycin resistance gene, *erm*(B) located on a mobile element, Tn*6215* was transferred from strain CD80 into CD062. The transfer was confirmed to be mediated by a bacteriophage called *Φ*C2 since a control mixture containing CD062 and CD80 filtrate without *Φ*C2 did not give rise to erythromycin-resistant derivatives of strain CD062 (Goh *et al.*, 2013).

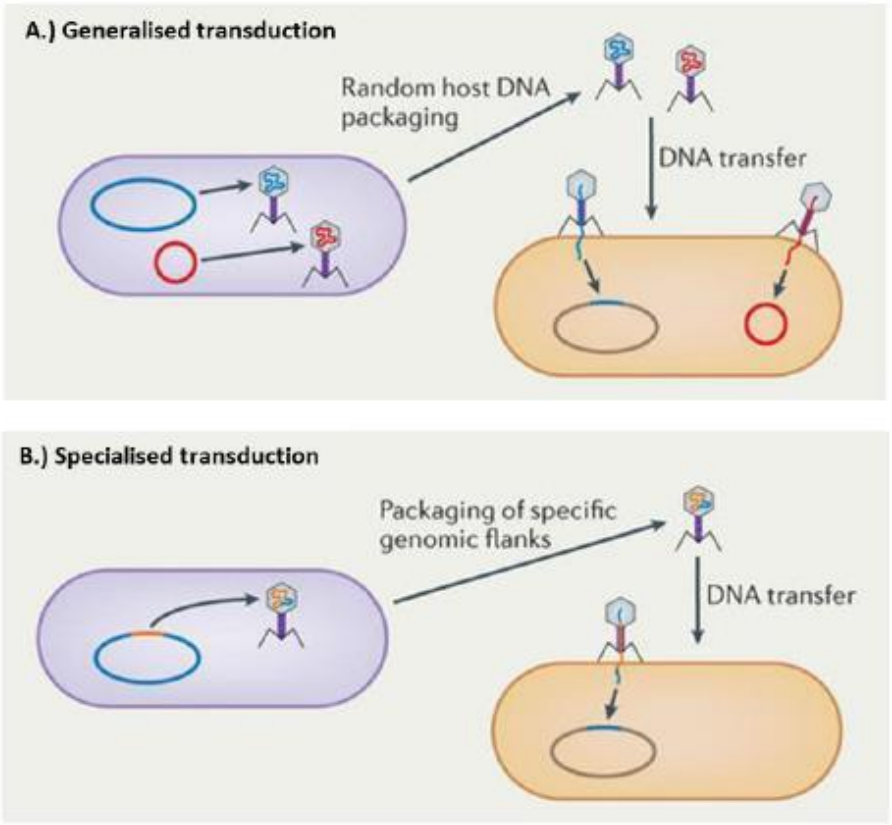


Figure 1.11 Types of transduction.

(Retrieved from (Salmond & Fineran, 2015)**.** A) Generalised transduction is the transfer of random DNA fragments of the host (shown in blue) to the neighbouring bacteria. B) Specialised transduction is the transfer of prophage DNA (shown in orange) and the bacterial DNA, located next to the prophage DNA (shown in blue) to a new recipient cell.

1.5.3. Conjugation

Conjugation is a form of HGT between bacterial cells that occurs via a close contact between donor and recipient (cell-to-cell contact). The process was first discovered in *Escherichia coli* for the fertility plasmid (F-plasmid) (Hayes, 1953). In 1947, it was shown that when two auxotrophic mutant strains of *E. coli* with specific nutritional requirements were grown together in a mixed culture, recombination of the mutant strains could occur which resulted in prototrophic isolates (Tatum & Lederberg, 1947). The recombination was mediated by a fertility factor (F-factor) present in at least one of the bacteria to facilitate the formation of mating pair (Lederberg *et al.*, 1952, Hayes, 1953). The F-factor was believed to be an autonomously replicating element capable of converting an F^- recipient strain into an F^+ donor strain and encoding all the proteins required for the process (Hayes, 1953, Adelberg & Pittard, 1965). The F-plasmid was also able to integrate into the chromosomal DNA leading to high frequency recombination (Hfr). This process leads to a transfer of part or even the entire donor chromosome which can recombine with the recipient chromosome after mating (Adelberg & Pittard, 1965). Further analysis on the recombinant cells revealed that linear DNA was transferred and integrated into the recipient genome. Some genes on the chromosome were transferred with higher frequencies than others depending on their distance from the origin of transfer (*oriT*) (Adelberg & Pittard, 1965). Hfr has been found in several Gram-negative bacteria such as *E. coli* (Lloyd & Buckman, 1995), *Rhodobacter sphaeroides* (Suwanto & Kaplan, 1992) and *Yersinia pseudotuberculosis* (Lesic *et al.*, 2012).

In Gram-positive organism *Lactococcus lactis*, a plasmid encoded lactose fermenting enzyme is present which turns lactose fermentation negative strains to strains with the

ability to ferment lactose (Gasson & Davies, 1980). It was demonstrated that conjugation occurred with higher frequencies in *L. lactis* cells with a clumping phenotype associated with CluA, a membrane bound protein encoded by a chromosomally located sex factor. The lactose fermentation plasmid itself was able to integrate into the chromosomal DNA within the sex factor region through recombination by the IS- element carried by the plasmid (Gasson *et al.*, 1992). The sex factor was responsible for the unidirectional transfer of the chromosomal DNA in an Hfr-like manner as genes located close to the sex factor in a clockwise orientation transferred with a higher frequency than those located further away from the *oriT* and in an anticlockwise orientation (van Kranenburg & de Vos, 1998).

1.5.3.1. Conjugative plasmids

Conjugative plasmids are self-replicating, double-stranded and generally circular units of DNA; however, examples of linear plasmids are also known including those found in Gram-positive filamentous bacteria of the genus *Streptomyces* (Lee *et al.*, 2011). For many years, conjugative plasmids have been associated with the dissemination of antibiotic resistance genes amongst bacterial species (Palmer *et al.*, 2010).

A sophisticated multiprotein secretion apparatus called a type IV secretion system (T4SS) is used by most conjugative plasmids to transfer DNA from donor to recipient (Smillie *et al.*, 2010). There are similarities among bacterial species at the initial steps of conjugation in which relaxase is required for preparing ssDNA and beginning the process of transfer (Zechner *et al.*, 2012). However, the actual translocation process including DNA transfer through the donor cell envelope and insertion into the recipient is different between Gram-positive and Gram-negative bacteria due to differences in

the structure of their cell envelopes (Goessweiner-Mohr *et al.*, 2014). The mechanism of T4SS in many Gram-positive bacteria involves the passage of ssDNA, except for the *Actinomycetales,* in which dsDNA is transferred (Vogelmann *et al.*, 2011).

Conjugation is initiated by close contact between a suitable donor and a recipient (Lawley *et al.*, 2003). Some Gram-positive bacteria can stimulate mating by producing a pheromone-like peptide as observed in many enterococcal strains (Hirt *et al.*, 2002, Chandler & Dunny, 2004). Once contact is established, a mating signal is generated, and the relaxase binds to the dsDNA and forms a relaxosome which is a complex of proteins that facilitates transfer by binding to the DNA (Smith & Thomas, 2004). This nucleoprotein complex nicks the plasmid DNA at a specific site known as origin of transfer (*oriT*) to give a single-stranded substrate suitable for transfer via mating pair formation (mpf) (Grohmann *et al.*, 2003).

Relaxase is the key enzyme to initiate conjugation and functions by catalysing a break in a specific phosphodiester bond in the *nic* site within the transfer origin, *oriT* (Grohmann *et al.*, 2003). Comparisons among a wide range of *oriT* regions have revealed that they all carry inverted repeats (IRs) that flank the *nic* site. These IR sequences form a hairpin structure to be recognized and cleaved by the relaxosome. The sequence of the IRs varies among the conjugative or mobilisable elements but their location relative to the *nic* site is similar (Guiney & Yakobson, 1983, Goessweiner-Mohr *et al.*, 2014). The most widely distributed relaxase belongs to the broad host range plasmid RP4/ RK2, first isolated in connection with an outbreak of antibiotic resistance in *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* strains in Birmingham in 1969 (Ingram *et al.*, 1973, Zatyka & Thomas, 1998).

Proteins involved in T4SS are all encoded by one or more transfer (*tra*) operons. Two major Tra proteins, TraE and TraJ have ATPase activities essential for fuelling the DNA translocation process and assembling the mating pair formation. TraJ binds to TraI and together they position TraJ to the cell membrane through protein-protein interaction. Therefore, TraI and TraJ form a two-partner T4SS coupling protein which is crucial in all T4SSs. TraJ also interacts with the relaxase, further confirming its important role as a coupling protein to link the relaxosome to the transfer channel (Goessweiner- Mohr *et al.*, 2014). Another Tra protein for which an absolute requirement in T4SS has been confirmed is peptidoglycan hydrolysing protein, TraG which is the first PG- hydrolysing protein characterized (Arends *et al.*, 2013). Other important Tra proteins such as TraM, TraL and TraO are involved in channel formation and adhesion (Figure 1.12) (Goessweiner-Mohr *et al.*, 2014).

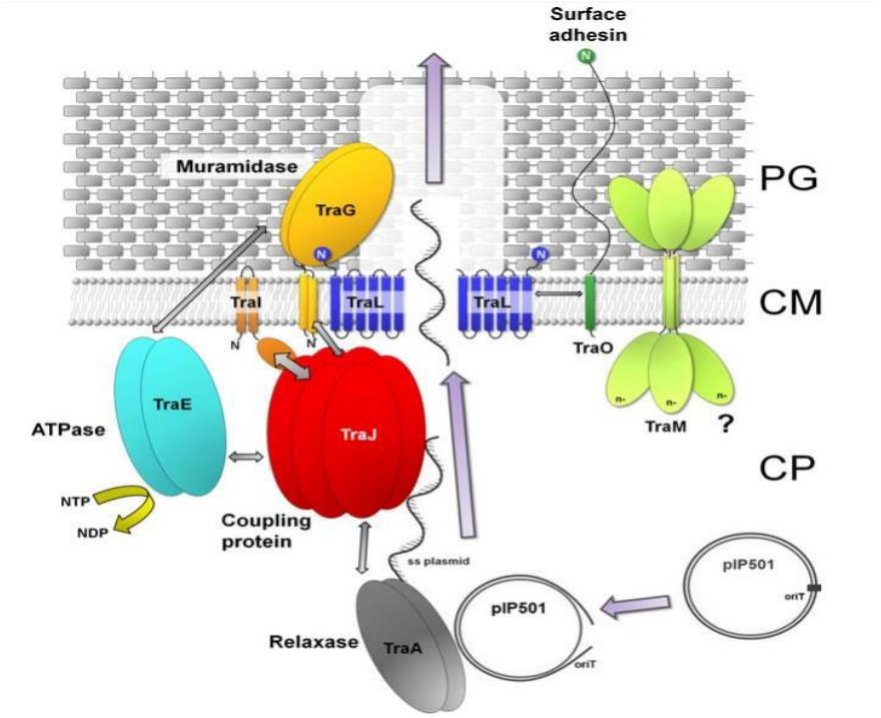


Figure 1.12 Schematic representation of T4SS in conjugative plasmid pIP501 isolated from *S. agalactiae* as a model for T4SS in Gram-positive bacteria.

(Retrieved from (Goessweiner-Mohr *et al.*, 2014)**.** First, TraA relaxase binds to the *oriT*. After being cleaved at the *nic* site, the single-stranded plasmid DNA is engaged with the transfer channel via TraJ-TraI coupling proteins. TraG hydrolyses the peptidoglycan layer to form punches (faded grey colour). The N-terminus of the T4SS proteins is marked (N). Arrows indicate protein-protein interactions. The thickness of the arrows shows the strength of the interactions. PG, peptidoglycan; CM, cytoplasmic membrane; CP, cytoplasm.

1.5.3.2. Conjugative transposons

Conjugative transposons (CTns), also known as integrative and conjugative elements (ICEs), can transfer into a recipient via conjugation and integrate into the host genome through site-specific recombination (Burrus *et al.*, 2002). They excise from the host genome and form a circular DNA molecule during transposition and conjugation (Scott *et al.*, 1988). The circular intermediate can then insert back into the site from which it has excised, transpose into a different site within the same chromosome, transfer to a recipient via conjugation or be degraded and lost from the host cell (Figure 1.13) (Clewell *et al.*, 1995). Like conjugative plasmids, CTns contain an *oriT* (Jaworski & Clewell, 1995). Nevertheless, unlike the conjugative plasmids, their replication occurs while integrated in the chromosome using the replication machinery of the host. However, a study by (Lee *et al.*, 2010) demonstrated that ICE*Bs*1, a CTn originally found in *B. subtilis,* replicates in a plasmid-like manner when it is in the circular state. Moreover, conjugative transposon Tn*916* can replicate autonomously using the rolling-circle mechanism. The *oriT* in Tn*916* functions as the origin of replication where the Tn*916*-encoded relaxase initiates the replication (Wright & Grossman, 2016).

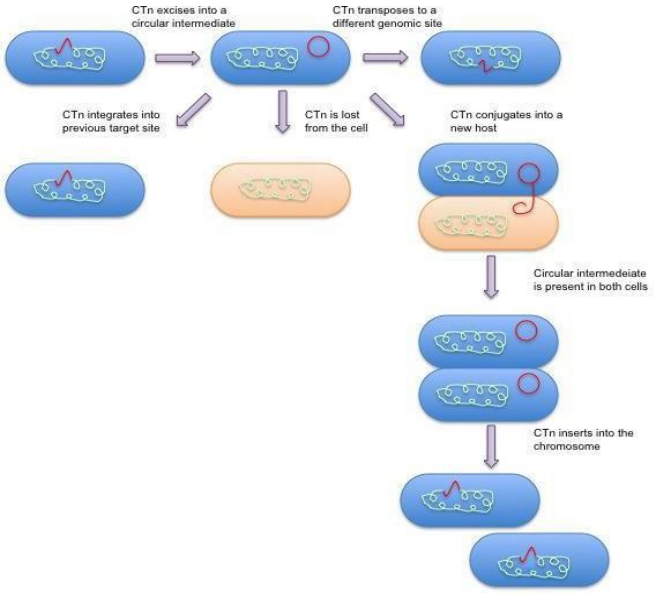


Figure 1.13 Schematic diagram of CTn fate.

(Retrieved from (Brouwer, 2013))**.** The chromosome is represented by a green circular molecule. The linear CTn integrated in the chromosome is represented by a red line. The circular intermediate of the CTn is represented by a red circle. A single strand of the CTn, nicked at the *oriT*, is transferred from donor into recipient. Blue cells represent a resistant phenotype, conferred by an antibiotic resistance gene on the CTn and orange cells represent a susceptible phenotype.

1.5.3.2.1. Tn*916*

Tn*916* is the first CTn discovered and was originally isolated from *Enterococcus faecalis* in 1981 by Franke and Clewell who characterized it as a transposon with the ability to transfer from plasmid-free donor strains into tetracycline-susceptible strains, and therefore it was hypothesized that this element was self-transmissible (Franke & Clewell, 1981). Since then, many Tn*916*-like elements have been discovered in several bacterial species and all have a similar backbone. Most of these elements confer resistance to tetracycline by encoding proteins responsible for protecting 30S ribosomal subunits in the mRNA translation complex (Roberts & Mullany, 2009). Tn*916*-like elements have a modular structure including accessory genes which are not involved in transfer and often encode functional proteins which contribute to the

environmental adaptability of the host (Roberts & Mullany, 2009). They can carry genes encoding antimicrobial peptides (Rauch & De Vos, 1992), adhesins, virulence associated genes (Seth-Smith *et al.*, 2012), chlorocatechol degradation (Ravatn *et al.*, 1998), and metabolism of sucrose, biphenyl and salicylate (Hochhut *et al.*, 1997, Nishi *et al.*, 2000). The accessory module is often flanked by genes for transcriptional regulation. Another functional module of the CTns is the integration module encoding the recombinase and excisionase proteins responsible for transposition of the element. The conjugation module encodes all the proteins required for conjugation (Figure 1.14, refer forward to page 57) (Roberts & Mullany, 2011).

1.5.3.2.2. Tn*1549*

Another family of CTns are Tn*1549*-like elements in which the conjugation module differs from Tn*916* (Figure 1.14, refer forward to page 57). Tn*1549* was originally discovered in *E. faecalis* strain 268-10 where it is integrated in the plasmid pAD1. The element carries the *vanB* operon conferring vancomycin resistance (Garnier *et al.*, 2000). Transfer of Tn*1549*- like elements from *Clostridium symbiosum* into *Enterococcus faecium* and *E. faecalis* has been shown in animal models (Launay *et al.*, 2006).

1.5.3.2.3. Excision and integration by site-specific recombination

In CTns, the process of site-specific recombination including excision and integration is mainly mediated by two protein families: serine or tyrosine recombinases (Grindley *et al.*, 2006). The latter mediates the integration and excision of Tn*916* (Taylor & Churchward, 1997, Garnier *et al.*, 2000). Excision begins with endonucleolytic cuts at

each end of the element generating single-stranded non-complementary hexanucleotides at each end called coupling sequences (Abbani *et al.*, 2005). The coupling sequences then join by covalent bonds forming a circular intermediate molecule with a heteroduplex at the joints. These heteroduplexes are then resolved by DNA repair or replication (Figure 1.15) (Caparon & Scott, 1989).

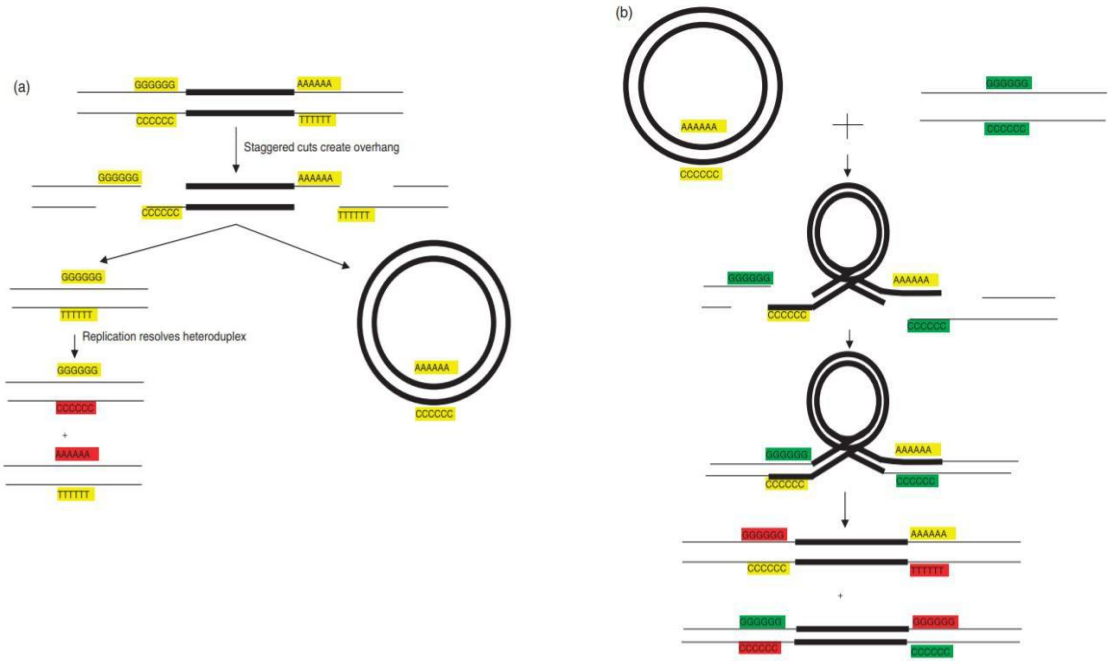


Figure 1.15 Tn*916* excision and integration.

(Adapted from (Caparon & Scott, 1989))**.** The thick lines are Tn*916* and the thin lines are the flanking regions. Nucleotides in yellow are from Tn*916* and those in green are from the target site. Nucleotides in red are the product of DNA repair through replication or mismatch repair. a) Excision: a staggered cut at the coupling sequence results in the excision of Tn*916* with non-complementary heteroduplex which is later resolved by DNA replication. b) Integration: staggered cleavage at the 3' end of the new target site and the heteroduplex at the joint of the circular intermediate molecule generates molecules that are subsequently ligated together, resulting in an inserted Tn*916* with heteroduplexes at each end. These are resolved by DNA replication resulting in two copies, each flanked by different DNA sequences.

1.5.3.3. Mobile genetic elements in *C. difficile*

1.5.3.3.1. Tn*5397*

Transfer of tetracycline resistance between *C. difficile* strains was first reported by Ionesco (1980) and Smith *et al.,* (1981). These workers showed that the transfer was resistant to DNase, could not be mediated by donor culture filtrate, and cell-to-cell contact was required, and therefore suggested that it was mediated by a conjugation-like mechanism (Ionesco, 1980, Smith *et al.*, 1981). When the first report of Tn*916* transfer in *E. faecalis* was published, it was hypothesized that the tetracycline resistance determinant could be encoded by a Tn*916*-like element (Franke & Clewell, 1981, Smith *et al.*, 1981). Transfer of the element from *C. difficile* strain 630 at a low frequency (5x 10^{-7} transconjugants per donor) was detected in the absence of a detectable plasmid (Wüst & Hardegger, 1983). Furthermore, a homology between this element and Tn*916* was confirmed via DNA hybridization in which at least six of seven *Hinc*II fragments of Tn*916* from *E. faecalis* hybridised with tetracycline resistant *C. difficile* strains. Therefore, a close relationship between Tn*916* and the tetracycline resistance determinant in *C. difficile* was suggested. Despite the great (95%) homology between two elements, differences were also observed such as the number of *Hind*III cleavage sites within the transposons (Hächler *et al.*, 1987). Finally, the element was shown to be a conjugative transposon and designated Tn*5397* (Mullany *et al.*, 1995). The sequencing also showed that Tn*5397* contained a group II intron; the first report of the presence of an intron in a conjugative transposon and the first report of a group II intron in Gram- positive bacteria (Mullany *et al.*, 1996). Despite bearing great structural similarities, the differences between Tn*916* and Tn*5397* are noticeable (Figure 1.14, refer forward to page 57).

A single serine recombinase, TndX is responsible for the entire recombination process of Tn*5397* (Wang *et al.*, 2000). When this element is integrated into the genome, it is flanked by a GA dinucleotide at each end. TndX recombinase mediates staggered cuts at these sites and results in the formation of dinucleotide overhangs at each end which then join after strand exchange with the target site to form the circular intermediate of Tn*5397* (Wang & Mullany, 2000).

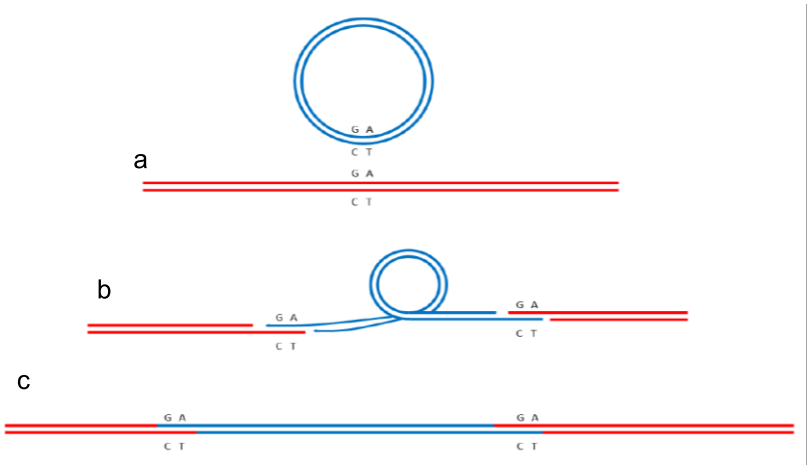


Figure 1.16 Schematic representation of Tn*5397* recombination.

(Adapted from (Wang & Mullany, 2000)) The blue lines represent Tn*5397* and the red lines represent the chromosomal DNA. The coupling sequences of the element and the specific target site are shown by the nucleotide pairs G-C and A-T, respectively. a) Tn*5397* is in the circular intermediate form and a suitable empty target site is present in the chromosome. b) Staggered cleavage of Tn*5397* and the chromosome by the serine recombinase TndX. c) Tn*5397* is ligated with the chromosomal DNA generating homoduplexes.

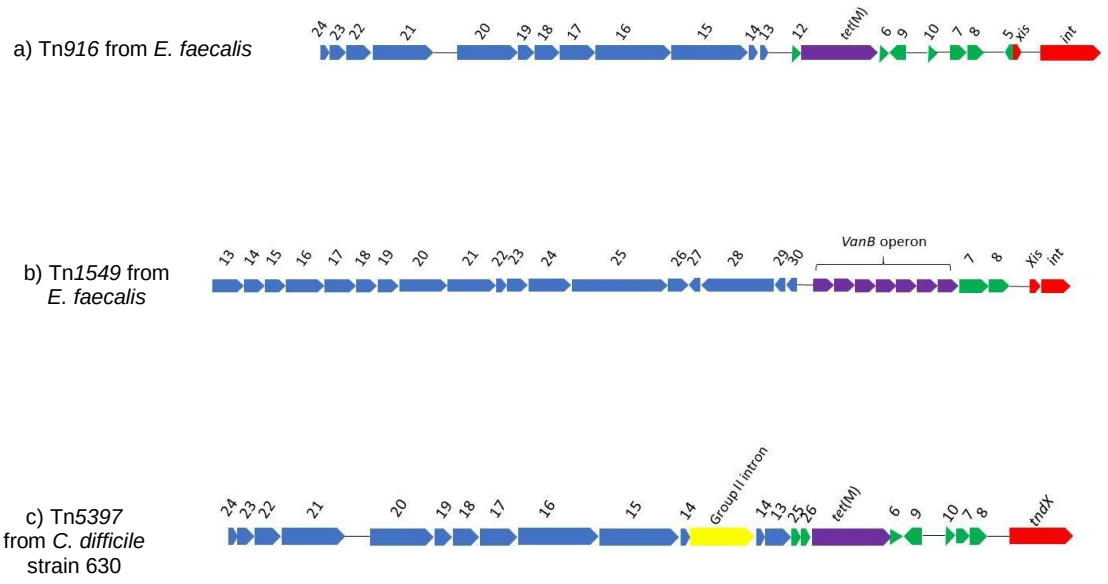


Figure 1.14 Schematic representation of Tn*916*, Tn*1549* and Tn*5397*.

a) Tn*916* from *E. faecalis*, blue arrows represent the ORFs of the conjugation module, green arrows represent the ORFs involved in transcriptional regulation, the purple arrow represents the *tet*(*M*) gene, red arrows represent the ORFs of the recombination module (integration and excision). b) Tn*1549* from *E. faecalis* strain 268-10, blue arrows represent the ORFs of the conjugation module, green arrows represent the ORFs involved in transcriptional regulation, the purple arrow represents the *vanB* operon, red arrows represent the ORFs of the recombination module. c) Tn*5397* from *C. difficile* strain 630, blue arrows represent the ORFs of the conjugation module, green arrows represent the ORFs involved in transcriptional regulation, the purple arrow represents the *tet*(*M*) gene, red arrow represents the ORF of the recombination module, yellow arrow represents the group II intron just downstream the *orf14.*

1.5.3.3.2. Other conjugative transposons in *C. difficile* 630

An investigation into the genome of *C. difficile* 630 revealed the presence of six other putative CTns (in addition to Tn*5397*) in this strain. The elements are named CTn*1*, CTn*2*, CTn*4*, CTn*5*, CTn*6* and CTn*7* based on their location within the genome. Tn*5397* was named CTn*3* even though mostly the initial designation is used in the literature and throughout this thesis (Sebaihia *et al.*, 2006). Like Tn*5397*, CTn*1*, CTn*6* and CTn*7* are related to Tn*916* (Figure 1.17). CTn*1* and CTn*7* both encode predicted ABC transporters on their accessory module; however, no substrates for these protein products have yet been identified. The accessory module of CTn*6* encodes several hypothetical proteins for which no clear roles have been shown. Both CTn*1* and CTn*6* elements encode for a tyrosine recombinase and excisionase which are predicted to mediate the recombination of their elements, whereas CTn*7* encodes a serine recombinase which presumably facilitates its recombination (Sebaihia *et al.*, 2006). CTn*2*, CTn*4* and CTn*5* all encoding ABC transporters on their accessory modules are part of the Tn*1549* family (Figure 1.18). CTn*4* encodes a tyrosine recombinase and excisionase for excision into the circular intermediate CTn and insertion into a suitable target site. CTn*2* and CTn*5* both encode serine recombinases for the recombination (Sebaihia *et al.*, 2006). Five of these six putative elements are predicted to encode ABC transporters; however, the substrate of these transporters have not yet been identified. It has been shown that the ABC transporter of CTn*7* has homology to magnesium ABC transporters (Sebaihia *et al.*, 2006).

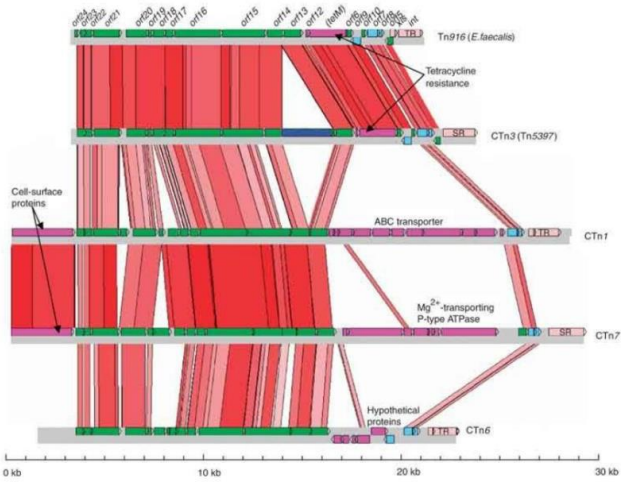


Figure 1.17 Comparison of Tn*916* to CTn*3* (Tn*5397*), CTn*1*, CTn*6* and CTn*7*.

(Retrieved from (Sebaihia *et al*., 2006))**.** Each module on the CTns is color coded: pink: recombination, purple: accessory function, green: conjugation, light blue: transcriptional regulation. The dark blue region in Tn*5397* is the group II intron. SR is serine recombinase, TR is tyrosine recombinase. Red lines between the elements represent protein similarity.

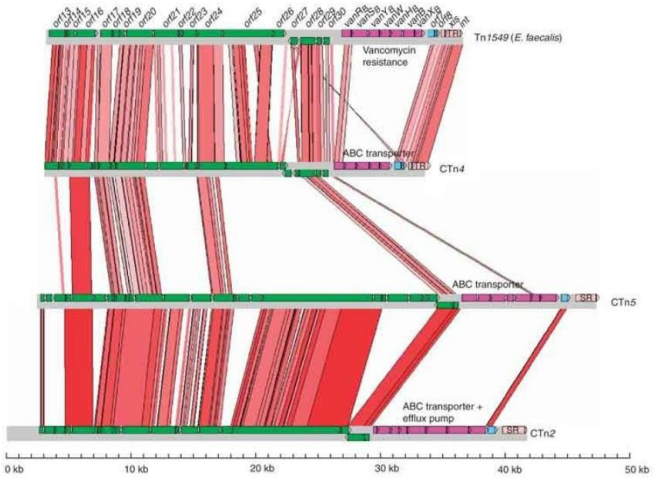


Figure 1.18 Comparison of Tn*1549* to CTn*2*, CTn*4* and CTn*5*.

(Retrieved from (Sebaihia *et al*., 2006))**.** Each module on the CTns is colour coded: pink: recombination, purple: accessory function, green: conjugation, light blue: transcriptional regulation. SR is serine recombinase, TR is tyrosine recombinase. Red lines between the elements represent protein similarity.

In order to determine whether these putative transposons are capable of excision, PCR reactions were performed using specific primer pairs to amplify junctions between the element and host genome, the joints of the element in a circular form and the repaired target site after excision. PCR products indicating the excision were produced with CTn*1*, CTn*2*, CTn*4*, CTn*7* (Sebaihia *et al.*, 2006, Brouwer *et al.*, 2011). CTn*6* is the only putative conjugative transposon in strain 630 for which no PCR product could be detected (Brouwer *et al.*, 2011). Furthermore, to investigate the transfer of these putative conjugative transposons, the ClosTron system (section 1.6.4) was used to mark the accessory modules of the transposons and create selectable markers. All marked elements (except for CTn*6*) transferred from strain 630 into the recipient strain CD37 at frequencies between 10^{-4} to 10^{-9} transconjugants per donor (Brouwer *et al.*, 2011).

1.5.3.4. Mobilisable elements in *C. difficile*

1.5.3.4.1. Tn*4453*

Mobilisable transposons are transposable genetic elements that also encode mobilisation functions but are not in themselves conjugative. They rely on co-resident conjugative elements to facilitate their transfer to recipient cells (Crellin & Rood, 1998). Chloramphenicol resistance in *C. perfringens* is conferred by a chloramphenicol acetyltransferase (CatP) protein that is encoded by the accessory module on a mobilisable transposon Tn*4451* (Lyras et al., 1998). An investigation into the *cat* gene in *C. difficile* strains revealed that this gene is located on a mobilisable transposon very similar to Tn*4451* and designated as *catD* (Wren *et al.*, 1988). The *catD* gene harbouring transposon in *C. difficile* has an 89% identity with the one in *C. perfringens*

and is designated Tn*4453* (Figure 1.19) (Wren *et al.*, 1988, Lyras & Rood, 2000). Insertion and excision of this element is mediated by a serine recombinase, TnpX. There are other genes located on the element including *tnpZ, tnpV, tnpY* and *tnpW*. The *tnpZ* gene is involved in mobilisation of the element and encodes the functional mobility (Mob) protein, but the function of the others is unknown (Adams *et al.*, 2002).

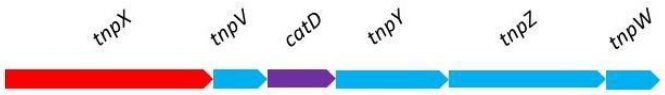


Figure 1.19 Schematic representation of Tn*4453*.

The red arrow represents *tnpX*, the purple arrow represents *catD.* Other ORFs are represented by blue arrows.

1.5.3.4.2. Tn*5398*

In addition to the transfer of tetracycline resistance from *C. difficile* 630 into *C. difficile* CD37, transfer of erythromycin resistance has also been observed at low frequencies (~ 10^{-8} per donor) (Wüst & Hardegger, 1983). It was later shown that the transfer of erythromycin resistance could also occur from *C. difficile* 630 into other bacterial species such as *Staphylococcus aureus* (Hächler *et al.*, 1987) and *B. subtilis* (Mullany *et al.*, 1995) in the absence of any detectable plasmid. Analysis of target sites of the element showed that it has a preferred insertion site in *C. difficile* but multiple sites in *B. subtilis* (Mullany et al., 1995). Thus, it was assumed that the element could be a conjugative transposon (Mullany *et al.*, 1995). Further studies revealed that *C. difficile* 630 contains two copies of adenine N-6- methyltransferase *erm*(B) genes designated

erm1(B) and *erm2*(B) separated by a 1.34 kb direct repeats (Figure 1.20) (Farrow *et al.*, 2000). Erm(B) confers erythromycin resistance through ribosomal protection but only *erm2*(B) is functional. An analysis to fully characterise the genomic arrangement of Tn*5398* revealed that this element does not encode genes for excision, integration or conjugation, and therefore does not fit into the standard category of conjugative, mobilisable or transposable elements (Farrow *et al.*, 2001). In addition to the two *erm*(B) genes, the element carries homologs of Tn*916* genes including *orf13*, *orf9* and *orf7* which are assumed to be involved in the regulation of the element. To be mobilised, Tn*5398* needs an *oriT* and a recognizable *nic* site. The presence of two potential *oriT* sites, which are located within the coding sequence of *orf298* has been confirmed in this element (Farrow *et al.*, 2001). The *nic* sites (TGGTGT) of these two potential *oriT* sites are identical to the *nic* sites found on Tn*916* and Tn*5397* (Farrow *et al.*, 2001).

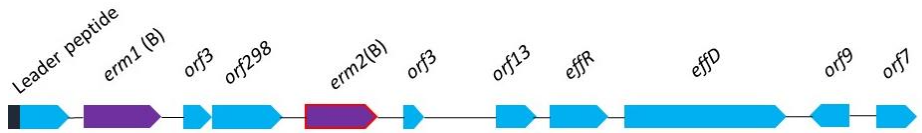


Figure 1.20 Schematic representation of Tn*5398*.

The purple arrows represent *erm1*(B) and *erm2*(B), the latter is functional in Tn*5398* (highlighted by red line). Other ORFs are represented by blue arrows.

1.5.4. Additional *C. difficile* MGEs

1.5.4.1. *skin*^{CD}

In both *B. subtilis* and *C. difficile*, an RNA polymerase sigma factor essential for sporulation is encoded by the *sigK* gene which is interrupted by a prophage-like element designated *sigK* intervening sequence or *skin*^{Bs} (in *B. subtilis*) and *skin*^{Cd} (in *C. difficile*) (Takemaru *et al.*, 1995, Haraldsen & Sonenshein, 2003). The transcription of the *sigK* gene requires excision of the *skin* element into a circular molecule by site-specific recombination at a late stage of sporulation (Amy *et al.*, 2015). In *B. subtilis*, the presence of the *skin* element is not required for efficient sporulation. However, *C. difficile* strains CD37 and ATCC9689 that do not naturally contain the *skin* element sporulate poorly (Haraldsen & Sonenshein, 2003). The element in strain 630 encodes a serine recombinase responsible for the excision of the element, a putative lipoprotein, a putative cell surface protein, two putative phage transcriptional regulators, seven phage proteins of unknown function and five hypothetical proteins (Figure 1.21) (Haraldsen & Sonenshein, 2003). A possible explanation for the presence of *skin*^{Cd} is that a prophage entered the *sigK* gene in an ancestral *C. difficile* strain and evolved to sense stress factors leading to the sporulation and phage lytic cycle (Reviewed in Mullany *et al.*, 2015). The exact role of *skin*^{Cd} is not clear, but it might be involved in the correct timing of SigK production and initiation of the sporulation pathway (Pereira *et al.*, 2013).

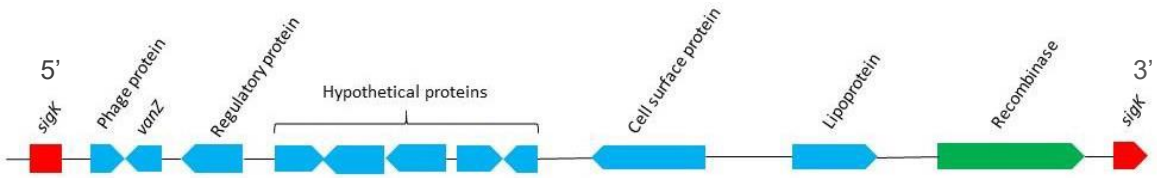


Figure 1.21 Schematic representation of *skin*^{Cd} in strain 630.

The green arrow represents the serine recombinase, other ORFs are represented by blue arrows. The interrupted *sigK* is represented by an interrupted red arrow.

1.5.4.2. IStron

Insertion sequence elements (IS-elements) are small genetic elements capable of mediating their own transposition into different target sites by encoding a transposase enzyme (Mahillon & Chandler, 1998). Group I introns are mobile genetic elements which encode ribozymes to catalyse their own splicing from a precursor mRNA (self-splicing), allowing exons to ligate and form mature mRNA (Hausner *et al.*, 2014). In 2000, a chimeric genetic element was found within the *tcdA* gene in *C. difficile* strain C34. This element had characteristics of both IS-elements and group I introns; therefore, it was designated IStron, Cd*Ist1* (Braun *et al.*, 2000, Hasselmayer *et al.*, 2004). The Cd*Ist1* element splices from the pre-mRNA molecule leading to the normal expression of the *tcdA* gene (Braun *et al.*, 2000). The element carries two CDS encoding a putative transposase (IS-element feature) and a ribozyme (group I intron feature). The presence of Cd*Ist1* in *C. difficile* was shown by southern blot and PCR analysis and confirmed by the whole genome sequencing of strain 630 (Braun *et al.*, 2000, Sebaihia *et al.*, 2006). A homolog with 63% sequence identity to Cd*Ist1* is present in *Bacillus cereus* (Tourasse *et al.*, 2006); however, similar IStron elements have not been reported in any other organism (Amy *et al.*, 2015).

1.6. Genetic manipulation tools

Historically, genetic manipulation of *C. difficile* has been proved to be extremely difficult since the transfer of foreign DNA by electroporation or chemical transformation has never been reported. However, remarkable progresses have been made through whole genome sequencing (WGC) projects and applying different genetic tools such as shuttle vectors, ClosTron and CRISPR-cas9, which all have resulted in significant advances in *C. difficile* researches (Mullany *et al.*, 1994, Purdy *et al.*, 2002, O'Connor *et al.*, 2006, Heap *et al.*, 2007, McAllister *et al.*, 2017, Wang *et al.*, 2018). These genetic manipulation tools have provided important information about the structure and organization of the *C. difficile* genome, genetic diversity, epidemiology, pathogenicity and evolution of this organism (Knight *et al.*, 2015, Muñoz *et al.*, 2018).

1.6.1. Shuttle conjugative Transposon: Tn*916*

The first study of gene cloning in *C. difficile* was performed by using Tn*916* as a shuttle conjugative transposon (Mullany *et al.*, 1994). In this work, a pBR322-based vector designated pCI195 (replicable in *E. coli*, non-replicable in *C. difficile*) carrying the *catP* gene and a 4.2 kb region of conjugative transposon Tn*919* (Casey *et al.*, 1991) was used to transform *B. subtilis* strain BS17 (CU2189:: Tn*916*ΔE (*tet*(*M*) gene is replaced by an *erm*(B) gene)). Chloramphenicol (encoded by pCI195) and erythromycin (encoded by Tn*916*ΔE) resistant transformants appeared on selective plates. Southern blot analysis showed that a single copy of pCI195 was integrated into Tn*916*ΔE via homologous recombination (transposon:: integrative plasmid). Filter mating was performed between *B. subtilis* transformant donor and *C. difficile* strain

CD37 recipient. Chloramphenicol and erythromycin resistant transconjugants appeared on selective plates. Southern blot analysis confirmed that Tn*916*ΔE::pCI195 was excised from *B. subtilis* genome and inserted into the *C. difficile* chromosome at a specific target site. The possibility of using conjugative transposons for gene cloning in *C. difficile* was further investigated by cloning a fragment of the *tcdB* gene into plasmid pCI195. The plasmid was then transferred into CD37 from *B. subtilis* strain BS17. DNA isolated from transconjugants was digested and probed with the cloned fragment of the *tcdB* gene from pCI195. Results confirmed the successful transfer since one hybridising fragment appeared with each transconjugant (Mullany *et al.*, 1994).

1.6.2. Other transposon-based mutagenesis systems

The elements from the superfamily *mariner* transposons are widely distributed in nature and found in rotifers, insects, fungi, plants and mammals (Robertson, 1993, Arkhipova & Meselson, 2005, Muñoz-López & García-Pérez, 2010). These elements are between 1 and 5 kb and encode a transposase which is flanked by two inverted repeats (Plasterk *et al.*, 1999, Muñoz-López & García-Pérez, 2010). The transposition of *mariner* elements is a non-replicative process and involves a cut-and-paste mechanism in which two transposase monomers bind to the inverted repeats at each end of the transposon, forming a structure called single-end complex. Then, the transposon ends are brought together by the transposase monomers and generate a second structure called paired-end complex which leads to the transposon excision. The transposase dimer recognizes a random TA dinucleotide sequence to carry out the insertion (Figure 1.22) (Bryan *et al.*, 1990).

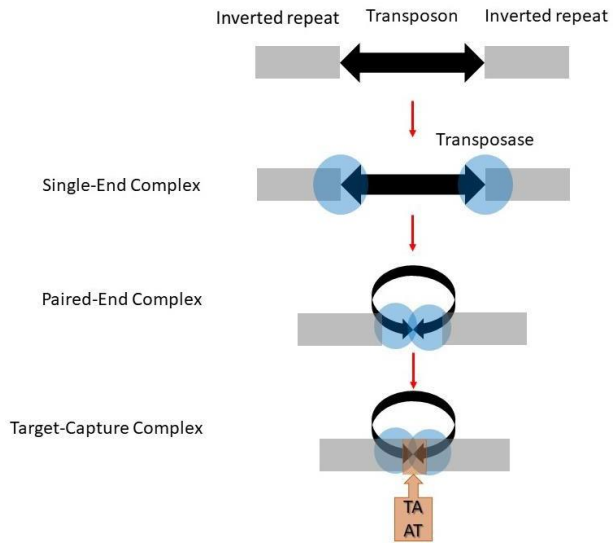


Figure 1.22 Schematic diagram of the cut-and-paste mechanism of transposition performed by the *mariner* elements.

Three stages of the cut-and - paste mechanism: Single-End complex, Paired-End complex and Target-Capture complex are shown in this diagram.

A *mariner*-based transposon system using Himar1 transposase for *in vitro* random mutagenesis has been developed in *C. difficile*. The facts that (i) Himar1 transposase is the only requirement for the cut-and-paste transposition mechanism and (ii) the element inserts into a TA rich target site make this element suitable for an organism with low GC content such as *C. difficile* (Cartman & Minton, 2010).

Pseudo-suicidal plasmids pMTL-SC0 and pMTL-SC1 were constructed to deliver *mariner*-based transposon into *C. difficile* R20291. These two *mariner* plasmids are identical except that the expression of *Himar1* in pMTL-SC1 is controlled by the *tcdB* gene promotor, but pMTL-SC0 lacks the inducible promotor (negative control). Both plasmids are pBP1-based (from *C. botulinum*), chloramphenicol (Cm)/ thiamphenicol (Tm) resistant (the resistance markers are located on the transposon), replicable in *E. coli* and carry the conjugative transfer gene *traJ* (Figure 1.23). Plasmids were transferred into R20291 and Tm^R colonies appeared on selective agar at the frequency of 4.5×10^{-4} for the pMTL-SC1 transconjugant culture.

PCR showed that (i) the transposon-based *catP* gene conferring Cm^R was present in the R20291 chromosome (confirming the transposition), (ii) plasmid-based *Himar1* transposase gene was not present in the genome (showing that the transposon was mobilised, and the plasmid was lost from the cell) (iii) plasmid-based P*tcdB* was not found in the genomic DNA (showing that plasmid did not integrate into the chromosome by homologous recombination). The randomness of the insertion was verified by sequencing and southern blot analysis (Cartman & Minton, 2010).

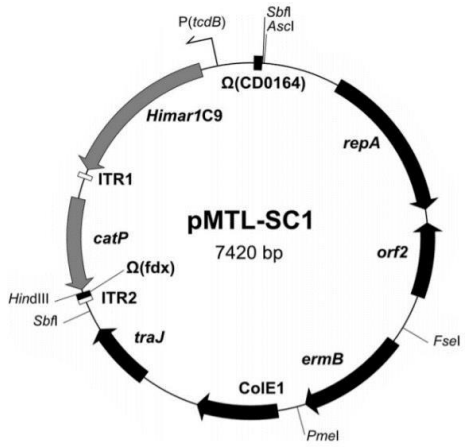


Figure 1.23 Map of plasmid pMTL-SC1.

(Retrieved from (Cartman & Minton, 2010))**.** Expression of the *mariner* transposase gene *Himar1* was controlled by the *tcdB* promotor, P*tcdB.* The plasmid backbone consists of the pBP1 replicon of *C. botulinum* (*repA* and *orf2*), the *erm*(B) gene, the Gram-negative replicon ColE1, and the conjugal transfer gene *traJ*. This vector is adapted from pMTL80000 modular system for *Clostridium* shuttle plasmids (Heap *et al.*, 2010).

Transposon mutagenesis approach has also been used in the hypervirulent strain R20291 to generate a comprehensive transposon library containing more than 70,000 mutants. Using transposon-directed insertion site sequencing (TraDIS), a set of 404 essential genes, required for growth *in vitro* has been identified. Furthermore, 798 genes essential for *C. difficile* sporulation and transmission have also been determined by using the same technique. All of these genes could be attractive targets for developing new drugs to treat CDI (Dembek *et al.*, 2015).

1.6.3. Transfer of replication-proficient plasmids into *C. difficile* from *E. coli* donor

The use of autonomously replicating (replication-proficient) plasmids has been investigated in *C. difficile.* The first mobilisable *oriT*-based shuttle vector to be used was developed by Purdy *et al.*, (2002). A non-conjugative plasmid containing an *oriT* from a broad-host-range conjugative plasmid RK2, an origin of replication derived from naturally occurring *C. difficile* plasmid pCD6 and an origin of replication derived from plasmid ColE1 was constructed (Figure 1.24). This construct was designated pMTL9301 and subjected to transfer from *E. coli* CA434 (HB101 carrying conjugative plasmid R702) donor at a frequency of 5.7×10^{-5} transconjugants per donor into *C. difficile* strain CD37. pMTL9301 was found to be relatively stable after transfer as only 8% of the cells lost the plasmid after 32 subcultures in the absence of antibiotic.

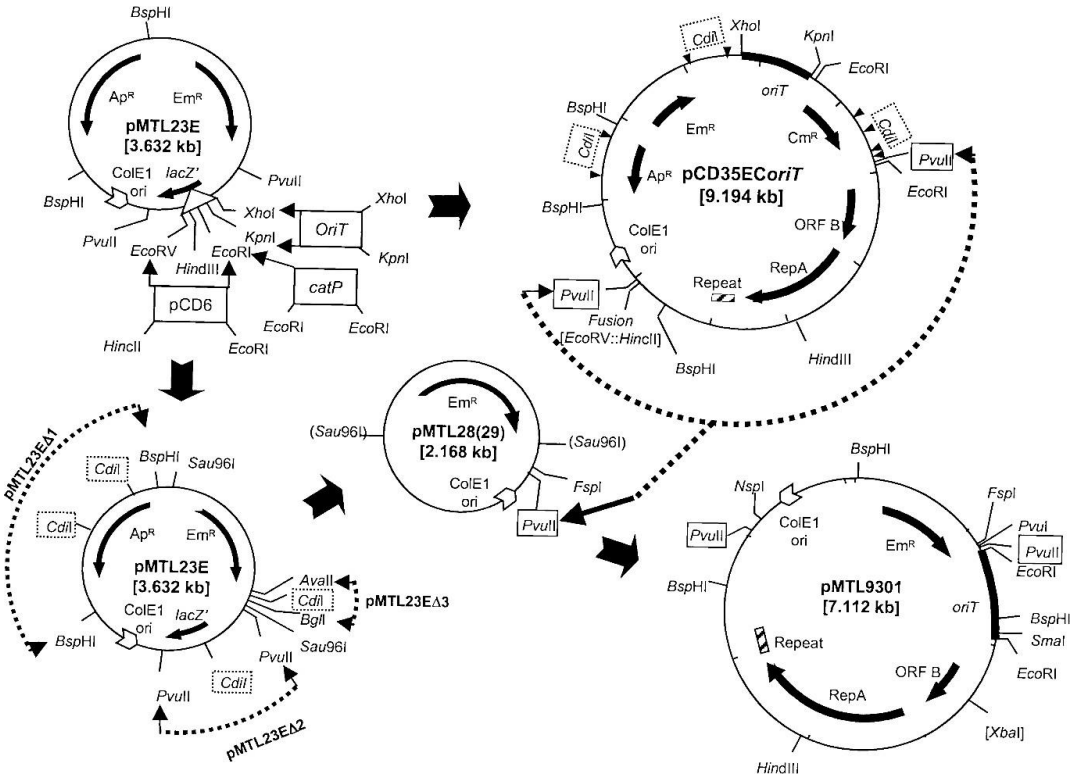


Figure 1.24 Schematic diagram of construction of replication-proficient shuttle vectors to be used in *C. difficile.*

(Retrieved from (Purdy *et al.*, 2002)). To generate the initial shuttle vectors a 2 kb fragment isolated from pCD6 was cloned into pMTL23E to generate pCD35E. A derivative of the pCD35E was also constructed containing the *catP* gene of the *C. perfringens* plasmid pJIR418 to yield pCD35EC. Mobilizable plasmid pMTL35EC*oriT* was then constructed through the insertion of a fragment harbouring *oriT* derived from the conjugative plasmid RK2. To construct pMTL9301, pMTL23E was reduced in size through the indicated sequential deletions to evade restriction modification system.

1.6.4. Use of ClosTron system: a group II intron derivative for targeted mutagenesis: directed gene inactivation

The ClosTron system is an efficient tool to produce targeted gene knock-outs in *C. difficile* and other clostridia (Heap *et al.*, 2007). This genetic approach is based on the TargeTron™ system which directs inactivation of specific genes (Zhong *et al.*, 2003). This system exploits mobile group II introns from the *ltrB* gene in *L. lactis* that can recognize and insert into the target site by base pairing (Figure 1.25). This system can be reprogrammed to insert into any desired target sequence (Karberg *et al.*, 2001, Zhong *et al.*, 2003). To enable screening of the mutants, the group II intron contains the *erm*(B) gene which itself is interrupted by a group I intron. The three elements are arranged in such a way so that only after successful integration of the group II intron, the group I intron splices out and the *erm*(B) gene is activated. This marker is called a Retrotransposition-Activated Marker (RAM) and is an important feature of the ClosTron (Mohr *et al.*, 2000).

Heap *et al.,* (2007) adapted the TargeTron system originally developed by (Zhong *et al.*, 2003) to introduce targeted mutagenesis in clostridia. The group II intron derived from *L. lactis* (Ll.ltrB), the *erm(B)* gene from the *E. faecalis* plasmid pAMβ1 and the group I intron from the *td* gene of phage T4 were all cloned into a pCD6-based *E. coli-Clostridium* shuttle vector pMTL007 (Figure 1.26) (Heap *et al.*, 2007). Moreover, an IPTG inducible promotor was cloned in; however, it was later replaced by the *tcdB* promotor in a revised version of the system (Heap *et al.*, 2010).

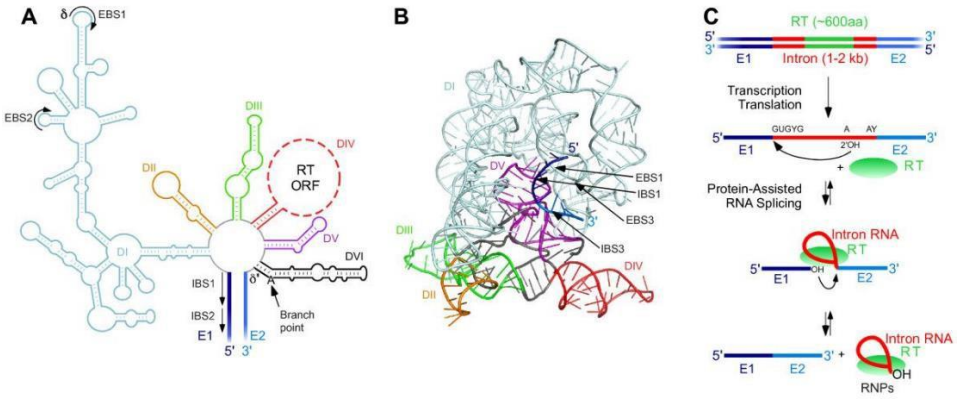


Figure 1.25 Group II intron structure and splicing mechanism; Retrohoming

(Retrieved from (Enyeart *et al.*, 2014)). A) Group II intron RNA secondary structure. The example shown is the *L. lactis* Ll.LtrB group II intron. Intron RNA domains are shown in different colours, and the 5' and 3' exons (E1 and E2, respectively) are thicker dark and light blue lines, respectively. The large 'loop' region of double helical domain (DIV), which encodes the group II intron reverse transcriptase (RT), is shown as a dashed line. B) Crystal structure of the group II intron (Toor *et al.*, 2010). Different domains are coloured as figure A. C) Group II intron RNA splicing and reverse splicing. Double-stranded DNA is indicated by double lines and RNA as a single line. E1 and E2 are shown in dark and light blue, respectively; the intron and intron RNA are shown in red; and the intron-encoded RT is shown in green.

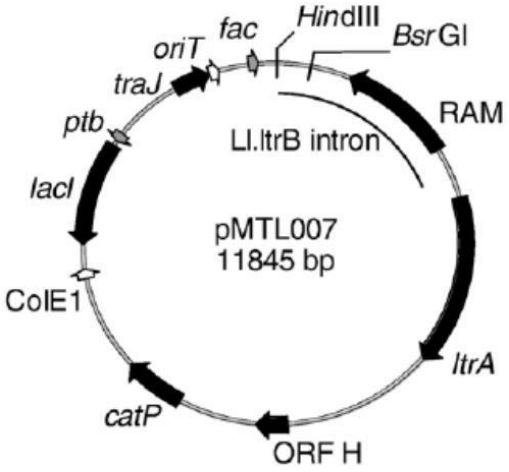


Figure 1.26 Schematic representation of Plasmid pMTL007

(Retrieved from (Heap *et al.*, 2007))**.** Group II intron derived from *L. lactis* (Ll.ltrB), *oriT/ traJ* required for the transfer, ColE1 required for replication in *E. coli* are shown. Plasmid pMTL007 is constructed based on pCD6; naturally occurring plasmid in *C. difficile* strain CD6.

1.6.5. CRISPR-Cas system

1.6.5.1. CRISPR-Cas system in *C. difficile*

Bacteria rely on diverse defence mechanisms that allow them to fight against viral infections and exposures to invading nucleic acids. In many bacteria and most archaea clustered regularly interspaced palindromic repeat (CRISPR) provides acquired immunity against viruses and plasmids by targeting nucleic acid in a sequence-specific manner (Jansen *et al.*, 2002). Generally, CRISPR-mediated immunity functions in three basic stages: Adaptation: acquisition of invading genetic elements referred to as protospacers and their incorporation into the host chromosome where they are subsequently referred to as spacers; Expression: CRISPR array transcription into CRISPR RNA (cr RNA) that together with specific CRISPR associated proteins (Cas) including endonucleases form a complex to attack foreign DNA; Target interference: crRNA containing one spacer flanked with repeat sequences serves as guide RNA to recognize foreign DNA by complementary base pairing. The recognition leads to the degradation of targeted nucleic acid (Marraffini, 2015).

CRISPR-Cas systems are grouped into six types, designated type I to type VI (Makarova *et al.*, 2015). The type I CRISPR-Cas system is the most diverse type and is divided into subtypes A to F and U. The subtype I-B CRISPR-Cas system is present in clostridia and mainly characterized by a subtype specific protein Cas8b that is part of the Cascade complex (S. Makarova *et al.*, 2011). The function of CRISPR array is summarized in figure 1.27.

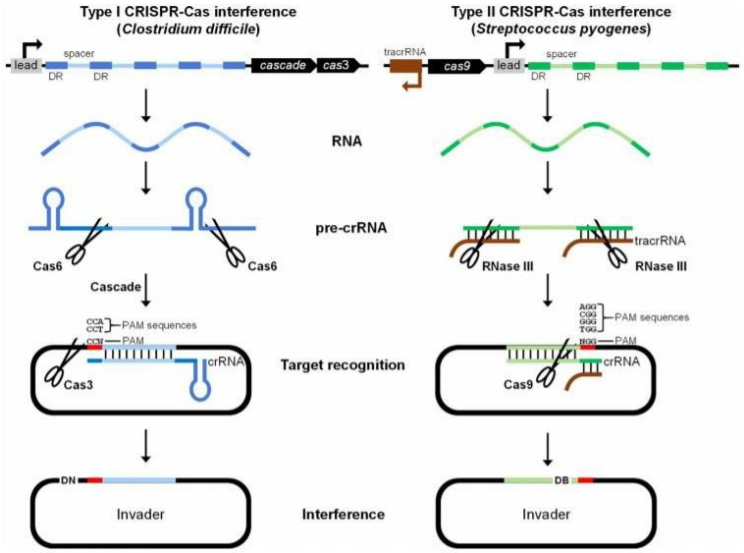


Figure 1.27 Comparison of Type I and Type II CRISPR-Cas systems.

(Retrieved from (Pyne et al., 2016)). CRISPR arrays composed of palindromic direct repeats (dark blue and dark green) and spacers (light blue and light green) are first transcribed into a precursor crRNA. The resulting transcript is cleaved and processed into individual mature crRNA by Cas6 endonuclease (Type I) or RNase III (Type II). A single guide RNA (gRNA) is the final mature transcript to invade nucleic acids via base pairing between the crRNA and the invader protospacer sequence. Type I interference requires a multiprotein Cascade complex (*cas6, cas8b, cas7, cas5* in *C. difficile*). Type I and Type II interference mechanisms require recognition of one of multiple protospacer adjacent motifs (PAM) sequences (red).

1.6.5.2. Using CRISPR-Cas system to generate *C. difficile* mutants

During the last decade, genome editing by CRISPR-Cas9 technology, the most popular biotechnological application of CRISPR, has been rapidly progressing (Hsu *et al.*, 2014, Barrangou & Horvath, 2017). Numerous CRISPR-based tools have been developed which proved to be remarkably efficient in prokaryotes and eukaryotes (Hsu *et al.*, 2014, Barrangou & Horvath, 2017). In prokaryotes, the presence of endogenous CRISPR-Cas systems simplifies the genome editing process since it does not require the introduction of additional factors to the system (Barrangou & Horvath 2017).

Several studies on the application of type I-B system in genome editing have recently been published. The first application of CRISPR-based genome editing in *Clostridium* sp. was reported by Pyne *et al.*, (2016). In this work, a plasmid vector harbouring an artificial CRISPR array composed of a specific protospacer to target the gene of interest in *Clostridium pasteurianum* was used. This approach resulted in a fast and markerless gene deletion (Pyne *et al.*, 2016). Further studies confirmed the efficiency of this method in other *Clostridium* species (Zhang *et al.*, 2018).

In order to investigate the role of selenoproteins in *C. difficile* physiology and growth, the CRISPR-Cas9 mutagenesis system was used in strain JIR8094 to delete the *selD* gene encoding selenophosphate synthetase (essential for incorporation of selenium into selenoproteins). In this work, a wild type *cas9* gene from *Streptococcus pyogenes* was cloned into pJK02 Cas9-producing plasmid (Figure 1.28). The expression of *cas9* was under the control of tetracycline-inducible *tetR* promotor. The Cas9 endoribonuclease was directed towards the gene of interest with the aid of guide RNA which was also cloned into the CRISPR vector and expressed under the control of native glutamate dehydrogenase (*gdh*) promoter. The complete CRISPR-Cas9 plasmid was transformed into *E. coli* HB101 containing conjugative plasmid, RK25 to be used as a donor for conjugation with *C. difficile* R20291. Transconjugants appeared on BHI medium were tested for mutation by PCR. A frequency of ~1 *selD* deletion in every 5 colonies was observed (~20% mutation frequency). Moreover, *C. difficile* R20291Δ*selD* showed a growth defect compared to the wild-type strain when tested in a protein rich medium. This work was the first successful application of the CRISPR-Cas9 system in *C. difficile* (Figure 1.29) (McAllister *et al.*, 2017).

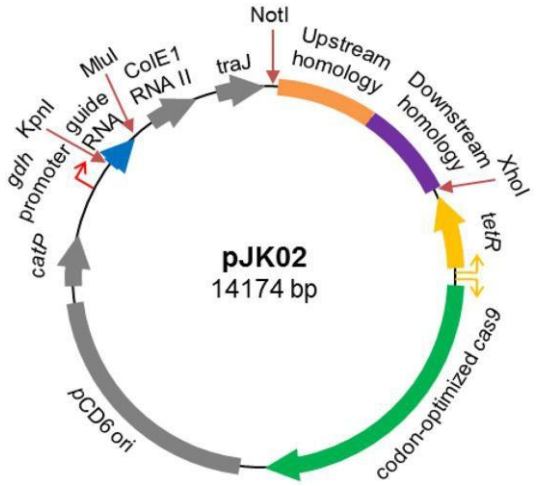


Figure 1.28 The first CRISPR-Cas9 vector used to introduce mutagenesis in the *C. difficile* genome.

(Retrieved from (McAllister *et al.*, 2017))**.** Major features are 1) targeting region for homologous recombination in purple and orange 2) gRNA under the expression of the *gdh* promoter, 3) The *tetR* promoter that regulates the expression of the *S. pyogenes cas9* gene

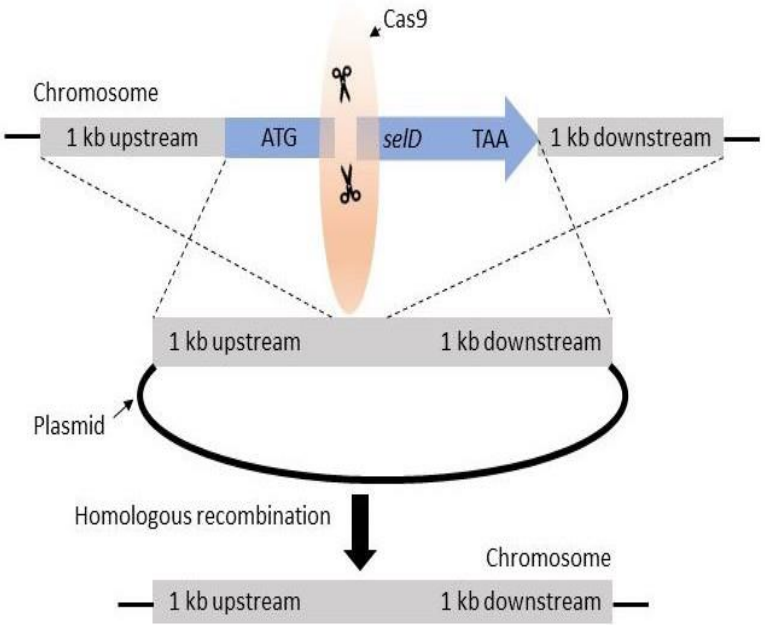


Figure 1.29 Schematic diagram of Cas9-mediated *C. difficile* mutant construction.

Deletion of the chromosomally-encoded *selD* gene was made by homologous recombination from a donor region located on the CRISPR-Cas9 plasmid (Figure 1.28) during repair of a Cas-9 mediated double- stranded DNA break. The location of the crRNA target region in *selD* is indicated by the cut DNA (Adapted from (McAllister *et al.*, 2017)).

1.7. Aim of the study

The aim of this study is to determine the effect of environmental changes on horizontal gene transfer in *C. difficile*.

Biochemical approach has initially been undertaken to describe the effect of different concentrations of pancreatic α-amylase on *C. difficile*. This approach was taken to characterise the bacterial response by assessing changes in colony morphology through secretion of exopolymers, subsequent biofilm formation and resistance to stresses. The effect of pancreatic α-amylase on horizontal gene transfer in *C. difficile* has also been determined following changes observed in the exopolymer secretion. Experiments were set up to test the hypothesis that any structural change in bacterial cell surface due to exposure to environmental stress could affect the frequency of MGEs transfer. Tn*5397* and pMTL9301 were chosen to test this and the frequencies of transfer were measured in the presence of pancreatic α-amylase. Subsequently, it was tested if the addition of deoxyribonuclease (DNase) could affect the transfer frequencies of these elements. The frequency of pMTL9301 transfer into *C. difficile* strain CD37 was significantly decreased when DNase was present. The molecular basis of this observation has extensively been studied in this thesis.

Chapter 2 Materials and methods

2.1. Bacterial strains and culture methods

Bacterial strains used in this study are listed in Table 2.1. *C. difficile* and *B. subtilis* strains were grown on Brain Heart Infusion (BHI) agar plates (Oxoid Ltd, Basingstoke, UK) supplemented with 5% defibrinated horse blood (BHIB) (E & O laboratories, Bonnybridge, UK) or in BHI broth. Broth and agar plates were pre-reduced before culturing *C. difficile*. All *C. difficile* strains were grown at 37 °C in anaerobic conditions (80% N2, 10% H2 and 10% CO2) in Macs-MG-1000-Anaerobic work station (Don Whitley scientific).

E. coli was grown on LB agar plates or in LB broth (Sigma-Aldrich Company Ltd, Dorset, UK). *E. coli* and *B. subtilis* strains were grown in aerobic conditions unless co-cultured with *C. difficile*.

When specified, deoxyribonuclease I (DNase) from bovine pancreas and/ or α-amylase from porcine pancreas was added to the mating mix to a final concentration of 50 µg/ml and 10 µM (700 µg/ ml), respectively.

All antibiotics, DNase and α-amylase were obtained from Sigma-Aldrich Company Ltd (Dorset, UK).

The storage of all bacterial isolates was made by adding an equal volume of 20% (v/v) of sterile glycerol to the broth overnight culture, resulting in 10% (v/v) glycerol stock. One ml aliquots of the glycerol stocks were kept at -80 °C.

Table 2.1 Bacterial strains used in this study.
Tc = tetracycline, Erm = erythromycin, Rif = rifampicin, ^{R} = resistant, ^{S} = sensitive

Bacterium	Ribotype	Properties	Source
C. difficile			
630	012	Tc^{R} Erm^{R} Rif^{S}	Wust *et al.*, (1982), Wüst & Hardegger, (1983)
630Δ*erm*	012	Tc^{R} Erm^{S} Rif^{S}	Hussain *et al.*, (2005)
R20291	027	Tc^{S} Erm^{S} Rif^{S}	Stabler *et al.*, (2006)
CD305	023	Isolated from a 74-year-old patient with severe symptoms in 2008	Dr. H. A. Shaw
CD37	009	Tc^{S} Erm^{S} Rif^{R}	Smith *et al.*, (1981)
E. coli			
DH5α	F^{-} *rec*A1 *end*A1 *hsd*R17 (rk^{-}, mk^{+}) *pho*A *sup*E44 λ^{-} *thi*$^{-}$1 *gyr*A96 *rel*A		Gibco, BRL
HB101	Hybrid of *E. coli* K12 and *E. coli* B, parent of CA434		Boyer & Roulland-Dussoix, (1969)
CA434	HB101 carrying the IncP conjugative plasmid R702		Williams *et al.*, (1990), Purdy *et al.*, (2002)
B. subtilis			
BS6A	*B. subtilis* CU2189:: Tn*5397*, Tc^{R}		Roberts *et al.*, (1999)
BS34A	*B. subtilis* CU2189:: Tn*916,*containing a single copy of Tn*916*		Roberts *et al.*, (2003)

2.2. Molecular techniques

2.2.1. Genomic DNA extraction

Genomic DNA was isolated using the Gentra Puregene Yeast/Bact. Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions; however, changes were made to the amount of the solutions that were used. Ten millilitres of overnight culture (16 h) was centrifuged for 5 min at 4,500 x *g*, and the cell pellet was resuspended in 1 ml PBS. The cell suspension was transferred to a microcentrifuge tube and centrifuged for 1 min at 16,000 x *g*. The cell pellet was resuspended in 600 µl cell suspension solution. Five microliters of lytic enzyme solution were added, and the tubes were inverted several times for mixing before they were incubated at 37 °C for 30 min. Tubes were centrifuged at 16,000 x *g* for 1 min and the supernatant was removed with a pipette. Cells were resuspended in 600 µl cell lysis solution and incubated at 80 °C for 5 min. Next, 5 µl of RNase A solution was added and the tubes were inverted 10 times to mix and incubated at 37 °C for 30 min. Samples were transferred to ice and 200 µl protein precipitation solution was added and vortexed for 20 sec after which the samples were incubated on ice for 30 min. The tubes were centrifuged for 3 min at 16,000 x *g* and the supernatant was poured into a microcentrifuge tube containing 600 µl isopropanol. The tubes were inverted approximately 50 times and centrifuged for 1 min at 16,000 x *g*. The supernatant was discarded, and the tubes were drained on tissue paper. The DNA pellet was washed by adding 600 µl 70% ethanol and inverting several times. The tubes were centrifuged for 1 min at 16,000 x *g* and the supernatant was discarded and the tubes drained on tissue paper. The samples were air dried for 15 minutes and 100 µl sterile distilled H_2O

was added. The DNA was incubated for 1 h at 65 °C and overnight at RT. Samples were analysed using a Nanodrop 1000 for quality and quantity and stored at -20 °C.

2.2.2. Plasmid extraction

Plasmid DNA purification was carried out using QIAprep Spin Miniprep Kit (Qiagen, Crawley, UK). This protocol was designed for purification of up to 20 µg of high copy plasmid DNA from 1-5 ml overnight cultures of *E. coli* in LB medium. All centrifugation steps were carried out at 17,900 x *g* (13,000 rpm) in a table-top microcentrifuge. The bacterial cells were pelleted, and the supernatant discarded. The pelleted cells were resuspended in 250 µl of Buffer P1 and transferred to a microcentrifuge tube. Buffer P2 (250 µl) was then added and the tube was inverted gently for 4-6 times. 350 µl of Buffer N3 was added to the mixture, and again the tube was immediately inverted 4-6 times. The tube was centrifuged for 10 min and the supernatants were applied to the QIAprep spin column by decanting or pipetting. The spin column was centrifuged for 1 min and the flow-through discarded. The spin column was washed by adding 750 µl of Buffer PE and centrifuging for 1 min. The flow-through was discarded and the spin column was centrifuged for an additional minute to remove residual wash buffer. The QIAprep spin column was then placed in a clean 1.5 ml microcentrifuge tube. The DNA was eluted by adding 30-50 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) or distilled water to the centre of the spin column membrane, left to stand for 1 min at room temperature (25 °C) and centrifuged for 1 min. The plasmid DNA was ready for further applications.

2.2.3. Oligonucleotide synthesis

All the oligonucleotides used in this study were synthesised by Sigma-Aldrich (Dorset, UK). The primers were designed either manually or by using DNAMAN and Primer3 web-based software (http://biotools.umassmed. edu/bioapps/primer3_www.cgi). The lists and sequences of primers in this study are shown in each chapter.

2.2.4. Standard Polymerase chain reaction (PCR)

PCR amplification was performed by using Biometra T3000 Thermocycler (Biometra, Glasgow, UK). The standard PCR reactions were prepared with a total volume of 30 µl, composing of 15 µl 2X Biomix Red (Bioline, London, UK), 2 µl of each primer (10 pmol/µl), 10 µl molecular biology grade water (molH_2O) and 1 µl DNA template (50-100 ng). Biomix Red contains the *Taq* DNA polymerase that can amplify 1 kb in 30 sec and generate 'A' overhang for TA cloning. The standard conditions were carried out as followings; (i) initial denaturation at 94 °C for 3 min (ii) denaturation at 94 °C for 1 min (iii) annealing at 50-65 °C (depending on primers) for 30 sec (iv) extension at 72 °C for 1-3 min (depending on the expected size of amplicons), repeated step (ii)-(iv) for 35 cycles (v) final elongation at 72 °C for 5 min and stored samples at 4 °C. The annealing temperature employed was 5 °C lower than the Tm° of the primer with the lower melting temperature.

2.2.5. Agarose gel electrophoresis

Agarose gel electrophoresis was performed to visualise the DNA products (genomic DNA, plasmids, PCR products, digestion products and ligation products). A standard concentration for the gel was 1-1.5% (w/v) agarose (Bioline, London, UK) prepared with 1X TAE (tris-acetate-EDTA) buffer. Gels were stained with GelRed (1:10,000 dilution) (Biotium, Cambridge, United Kingdom). The DNA products (except the PCR products amplified by Biomix Red) were mixed with 5X loading buffer (Bioline, London, UK) and loaded into the gel wells. One microliter of either HyperLadder 1 kb (Bioline, London, UK) or 1 kb extension ladder (Life Technologies, Paisley, UK) was also added as a size reference. The electrophoresis was run at 50-100 V for 60-90 min. Gels were visualised under UV excitation using an Alpha Imager (Alpha InnoTech, Exeter, UK) and the image was captured by AlphaView software (Alpha InnoTech, Exeter, UK).

2.2.6. PCR product purification

Purification of PCR products was performed by using the QIAquick PCR Purification Kit (Qiagen, Crawley, UK). This protocol was conducted to clean the PCR products by removing primers, enzymes, salts and other impurities. All the centrifugation steps were done at 14680 x *g* (13000 rpm, Eppendorf centrifuge 5415 D). Five volumes of Buffer PB was added to 1 volume of the PCR products. After mixing the solution, the mixture was transferred to the QIAquick spin column and centrifuged for 1 min. The flow-through was discarded and 700 µl of Buffer PE was then added to the column. After 1 min centrifugation, the flow-through was discarded from the collection tube, and additional centrifugation was done for 1 min.

The spin column was then transferred to a new sterile 1.5 ml microcentrifuge tube. The DNA was eluted by adding 30 µl of molecular biology grade water to the centre of the membrane, left to stand for 3 min and centrifuged for 1 min. The purified DNA was kept in -20 °C freezer.

2.2.7. DNA extraction from agarose gels

This was performed with QIAquick Gel Extraction Kit (Qiagen, Crawley, UK). This protocol was used to purify and retrieve only specific DNA bands of interest on an agarose gel. All the centrifugation steps were done at 14680 x *g* (13000 rpm, Eppendorf centrifuge 5415 D). The DNA was subjected to the agarose gel electrophoresis (section 2.2.5). The DNA with the size of interest was then excised from the gel by visualising under UV light and cut with a clean scalpel. The gel slice was transferred to a 1.5 ml microcentrifuge tube and weighed. Three volumes of Buffer QG was then added to 1 volume of gel and incubated in a heat block at 50 °C. The tube was occasionally vortexed to help dissolve the gel. After the gel was completely dissolved, 1 volume of isopropanol was added and mixed. The mixture was then transferred to the QIAquick spin column and centrifuged for 1 min. After discarding the flow through, 500 µl of buffer QG was added and centrifuged for 1 min. The flow-through was then discarded and 700 µl of buffer PE was added to the column. After 1 min centrifugation, the flow through was discarded and the column centrifuged again for 1 min. The spin column was then transferred to a new sterile 1.5 ml microcentrifuge tube. The DNA was eluted by adding 30 µl of molecular biology grade water to the centre of the membrane, left to stand for 3 min and centrifuged for 1 min. The extracted DNA was kept in -20 °C freezer.

2.2.8. Restriction endonuclease reaction

DNA digestion was performed by using restriction enzymes (NEB, Hitchin, UK). The standard digestion reactions were prepared in a 10 µl total volume, containing 1 µl restriction enzymes (20 U), 1 µl 10X digestion buffer, 1-5 µl DNA samples and topped up with molecular biology grade water. The reactions were incubated at 37 °C for at least 1h for complete digestion unless stated otherwise. The digestion products were then either purified by QIAquick PCR Purification Kit (Qiagen, Manchester, UK) with the same protocol as in section 2.2.6 or visualised on an agarose gel as described in section 2.2.5.

2.2.9. DNA ligation reaction

Ligation of DNA was carried out using T4 DNA ligase (NEB, Hertfordshire, UK) according to the manufacturer's instructions. Ligation was carried out using a molar ratio of 1:3 vector to insert. The ligation mixture was incubated overnight at 4 °C with regular T4 DNA ligase or incubated for 10 min at room temperature (25 °C) with Quick T4 DNA ligase, dependent on the requirement of the experiment.

2.3. Transformation into competent *E. coli* CA434

Competent cells were prepared by growing an overnight culture in LB broth and using 2 ml to inoculate a further 100 ml of LB broth. Cells were grown to OD_{600} ~ 0.3. The broth culture was centrifuged at 4,500 x *g* for 5 min at 4 °C. The cells were resuspended in 10 ml ice cold 100 mM $CaCl_2$ and incubated on ice for 20 min. The

cells were centrifuged again, as above, and resuspended in 0.5 ml $CaCl_2$. After 15 min incubation on ice, 70 µl DMSO was added, and the cells were incubated on ice for 15 min. Following this, another 70 µl DMSO was added. Cells were subjected to snap freezing using an ethanol bath with dry ice. The cells were stored at -80 °C. The protocol is based on the papers by (Mandel & Higa, 1970) and (Cohen *et al.*, 1972).

Transformation was performed as described for the p-GEM-T vector system (Promega, Southampton, UK). Competent cells were thawed on ice and 50 µl of cells were added to a tube containing the target DNA. The cells were incubated on ice for 30 min after which the cells were heat-shocked at 42 °C for 45 sec after which the tube was placed on ice again for 2 min. 950 µl of SOC medium (Sigma Aldrich Ltd, Gillingham, UK) was added to the tube and the cells were incubated at 37 °C for one hour with shaking at 200 rpm. After this, the cell suspension was transferred onto selective LB agar (Sigma Aldrich Ltd, Gillingham, UK.) plates and incubated over night at 37 °C.

2.4. Filter mating

This method is based on that described by Mullany *et al.*, (1990). Cultures of both donor and recipient strains were grown overnight for 16 h. These were used to start a 10 ml culture of the donor strain and a 50 ml culture of the recipient strain, both at an OD_{600} ~ 0.1. These were grown shaking at 50 rpm. After 4-6 h, when the OD_{600} was between 0.5 and 0.6, the cells were centrifuged for 10 min at 4,500 x *g* and the pellets were resuspended in 500 µl BHI broth. The two cultures were mixed and 200 µl of the mixture was spread onto each of four 0.45 µm pore size cellulose nitrate filters (Sartorius, Epsom, UK), on BHIB plates. After 24 h the filters were placed into 25 ml

tubes and 1 ml BHI broth was added. The tubes were vortexed, and the resulting cell suspension was spread on plates selective for transconjugants. In filter- mating using *B. subtilis* as recipient, these plates are incubated aerobically in order to select against the *C. difficile* donor. Serial dilutions were spread on selective plates to count the number of donor and recipient cells harvested from the filters. After 72-96 h the putative transconjugants were counted and sub-cultured onto fresh selective plates.

Identity of the transconjugants was confirmed by PCR using a primer pair that is specific for the recipient strain and a primer pair that is specific for the transferring element; these are specified in the relevant chapters.

2.5. DNA sequencing reactions

The sequencing of plasmids and PCR products were performing by Genewiz, formerly Beckman Coulter Genomics (Essex, UK). The concentration of DNA products was determined by NanoDrop^{TM} 1000 Spectrophotometer (Thermo Scientific, Surrey, UK). Ten microliters of plasmids (100 ng/µl) and 5 µl of PCR products (50 ng/µl) were prepared for sequencing with appropriate primers.

2.6. DNA sequence analysis

The sequencing results were analysed by using BioEdit version 7.2.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and DNAMAN. For the samples sequenced with more than one reaction, the sequences were combined by using the CAP contig function in the BioEdit software (Huang, 1992). The comparison of DNA sequences to the nucleotide and protein database by using the National Centre for

Biotechnology Information (NCBI) tools, BlastN and BlastX, respectively (Altschul *et al.*, 1990). The sequence alignment was performed by using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers *et al.*, 2011).

2.7. Statistical Analysis

Statistical analysis was conducted on data using Microsoft Excel 2016. The statistical test used was the 2 paired students T-test, equal variance was assumed. For environmental stress analysis, one-way or two-way ANOVA were used, and again equal variance was assumed. In both cases, *P* values < 0.05 were considered significant and *P* values < 0.001 were considered highly significant.

Chapter 3 Exposure to pancreatic α-amylase promotes exopolymer secretions in *C. difficile*

3.1. Introduction

3.1.1. *C. difficile* extracellular polymeric substance (EPS)

3.1.1.1. Definition of EPS

The abbreviation “EPS” was first introduced by Wingender *et al.,* (1999) as a general term to refer to various macromolecules such as polysaccharides, proteins, lipids, phospholipids, nucleic acids (DNA and/ or RNA) and other compounds such as metals secreted by bacterial cells (Wingender *et al.*, 1999). EPS is a complex high-molecular-weight polymer mixture which protects bacterial cells from dehydration, desiccation and toxic materials (Li *et al.*, 2016). In addition, it can be consumed by starved cells as a carbon or energy source in conditions of nutrient deficiency (Zhang & Bishop, 2003). It also has a significant role as a strong adhesive to bind bacterial cells together to form microbial aggregates and biofilms (Bales *et al.*, 2013). The major components of EPS are carbohydrates, proteins and DNA which can vary in quantity and composition based on different factors such as culture media, growth phase, environmental and host signals (Nielsen *et al.*, 1996).

The key component of *C. difficile* biofilm is the EPS matrix (Dawson *et al.*, 2012). As well as protecting vegetative cells and spores from environmental stresses, the EPS facilitates cell-to-cell communication and interaction by immobilising cells and keeping them very close to each other (reviewed in Flemming *et al.*, 2007). The presence of extracellular immunogenic polymers on bacterial cell surfaces and their accessibility to the adaptive mucosal immune system makes them favourable vaccine candidates; therefore, the polysaccharide and protein contents of *C. difficile* EPS are being

investigated to design vaccines targeting the human immune response against *C. difficile* infections (CDI) (Laverde *et al.*, 2014, Leuzzi *et al.*, 2014).

3.1.1.1.1. *C. difficile* extracellular carbohydrate

In addition to peptidoglycan (PG) which is a largely conserved cell wall structure composed of glycan molecules cross-linked by short peptide chains, three anionic polysaccharides (PSs), specific to *C. difficile* have also been identified (Kirk *et al.*, 2016). The presence of PS-I, a pentaglycosyl phosphate and PS-II, a hexaglycosyl phosphate were first reported in *C. difficile* strains MOH900 and MOH718 (Ganeshapillai *et al.*, 2008). By using nuclear magnetic resonance (NMR), the third *C. difficile* specific polysaccharide that is a novel lipoteichoic acid (LTA) was also discovered in clinical and environmental isolates of *C. difficile* (Reid et al., 2012). Investigations into *C. difficile* polysaccharides revealed that unlike PS-I which is only associated with hypervirulent ribotype 027 isolates, PS-II and PS-III are more widely distributed across *C. difficile* strains with PS-III having a highly conserved structure among all strains studied (Figure 3.1) (Ganeshapillai *et al.*, 2008, Reid *et al.*, 2012).

In recent years, *C. difficile* surface polymers have been investigated extensively to be used as vaccine candidates due to their antigenic properties and potential to control infection and colonization (Ghose & Kelly, 2015). It has been shown that anti-PS-I IgG is elevated in horse sera following exposure to both native and synthetic PS-I (Jiao *et al.*, 2013). The first dual vaccine containing synthetic PS-I conjugated with a subunit of *C. difficile* toxin B was designed as a promising therapeutic agent against CDI (Jiao *et al.*, 2013). However, since PS-I is not widespread amongst *C. difficile* strains, PS-II and PS-III are more attractive options to develop polysaccharide-based vaccines.

Anti-PS-II antibodies can be detected in humans and pigs following exposure to *C. difficile* (Oberli *et al.,* 2011). Therefore, PS-II-based vaccines conjugated with carriers such as CRM_{197} (non-toxic mutants of diphtheria toxin) have been designed that have the ability to confer immunity in mice (Oberli *et al.*, 2011, Monteiro *et al.*, 2013).

3.1.1.1.2. *C. difficile* extracellular protein

The bacterial cell surface layer (S-layer) is a crystalline or para-crystalline array covering the entire cell (Fagan & Fairweather, 2014). It is found in a broad range of bacteria, including environmental species and pathogens as well as many Archaea (de la Fuente-Núñez *et al.*, 2012). The best characterised examples of *C. difficile* extracellular proteins are Cwp84, a cysteine protease which cleaves the SlpA precursor (Janoir *et al.*, 2007), Cwp66, a putative adhesin (Waligora *et al.*, 2001) and CwpV, a phase variable protein (Emerson *et al.*, 2009). Cwp84 and Cwp66 have proteolytic and adhesive activities, respectively crucial for bacterial attachment and dissemination during infection (discussed further in chapter 1) (Figure 3.1) (Waligora *et al.*, 2001).

SLPs appear to be crucial in bacterial attachment to the host cells as chemical removal or treatment with anti- SLP antibodies disrupts *C. difficile* attachment to human HeLa cells (Oatley *et al.*, 2018). In addition to playing an important role in bacterial adhesion, SLPs have immunogenic properties as anti-SLP antibody has been detected in patients with *C. difficile* associated diarrhoea (CDAD) (Wright *et al.*, 2008, Bruxelle *et al.*, 2016). SLPs induce maturation of dendritic cells (DCs) and secretion of interleukin-12 (IL-12) and tumour necrosis factor α (TNFα) in mice, followed by the subsequent phagocytosis and bacterial cell death (Ryan *et al.*, 2011).

3.1.1.2. Mucoid bacteria

The components of culture medium play a key role in determining the character and morphology of bacterial colonies. Some bacterial species (e.g., *Klebsiella* spp.) produce mucoid colonies which are shiny, flowing and slimy with irregular edges (Kandi, 2015). Typically, overexpression of the bacterial EPS due to variations in growth conditions including environmental and host signals, temperature, stress, pH, DNA damage, and nutrient limitations can result in mucoid colony formation both *in vivo* and *in vitro* (Boucher *et al.*, 1997).

It has been observed that when hog pancreatic α-amylase was incorporated into charcoal Muller Hinton agar (MHA), a change in colony morphology occurred in *Campylobacter jejuni* strains. The colonies appeared larger and more mucoid than those grown on MHA without amylase. This response was specific to hog pancreatic α-amylase and was not evident when amylase from *Aspergillus oryzae* or human salivary amylase was used. Moreover, the mucoid colonies were shown to contain increased quantity of carbohydrate and form significantly higher amounts of biofilm compared to nonmucoid ones. Exposure to α-amylase protected *C. jejuni* from environmental stresses, indicating that increased carbohydrate secretion possibly plays an important role in persistent *C. jejuni* infection by increased biofilm formation, increased interaction with human cell lines and increased ability to colonize chickens (Jowiya *et al.,* 2015).

Another example of mucoid colony formation in association with increased secretion of the EPS has been observed in *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis. Phenotypic switching in *P. aeruginosa* acts as a strong virulence factor, allowing the bacteria to survive despite the chronic inflammatory response.

Mucoidy in this strain is caused by a mutation in the *mucA* gene. Because of this mutation, *P. aeruginosa* produces increased amounts of EPS to protect itself from the innate clearance mechanism in the lung (Boucher *et al.*, 1997).

Mucoidy, overexpression of EPS and biofilm formation have been shown to be linked in numerous gut pathogens (Jowiya *et al.*, 2015). Dapa *et al.,* 2013 showed that *C. difficile* can produce biofilms comprised primarily of extracellular proteins, polysaccharides and DNA. This suggests that EPS overexpression and the resultant mucoid colony formation by this anaerobic gut pathogen may play a role in CDI recurrence.

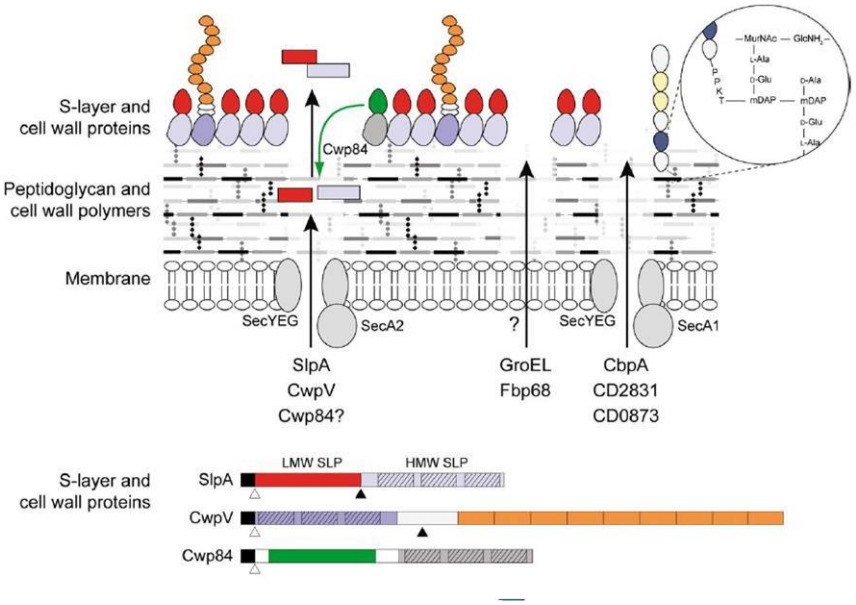


Figure 3.1 Organization of the *C. difficile* cell envelope.

(Retrieved from (Kirk *et al.*, 2016))**.** *C. difficile* has a proteinaceous surface layer (S-layer). The S-layer is covered and functionalized by members of the CWP family; shown are the phase variable anti-phage CwpV and cysteine protease Cwp84. Secretion of the SlpA precursor and CwpV is mediated by the accessory Sec system; ATPase SecA2. Following the SlpA precursor translocation through the cell membrane and secretion, Cwp84 (green arrow) cleaves the pre-SlpA into HMW and LMW SLPs. These subunits form a heterodimer which is the basis of the S-layer.

3.2. Aims and objectives

The hypothesis examined in this chapter is that the intestinal pathogen *C. difficile* can detect and respond to mammalian pancreatic α-amylase by overexpression of EPS.

Objectives

- To determine the colony morphology of *C. difficile* in the presence of pancreatic α-amylase
- To determine the amounts of carbohydrate secreted by *C. difficile* in the presence of pancreatic α-amylase
- To determine the amounts of protein secreted by *C. difficile* in the presence of pancreatic α-amylase

3.3. Materials and methods

3.3.1. Strains and culture media

The list of bacterial strains used in this chapter are shown in table 3.1. *C. difficile* strains were grown in brain heart infusion (BHI) agar or broth from Oxoid Ltd (Basingstoke, UK), supplemented with 5% defibrinated horse blood (E and O Laboratories, Bonnybridge, Scotland, UK) and incubated in an anaerobic atmosphere (80% nitrogen, 10% hydrogen and 10% carbon dioxide) in Macs-MG-1000-Anaerobic work station (Don Whitley scientific). Media were supplemented when required with pancreatic α-amylase from porcine pancreas obtained from Sigma-Aldrich (Dorset, UK) to final concentrations of 0.1 µM, 1 µM and 10 µM. *C. difficile* selective supplement obtained from Oxoid Ltd (Basingstoke, UK) was added to the plates supplemented with α-amylase.

Table 3.1 Bacterial strains used in this study.
Tc = tetracycline, Erm = erythromycin, Rif = rifampicin, ^{R} = resistant, ^{S} = sensitive

Bacterium	Ribotype	Properties	Source
630Δ*erm*	012	Tc^{R} Erm^{S} Rif^{S}	Hussain *et al.*, (2005)
R20291	027	Tc^{S} Erm^{S} Rif^{R}	Stabler *et al.*, (2006)

3.3.2. Preparation of basal defined medium

Composition of basal defined medium (BDM) was described by (Karasawa *et al.*, 1995). A slight amendment was made to the protocol by replacing individual amino acids with essential and non-essential amino acid mixtures obtained from Sigma-Aldrich (Dorset, UK) (Appendix 1). BDM agar plates were prepared by adding pancreatic α-amylase and *C. difficile* selective supplement. They were then inoculated with *C. difficile* and incubated at 37 °C in an anaerobic condition.

3.3.3. Growth curves

C. difficile strains R20291 and 630Δ*erm* were revived from frozen stock onto BHI agar and grown for 48 h. A single colony was inoculated into 10 ml BHI broth (Oxoid) and grown overnight at 37 °C in an anaerobic condition with shaking at 50 rpm. The overnight culture (OD_{600} ~ 0.8) was diluted into 50 ml BHI broth with or without pancreatic α-amylase (0.1, 1 and 10 µM) to OD_{600} ~ 0.1 and incubated at 37 °C under anaerobic conditions with shaking at 50 rpm. The optical density at 600 nM (OD_{600}) was recorded hourly.

3.3.4. EPS purification

The growth was removed from BHI agar plates supplemented with or without pancreatic α-amylase and suspended in 5 ml phosphate buffered saline (PBS) (Sigma-Aldrich) or 1 M sodium chloride (NaCl). For proteomics analysis, the preparation was done in the presence and absence of 1 µl protease inhibitor cocktail powder (catalogue number: P2714, Sigma, UK) prepared according to the supplier's instruction.

The bacterial suspension was washed at 200 rpm on a rotary platform for 1.5 h at 30 ºC then vortexed for 15 min and washed at 200 rpm for a further 1.5 h at room temperature. The bacteria were removed by centrifugation at 1,800 x *g* for 15 min and the material in the supernatant was precipitated by addition of 4 volumes of ice-cold acetone and left at 4 °C overnight. The precipitate was recovered by centrifugation at 450 x *g* for 5 min, washed in water and dried in a Speedvac SPD1010 (Thermo Scientific). Finally, the pellet was dissolved in 1 ml of distilled water and, if not used immediately, was stored at -70 °C.

3.3.4.1. Carbohydrate assay

Total carbohydrate was measured using the phenol-sulphuric acid assay (DuBois *et al.*, 1956) with glucose as the standard.

3.3.4.2. Protein assay

The acetone-precipitated material was assayed for protein using a Micro BCA Protein Assay Kit (Pierce, Thermo Scientific) according to the manufacturer's instruction.

3.4. Results

3.4.1. Pancreatic α-amylase induces a colony morphology change in *C. difficile*

When hog pancreatic α-amylase was incorporated into BHI agar, a change in colony morphology was observed (Figure 3.2). *C. difficile* strains R20291 and 630Δ*erm* colonies grown on BHI agar supplemented with α-amylase were mucoid and larger than those grown on BHI agar without α-amylase. This response was specific to hog pancreatic α-amylase and was not evident in the presence of amylase from *Aspergillus oryzae* or human salivary amylase. Furthermore, porcine mucin, porcine bile, porcine pancreatic trypsin and bovine serum albumin (BSA) were also tested at a range of different concentrations and no change in colony morphology was observed (Table 3.2). The fact that mucoidy was only observed in the presence of hog pancreatic α-amylase shows that *C. difficile* can detect and respond to this specific amylase. No difference was observed between strains in the degree of mucoidy in response to enzyme supplementation (no visual difference). The lowest concentration at which mucoidy was visible was 10 μM (Figure 3.2). The hog pancreatic α-amylase preparation contained only pancreatic α-amylase and no other proteins (Appendix 2).

Table 3.2 Appearance of mucoidy of *C. difficile* in response to medium supplemented with pancreatic enzymes.

Mucoidy and non-mucoidy are marked with (+) and (-), respectively. The experiment is repeated three times with similar results.

Bacterium	Pancreatic α-amylase	α-amylase from *A. oryzae*	Human salivary α-amylase	BSA	Trypsin	Mucin	Porcine bile
R20291	+	-	-	-	-	-	-
630Δ*erm*	+	-	-	-	-	-	-

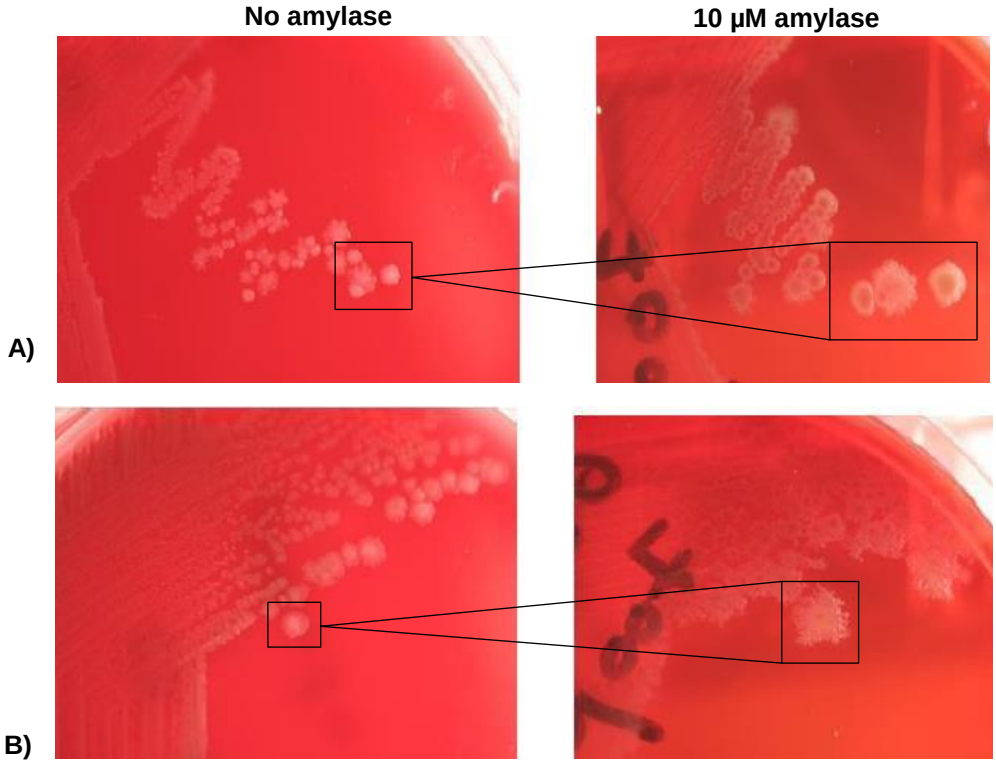


Figure 3.2 *C. difficile* colony morphology change in the presence of 10 μM pancreatic α-amylase.

A) R20291, B) 630Δ*erm*.

Colonies are mucoid and larger in the presence of amylase than those grown in the absence of amylase. The difference in size is more evident in strain 630Δ*erm,* but the degree of mucoidy is similar between the strains.

3.4.2. Starch is not required for amylase-induced mucoidy

To investigate whether the mucoid phenotype is due to α-amylase hydrolysing starch molecules as its substrate, *C. difficile* was grown on BDM (for method see section 3.3.2). BDM lacks starch and contains glucose and amino acids as energy sources. *C. difficile* strains R20291 and 630Δ*erm* formed mucoid colonies when cultured on α-amylase-supplemented BDM. We observed that the mucoidy was lost when the mucoid colonies were re-streaked on BDM without α-amylase. These results suggest that *C. difficile* detects and responds to pancreatic α-amylase as an environmental signalling factor and the change in colony morphology is not due to amylase enzymatic activity on the starch.

3.4.3. Growth promotion by pancreatic α-amylase

Since the colonies produced by *C. difficile* on BHI plates supplemented with pancreatic α-amylase were larger as well as being more mucoid, the growth of *C. difficile* R20291 and 630Δ*erm* in the presence and absence of varying concentrations of hog pancreatic α-amylase was compared in BHI broth. *C. difficile* R20291 culture supplemented with α-amylase had a higher optical density at every time point; however, this was not statistically significant ($P > 0.05$) (Figure 3.3.A). *C. difficile* 630Δ*erm* culture supplemented with 10 μM pancreatic α-amylase showed statistically significant increased rate of growth ($P < 0.05$) (Figure 3.3.B).

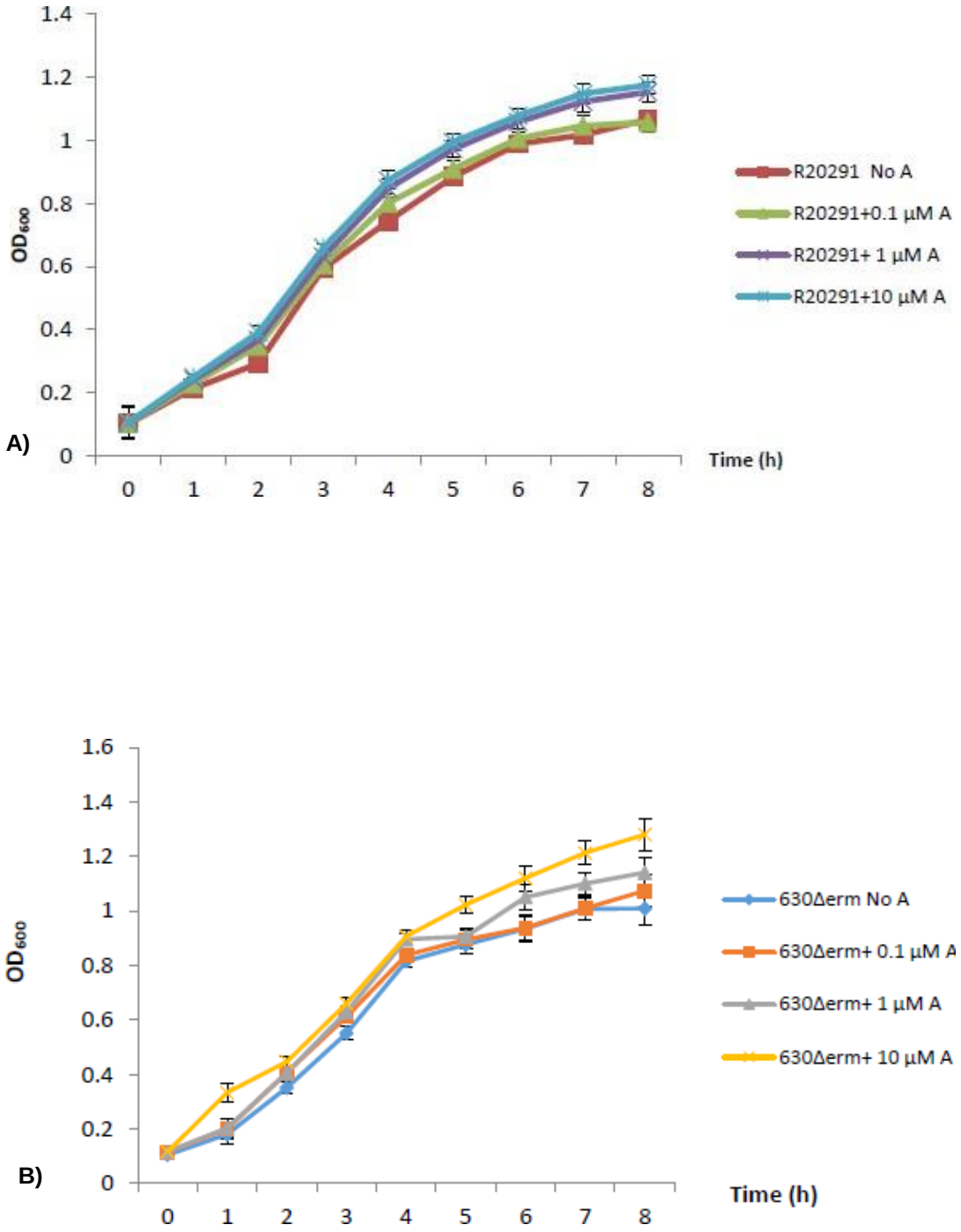


Figure 3.3 Growth of *C. difficile* strains A) R20291 and B) 630Δ*erm* at 37 °C in BHI broth with varying concentrations of pancreatic α-amylase.

The optical density (OD_{600}) was measured at hourly intervals. Statistical significance was analysed using one-way ANOVA. *P* value < 0.05 was considered significant. Data shown is the mean and standard deviation from three independent experiments. A = amylase

3.4.4. Determining the extracellular carbohydrate content of *C. difficile* exposed to pancreatic α-amylase

To determine if the mucoid phenotype observed in the presence of α-amylase is a result of increased carbohydrate secretion, the EPS was prepared from *C. difficile* strains R20291 and 630Δ*erm* (section 3.3.4) grown on increasing concentrations of α-amylase and the carbohydrate content was measured (section 3.3.5). Extraction of extracellular carbohydrate was compared using PBS (Figure 3.4) or 1 M NaCl (Figure 3.5) as solvents. Using the latter, carbohydrate extraction was significantly higher in the presence of pancreatic α-amylase (Figure 3.5).

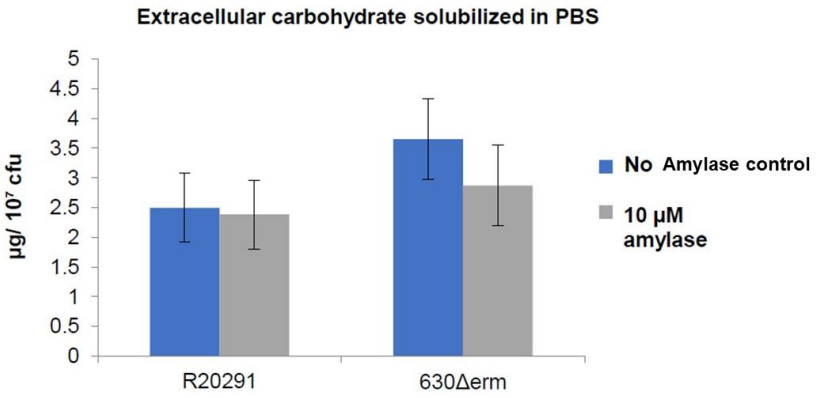


Figure 3.4 Total amounts of water-soluble extracellular carbohydrate secreted by R20291 and 630Δ*erm* was measured using phenol-sulphuric acid assay.

No significant difference was observed when EPS was purified with PBS. The statistical test used was the two-way ANOVA. *P* value < 0.05 was considered significant. Data shown in the mean and standard deviation from three independent experiments.

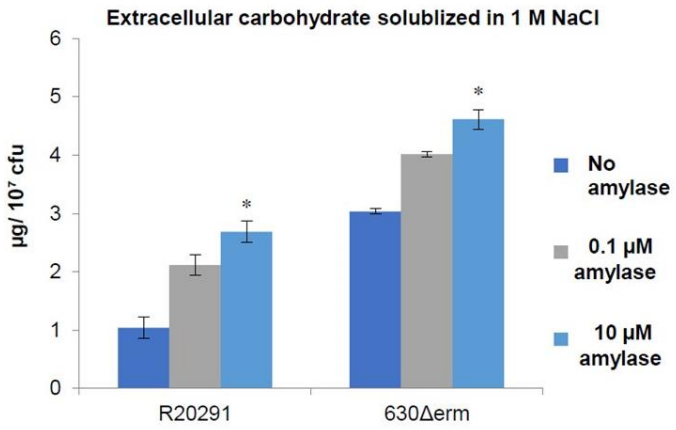


Figure 3.5 Total amounts of water-soluble extracellular carbohydrate secreted by R20291 and 630Δ*erm* was measured by phenol-sulphuric acid assay.

1 M NaCl was used to extract EPS. Results showed a significant increase in the amounts of carbohydrate in the presence of 10 µM amylase. The statistical test used was the two-way ANOVA. *P* value < 0.05 was considered significant. The data shown are representative of three independent experiments performed in triplicate.

3.4.5. Determining the extracellular protein content of *C. difficile* exposed to pancreatic α-amylase

To determine if the mucoid phenotype observed in the presence of α-amylase is a result of increased protein secretion, the EPS was prepared from *C. difficile* strains R20291 and 630Δ*erm* (section 3.3.4) grown on increasing concentrations of α-amylase and the protein content was determined (section 3.3.6). Extraction of extracellular protein was compared using PBS (Figure 3.6) or 1 M NaCl (Figure 3.7) as solvents. Using the latter, protein extraction was significantly higher in the presence of pancreatic α-amylase (Figure 3.7).

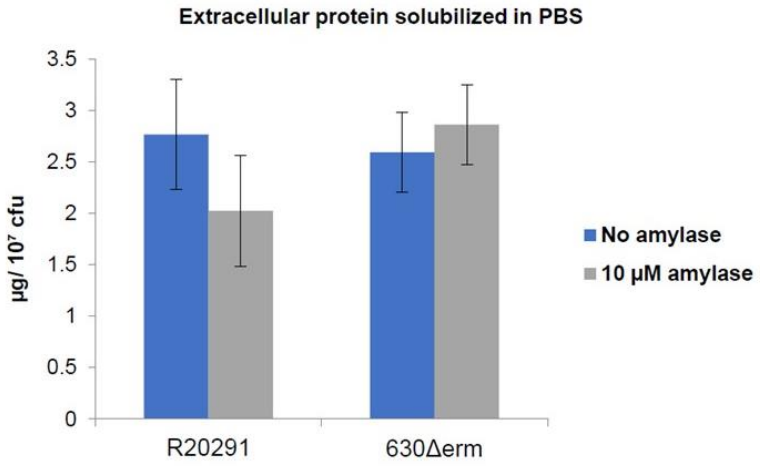


Figure 3.6 Total amounts of extracellular protein secreted by R20291 and 630Δ*erm* was measured.

No significant difference was observed when EPS purified with PBS. The statistical test used was the two-way ANOVA. *P* value < 0.05 was considered significant. Data shown in the mean and standard deviation from three independent experiments.

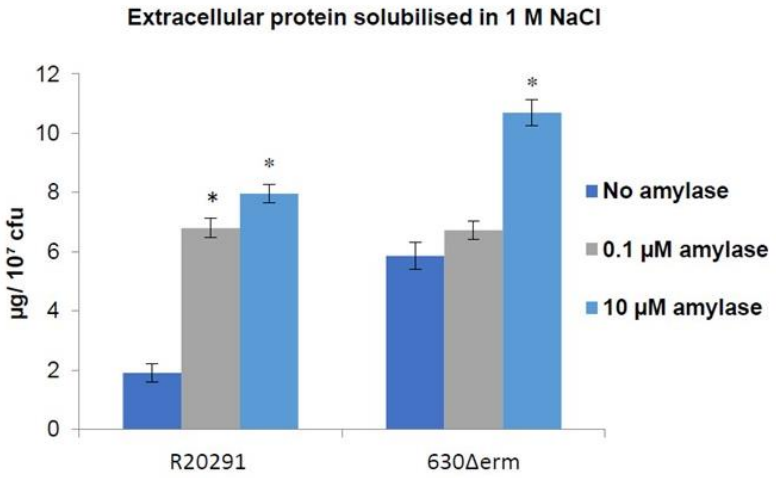


Figure 3.7 Total amounts of extracellular protein secreted by R20291 and 630Δ*erm*.

1 M NaCl was used to extract EPS. Results showed a significant increase in the amounts of protein in the presence of 0.1 and 10 µM amylase. The statistical test used was the two-way ANOVA. *P* value < 0.05 was considered significant. The data shown are representative of three independent experiments performed in triplicates.

3.5. Discussion

Overexpression of EPS and the subsequent conversion to the mucoid phenotype is well-described in numerous bacterial species particularly those involved in cystic fibrosis (Zlosnik *et al.*, 2011). Furthermore, infection with a non-mucoid phenotype can be more rapidly eliminated following antibiotic treatment than infection with mucoid isolates (Zlosnik *et al.*, 2011).

Several Gram-negative bacteria including *Campylobacter jejuni, Burkholderia cepacia, Pseudomonas aeruginosa, Vibrio cholerae* and *Vibrio parahaemolyticus* are able to express mucoidy as a result of increased EPS secretion (Enos-Berlage & McCarter, 2000, Lee *et al.*, 2005, Bartholdson *et al.*, 2008, Jowiya *et al.*, 2015). In the case of *B. cepacia,* increased EPS production is due to interaction with onion skin which the organism encounters within its life cycle (Bartholdson *et al.*, 2008).

The mucoid phenotype in Gram-positive bacteria is best characterised in some strains of *Staphylococcus aureus* (e.g., strains Smith diffuse and M which produce abundant polysaccharide intercellular adhesion (PIA) and poly-*N*-acetylglucosamine (PNAG)) (Lister & Horswill, 2014). These highly mucoid, heavily encapsulated *S. aureus* strains are more resistant to phagocytosis than non-mucoid strains in animal models (Koenig, 1962). In a separate study (Lee *et al.*, 1987), a correlation between the amounts of surface associated polysaccharide and staphylococcal virulence was found. It was shown that strains with lower amounts of extracellular polysaccharide were cleared more easily from the bloodstream in animal models than those that were highly mucoid and encapsulated.

To link mucoid colony formation by *C. difficile* strains to increased secretion of EPS in response to pancreatic α-amylase, we undertook a biochemical approach to quantify the change in carbohydrate and protein production following supplementation of the culture media with varying concentrations of pancreatic α-amylase.

Owing to the facts that pancreatic α-amylase induced mucoidy in *C. difficile* strains, we hypothesized that the increased secretion of carbohydrates and proteins in EPS was responsible for the morphology change in R20291 and 630Δ*erm*. However, it would be advantageous to further extend this analysis to look for the presence of lipids and nucleic acids and their response to pancreatic α-amylase as a host signal. Our results indicated that mucoidy was not caused by amylase enzymatic activity on starch molecules since it also appeared on amylase-supplemented basal defined medium (BDM) which lacks starch. Mucoidy was lost after sub-culturing mucoid colonies on amylase-free BDM, indicating that signals received from the presence of amylase are required for mucoid colony formation and the presence of starch, the substrate of amylase, is not essential. By ruling out the role of digestive activity of α-amylase in mucoid colony formation, we suggest that detection of the amylase signal by *C. difficile* is a regulatory pathway which contributes to the phenotypic change.

C. difficile is able to recognize and respond to a number of biological compounds that it encounters in the human GI tract including bile salts (Sorg & Sonenshein, 2008, Francis *et al.*, 2013), short chain fatty acids (Antharam *et al.*, 2013), organic acids (Ferreyra *et al.*, 2014), mucin constituent MUC1 (Linden *et al.*, 2008) and sugar compounds (Ng *et al.*, 2013) . The results of this chapter show that *C. difficile* is also able to recognise mammalian pancreatic α-amylase specifically and respond with the increased production of exopolymers.

A well-studied example of *C. difficile* strains detecting and responding to the host signals is the recognition of bile salts such as taurocholate and glycocholate (primary bile salts conjugated with taurine and glycine) by their spores in the human gut. Spore germination requires the release of Ca^{++}-dipicolinic acid (DPA) from the spore core and the subsequent hydrolysis of spore cortex (Wilson, 1983). It has been determined that a germination-specific protein, CspC which is a bile acid germinant receptor mediates bile acid recognition and the subsequent spore germination in *C. difficile.* Mutation in the *cspC* disrupts the initiation of spore germination (Sorg & Sonenshein, 2008). Based on our results, it is possible that a similar recognition mechanism is involved in detecting the presence of pancreatic α-amylase signal by *C. difficile*.

In addition, strain R20291, but not 630Δ*erm*, showed a significant increase in protein secretion ($P < 0.05$) when cultured with 0.1 µM amylase. Although 10 µM is the concentration at which mucoidy was evident, we assume that both *C. difficile* strains can detect the presence of 0.1 µM amylase; however, the response may not be presented as overexpression of exopolymers or morphological changes. Further studies are needed to determine *C. difficile* alternative behavioural responses to the varying concentrations of signalling molecules. Data obtained from numerous studies show that not only can *C. difficile* detect the presence of biological compounds in the gut, but also any slight changes in their concentration could trigger the response and facilitate bacterial colonization. For instance, the effects of varying concentrations of bile salts on *C. difficile* spore germination have been investigated by exposing spores to taurocholate at concentrations ranging from 0.001% to 10%. 0.001% was the lowest concentration which resulted in colony formation by approximately 0.0002% of the total number of spores indicating that *C. difficile* can still detect the environmental signals, albeit at a low level (Sorg & Sonenshein, 2008).

C. difficile encounters both bile salts and pancreatic α-amylase at the same point in the small intestine. The pancreatic duct connects to the common bile duct before the hepatopancreatic ampulla, after which both ducts open into the intestine (Hopkins & Wilson, 2018). It has been suggested that encountering bile salts would benefit *C. difficile* to invade epithelial cells within the human GI tract. (Kochan *et al.*, 2017). The idea of the bacterium using biological compounds as environmental signals is supported by Lindén *et al.*, (2008), who suggested that *C. difficile* uses mucin (MUC2) to regulate expression of genes associated with pathogenicity. The results presented in this chapter indicate a similar role for pancreatic α-amylase in that α-amylase-induced EPS expression may promote *C. difficile* aggregation, adherence to epithelial cells, biofilm formation and the subsequent colonization as the initial steps of infection (discussed further in chapter 4). Interestingly, *C. difficile* is amongst the most frequently isolated microorganism associated with hyperamylasemia (high blood amylase level) during acute gastroenteritis (Tositti *et al.*, 2001). We assume that the overexpression of *C. difficile* surface associated antigenic polysaccharides and proteins in the presence of pancreatic α-amylase could be linked to survival in the gut and pathogenicity.

In addition to bile acids and α-amylase, *C. difficile* can also detect signals from short chain fatty acids (SCFAs) in the human gut. Antharam *et al.,* (2013) showed that several species of bacteria with the ability to produce SCFAs particularly butyric producing anaerobic bacteria (e.g. *Ruminococcaceae* and *Lachnospiraceae*) were absent or reduced in number during *C. difficile* infection. Butyric acid is known to have anti-inflammatory effects by increasing antimicrobial peptide level and mucin production.

Furthermore, it decreases the permeability of the epithelial cells by inducing the expression of tight junction proteins (Cook & Sellin, 1998, Wong *et al.*, 2006).

Therefore, depleted butyrogenic bacteria and subsequent butyric acid level in the human gut can be detected by opportunistic pathogens such as *C. difficile* and lead to successful colonization and infection. Hence, replenishing reduced number of butyrate-producing bacteria can aid the design of novel therapeutic approaches to tackle *C. difficile* infection (Antharam *et al.*, 2013).

Another finding of this study is the ability of pancreatic α-amylase to promote *C. difficile* growth. The results presented here have shown that supplementation of the pancreatic α-amylase into *C. difficile* culture media resulted in increased growth rate in liquid culture. Although the difference in growth rates with and without amylase is not statistically significant in strain R20291 ($P > 0.05$), it is likely that it is biologically relevant. Increase in bacterial growth rate in response to intestinal secretions has been documented. For instance, norepinephrine; a neurotransmitter hormone in the body, has been shown to significantly increase the rate of growth in *C. jejuni* (Cogan et al., 2007). Moreover, *Helicobacter pylori* growth rate is stimulated by gastrin; a gastrointestinal hormone produced by g cells in the duodenum and in the pyloric antrum of the stomach (Chowers *et al.*, 1999). We hypothesize that a similar response mechanism to the GI tract growth stimulating components is present in *C. difficile* strains to promote the growth.

In general, results obtained from this chapter show that *C. difficile* can respond specifically to mammalian pancreatic α-amylase by producing a mucoid phenotype through secretion of exopolymers. These results highlight the importance of looking beyond well-documented virulence factors of *C. difficile* (including toxins and colonization factors such as fibronectin-binding proteins, SLPs, CWPs and flagella) and measuring the bacterial response to the host factors when determining the relationship between *C. difficile* pathogenicity and human health.

Furthermore, it is crucial to highlight the potential of our observations in this chapter because of their importance in the understanding of the adaptation and subsequent evolution of *C. difficile*. It has been revealed that horizontal gene transfer is a driving force in the evolution of microorganisms. Horizontal transfer rates are typically higher in biofilm communities compared with those in planktonic states (Madsen *et al.*, 2012). If the increased secretion of extracellular polymers in *C. difficile* in response to environmental stresses (in this case, pancreatic α-amylase) leads to more bacterial attachment and biofilm formation, it is possible that the rate of antibiotic resistance and/ or virulence genes transfer is also increased within the cells encapsulated in EPS.

Chapter 4 Pancreatic α-amylase disrupts/ inhibits *C. difficile* biofilm formation

4.1. Introduction

4.1.1. Biofilms

4.1.1.1. Definition of biofilm

Biofilms are heterogenous microbial communities that can form on solid surfaces, at air-liquid interfaces (i.e., pellicle biofilms), or in liquid as cellular clusters (i.e., flocs) (Hall-Stoodley *et al.*, 2004, Marti *et al.*, 2011, Nadell *et al.*, 2016). The ability to form biofilms is widespread across many bacterial genera; both Gram-positive and Gram-negative and on many surfaces in natural and industrial environments (Vlamakis *et al.*, 2013). Depending on the environmental conditions at which the biofilms form, they contain cells of one (mono-species), two (dual-species) or many bacterial species (multi-species). A typical biofilm can be composed of 10-25% cells and 75-90% extracellular polymeric substances (EPSs) (Zhao *et al.*, 2017).

Biofilm formation enhances the ability of several pathogens to colonize and establish an infection by providing them with an enclosed environment to escape host immune response and resist antimicrobial agents (Mah & O'Toole, 2001). They also have the potential to be problematic in many man-made materials such as medical devices and industrial settings, leading to rising costs in health care and industrial sectors (Garrett *et al.*, 2008).

Biofilms also harbour some beneficial properties which could be applied in many areas including biochemistry, bioremediation and electrochemistry (Erable *et al.*, 2010). For example, biofilms produced by *Geobacter sulfurreducens* and *Rhodoferax ferrireducens* act as an electron transporter (electro-active biofilm) and can be used to

reduce excessive levels of heavy metals (e.g., chromium and uranium) in contaminated soil and water (Reimers *et al.*, 2001, Mitra & Mukhopadhyay, 2016).

C. difficile produces biofilms *in vitro* (Dawson *et al.,* 2012, Đapa *et al.,* 2013) and *in vivo* (Soavelomandroso *et al.,* 2017), an ability which could be significant for colonization, adherence to the epithelial cells and persistence in the host gastrointestinal (GI) tract and may be involved in the recurrence of *C. difficile* infection (CDI) in some patients.

4.1.1.2. Biofilm structure

The architecture and structure of the biofilm is provided by EPS which in many cases is highly hydrated and strongly attached to the underlying surface (Limoli *et al.*, 2015). The composition of the EPS matrix is heterogenous within both mono and multi-species biofilms and provides biofilms with 50% to 90% of the total organic carbon (Reviewed in Madsen *et al.*, 2018). The liquid phase of the EPS is the viscous section of the biofilm, providing it with resilience against physical stresses and environmental pressures (Renner & Weibel, 2011). Furthermore, spaces between bacterial cells are filled with the EPS molecules which shape the structure of the biofilm and determine the living conditions of the cells (Persat *et al.*, 2015).

Many bacterial species (e.g., *Klebsiella pneumoniae*) living in the deep parts of the biofilm phenotypically differ from planktonic cells since they have limited access to nutrient sources, oxygen and other environmental growth factors. Consequently, they are slow or non-growing and have reduced susceptibility to antimicrobial agents (Wentland *et al.*, 1996, Becker *et al.*, 2001).

The chemical and physical properties of the EPS vary significantly among biofilms (Jahn & Nielsen, 1995). All major macromolecules including polysaccharides, proteins, nucleic acids, peptidoglycans, and lipids can be found in a biofilm (reviewed in Flemming *et al.*, 2007). Polysaccharide is among the predominant components of the EPS matrix mediating essential biofilm properties including adhesion, aggregation of the bacterial cells, water retention, nutrient delivery and protection against environmental stresses (Limoli *et al.*, 2015).

Extracellular DNA (eDNA) plays a key role during early stages of biofilm formation by some bacterial species such as *Listeria monocytogenes* (Harmsen *et al.*, 2010, Harmsen *et al.*, 2010). Different mechanisms such as autolysis and active secretion as well as membrane vesicle-mediated DNA release are associated with the presence of eDNA in the biofilm matrix (Ibáñez de Aldecoa *et al.*, 2017). It has been hypothesized that the presence of eDNA provides mature biofilms with stability and integrity since the addition of DNase to some bacterial biofilms (e.g., *P. aeruginosa*) disrupts the structure (Whitchurch *et al.*, 2002).

4.1.1.3. Main roles of EPS in the biofilm

4.1.1.3.1. EPS and antibiotic resistance

Microorganisms inside the biofilm are more resistant to antimicrobial agents than their planktonic counterparts (Jefferson, 2004, Qi *et al.*, 2016). The significant increase in antimicrobial resistance in sessile bacteria results from the EPS acting as a diffusion barrier. This feature has been seen in the case of tobramycin, a polycationic aminoglycoside that interacts with polyanionic *P. aeruginosa* EPS matrix and gets trapped before reaching the core (Taylor *et al.*, 2014).

However, antibiotics that do not react with the EPS due to their electric charges diffuse easily through the matrix (Mah, 2012). For example, fluorescence-labelled vancomycin diffuses through the biofilm matrix of *Staphylococcus aureus* human isolates and reaches the bacterial cells within the biofilm. Therefore, EPS acting as a physical diffusion barrier alone is not enough for antibiotic resistance in bacterial biofilms (Daddi Oubekka *et al.*, 2012).

Additionally, the availability of oxygen may also affect susceptibility to the antimicrobials as agar-enclosed *E. coli* biofilm shows a reduced susceptibility to aminoglycoside antibiotics because of decreased uptake by the oxygen-deprived cells (Tresse *et al.*, 1995).

4.1.1.3.2. EPS and horizontal gene transfer (HGT)

Enhanced resistance of biofilms to antibiotics can also be conferred by an increased rate of horizontal gene transfer. The EPS matrix provides a stable optimum condition for conjugation which is one of the common mechanisms of HGT and requires cell-to-cell contacts between donors and recipients (Madsen *et al.*, 2018). For example, the transfer ratio of plasmid pB10 harbouring antibiotic resistance genes from *Pseudomonas putida* to *E. coli* increases from 2/1000 (transconjugants/ cfu) in planktonic culture to 8/100 within the biofilm (Van Meervenne *et al.*, 2014).

4.1.1.4. Biofilm formation and development

Biofilm formation is divided into three main stages: initial attachment (adherence), maturation and dispersal (Watnick & Kolter, 2000). The initiation of biofilm formation requires changes in gene expression which lead to a transition of the bacterium from a planktonic form (free swimming) to a sessile form (O'Toole *et al.*, 2000).

To understand the process of biofilm formation, it is essential to investigate the properties of both bacterial cell surfaces and abiotic or biotic surfaces to which the bacterial cells attach. The characteristics of the surface have a significant impact on biofilm formation that is normally enhanced on rougher and more hydrophobic surfaces (Donlan, 2002). Moreover, the topography and characteristics of the bacterial cell surface play a significant role in biofilm formation. For example, the presence of flagella, pili or fimbria impact the amount of biofilm, as bacterial cells must overcome the repulsive forces of the surface materials and these extracellular structures help the cells to remain attached to the surface until the permanent attachment mechanism is involved (Koczan *et al.*, 2011, Gu *et al.*, 2016).

4.1.2. Biofilm formation by *C. difficile*

Biofilm formation by a few clostridial species, such as mono-species biofilm of *Clostridium perfringens* and multi-species biofilm of *C. difficile* alongside other anaerobic gut bacteria, has been reported (Varga *et al.*, 2008, Donelli *et al.*, 2012). Type IV pilus (T4P) plays an important role in *C. perfringens* gliding, adhesion and biofilm formation (Craig *et al.*, 2004, Varga *et al.*, 2008). The role of T4P in *C. difficile* early biofilm formation has been determined in *C. difficile* strains R20291 and 630Δ*erm* (Maldarelli *et al.*, 2016).

Using field emission scanning electron microscopy to examine the biofilm formed on a biliary stent *in vitro,* a multi-species biofilm of *C. difficile*, *Clostridium baratii*, *Clostridium fallax*, *Clostridium bifermentans*, *Finegoldia magna*, and *Fusobacterium necrophorum* was reported (Donelli *et al.*, 2012). This was the first study showing biofilm formation by *C. difficile* although in combination with other anaerobic species. Dawson *et al*., (2012) described the first mono-species biofilm formed by *C. difficile* strains 630 and R20291 *in vitro*. R20291 produced significantly higher amounts of biofilms as measured by crystal violet (CV) assay and higher numbers of bacteria within the biofilm than those produced by 630.

Biofilm formation *in vitro* was further investigated and shown to contribute to *C. difficile* tolerance to oxygen and antibiotics, including metronidazole and vancomycin used to treat infected patients (Semenyuk *et al.*, 2015, Mathur *et al.*, 2016, James *et al.*, 2018). Since then, the composition of the *C. difficile* biofilm has also been analysed by labelling with antibodies against whole bacteria and treating the biofilm with proteinase and DNase. Results showed that the *C. difficile* biofilm matrix is composed of proteins, surface polysaccharides (primarily PSII) and eDNA (Đapa *et al.*, 2013).

In addition to the biofilm components, different factors regulating biofilm formation in *C. difficile* have also been characterised. For example, surface-associated Cwp84 which is involved in S-layer maturation, negatively regulates biofilm formation by a mechanism which is still unknown (Pantaléon *et al.*, 2015). In contrast, an autoinducer (AI-2) signalling molecule of quorum sensing, LuxS (Carter *et al.*, 2005) positively regulates *C. difficile* biofilm formation (Dawson *et al.*, 2012, Đapa *et al.*, 2013).

The first *in vivo* mono-species biofilm formed by *C. difficile* has recently been identified by (Soavelomandroso *et al.*, 2017) in a mouse model. These workers showed that distribution of bacterial cells on the epithelial cells varies between *C. difficile* strains in which 630Δ*erm* distributes mostly as single cells, whereas R20291 tends to form numerous bacterial aggregates. More research is required to correlate this observation with R20291 hypervirulence and the recurrence of CDI.

A genome-wide study to compare gene expression between 630Δ*erm* planktonic cells and biofilm-associated cells has been conducted by (Poquet *et al.*, 2018). 751 genes (20% of *C. difficile* genome) are differently expressed between biofilm and planktonic cells and almost half of them are up-regulated (338 genes). Many up-regulated genes are involved in translation and metabolic pathways including central metabolism, whereas few down-regulated genes are involved in sporulation indicating that *C. difficile* biofilm cells are metabolically active.

4.1.3. Environmental stresses and biofilm formation

The stresses encountered by *C. difficile* from its natural reservoir in the animal and human GI tract to hospital environment, soil, food and water are varied involving changes in gut metabolites, temperature, pH and oxygen concentrations (Janezic *et al.*, 2016). Examples of proteins which have an integral role in the ability of *C. difficile* to adapt to environmental changes include Spo0A, the master regulator for sporulation. This protein also plays a significant role in *C. difficile* biofilm formation (Dawson *et al.*, 2012). Sporulation and biofilm formation are linked in other bacterial species such as *B. subtilis* in which the switch between biofilm formation and sporulation depends on the concentration of phosphorylated Spo0A (Spo0A-P).

Intermediate levels of Spo0A-P induce biofilm formation, while high levels induce sporulation in the cell (Vlamakis *et al.*, 2013). It has been shown that mutation in the *spo0A* gene results in decreased adhesion to the abiotic surfaces in early stages *of C. difficile* biofilm formation (Dawson *et al.*, 2012, Đapa *et al.*, 2013). Therefore, it is suggested that the Spo0A regulator acts as a switch between different pathways including toxin production (Deakin *et al.*, 2012), biofilm formation and sporulation in *C. difficile* depending on the environmental stress factors (Đapa *et al.*, 2013).

C. difficile biofilm formation in the presence and absence of 0.1 M glucose and 0.3 M sodium chloride (NaCl) was investigated by (Đapa *et al.*, 2013). Both strains R20291 and 630 formed higher amounts of biofilm in BHI supplemented with 0.1 M glucose. In the absence of glucose, strain 630 formed significantly lower amounts of biofilm compared to what was seen in the presence of glucose. However, the presence of glucose did not significantly affect biofilm formation in strain R20291. The presence of NaCl inhibited biofilm formation in both strains. It has therefore been suggested that *C. difficile* biofilm formation responds to the presence of stress factors by over or under expression of biofilm-related EPS molecules.

In addition, mucoid growth of bacteria (discussed in chapter 3) which can be defined as ‘biofilms’ has been shown to increase survival in the presence of environmental stresses. Mucoid strains of *P. aeruginosa* which are characterised by excessive amounts of exo-polysaccharide alginate showed a significant increase in viability within chlorinated water in comparison to non-mucoid cells. When alginate was removed, survival was reduced (Dingemans *et al.*, 2016).

4.2. Aims and objectives

The hypothesis examined in this chapter is that biomass formation by *C. difficile* can be induced in response to pancreatic α-amylase (owing to the fact that without a microscopic examination or image analysis techniques, 'biofilm' would not be a precise terminology to describe sessile bacterial cells, in this work, we use the word 'biomass' to define *C. difficile* cells adhered to the surface)

Objectives

- To determine the effect of pancreatic α-amylase on *C. difficile* strains R20291 and 630Δ*erm* biomass formation
- To determine the role of Spo0A in biomass formation by including 630Δ*erm*Δ*spo0A* in our experiments
- To determine the role of hydrophobicity of different abiotic surfaces and surface roughness on *C. difficile* biomass formation

4.3. Materials and methods

4.3.1. Strains and culture media

The list of bacterial strains used in this chapter are shown in table 4.1. *C. difficile* strains were grown in brain heart infusion (BHI) agar or broth from Oxoid Ltd (Basingstoke, UK), supplemented with 5% defibrinated horse blood (E and O Laboratories, Bonnybridge, Scotland, UK) and incubated in an anaerobic atmosphere (80% nitrogen, 10% hydrogen and 10% carbon dioxide) in a Macs-MG-1000- Anaerobic work station (Don Whitley scientific). Media were supplemented when required with pancreatic α-amylase from porcine pancreas obtained from Sigma- Aldrich (Dorset, UK) to final concentrations of 0.1 µM, 1 µM and 10 µM. *C. difficile* selective supplement obtained from Oxoid Ltd (Basingstoke, UK) was added to the plates supplemented with α-amylase.

Table 4.1 Bacterial strains used in this study.
Tc = tetracycline, Erm = erythromycin, Rif = rifampicin, ^{R} = resistant, ^{S} = sensitive

Bacterium	Ribotype	Properties	Source
630Δ*erm*	012	Tc^{R} Erm^{S} Rif^{S}	Hussain *et al.*, (2005)
R20291	027	Tc^{S} Erm^{S} Rif^{R}	Stabler *et al.*, (2006)
630Δ*erm*Δ*spo0A*	012	630Δ*erm* containing *spo0A*::*erm*(B)	Heap *et al.*, (2007); Deakin *et al.*, (2012)

4.3.2. Biofilm assay

Biofilms were grown using a method adapted from (Dawson *et al.*, 2012). Briefly, a single colony of each *C. difficile* strain (Table 4.1) was inoculated into 10 ml pre-reduced BHI broth and grown for 18 h at 37 °C under anaerobic conditions with shaking at 50 rpm. The overnight cultures were diluted to an OD_{600} ~ 0.1 and 100 µl of each culture was added to 1 ml pre-reduced BHI broth inside each well of 24-well tissue culture plates (treated and/ or non-treated, Sarstedt, Germany) and 24-well tissue culture plates containing glass coverslips. To test biofilm growth in the presence of amylase, 0.1, 1 and 10 µM hog pancreatic α-amylase (Sigma-Aldrich) was supplemented into one set of wells and incubated for 6 days at 37 °C under anaerobic conditions.

For the crystal violet assay, BHI broth was removed by pipetting and the wells were washed twice with 1 ml pre-reduced PBS. Crystal violet (CV, Sigma. UK, 0.1% w/v) was added to the wells and incubated for 30 min at room temperature. The excess crystal violet was removed from the wells by three washes with 1 ml PBS. Crystal violet was extracted by adding methanol to the wells and incubation for 15 min at room temperature. The OD_{595} was measured using a spectrophotometer (Pharmacia biotech ultrospec 2000, Italy).

4.4. Results

4.4.1. *C. difficile* R20291 exhibits greater biomass (number of adhered cells) production than 630Δ*erm* and 630Δ*erm*Δ*spo0A*

The ability of *C. difficile* strains (Table 4.1) to form biomass on glass coverslips, treated tissue culture plates and non-treated tissue culture plates was assessed. Quantification of biomass formation using the crystal violet assay showed that the total biomass produced by the *spo0A* mutant derivative of 630Δ*erm* was significantly ($P < 0.001$) lower than that of 630Δ*erm* on all abiotic surfaces tested. Moreover, strain R20291 produced higher amounts of biomass compared to strain 630Δ*erm* after 6 days of growth at 37 °C ($P < 0.05$). In general, our results show that R20291 produced higher amounts of biomass than 630Δ*erm* and mutation of the *spo0A* gene significantly decreased the amounts of biomass formation (Figure 4.1). These results are in agreement with those reported by (Đapa *et al.*, 2013) in that R20291 produced significantly higher amounts of biofilms compared to strain 630 and the *spo0A* mutant derivative of R20291 demonstrated significantly decreased amounts of biofilm compared to the wild-type strain. Furthermore, our results indicate that *C. difficile* biomass formation on different abiotic surfaces vary depending on the hydrophobicity and surface roughness, with more hydrophilic treated tissue culture plates providing an optimal condition to grow biomass than hydrophobic non-treated polystyrene plates (Figure 4.1).

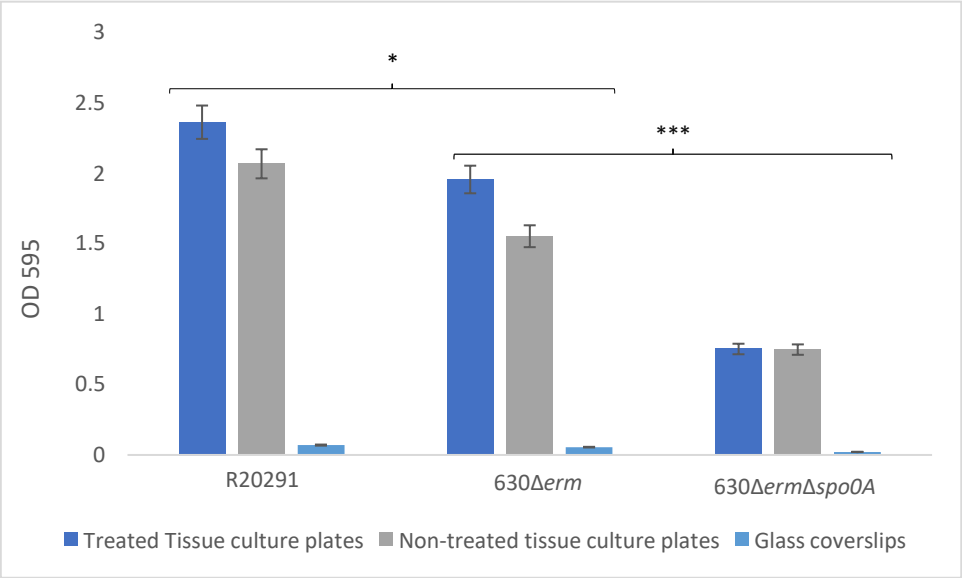


Figure 4.1 Biomass formation by *C. difficile* strains (1) R20291, (2) 630Δ*erm* and (3) 630Δ*erm*Δ*spo0A* on glass coverslips, treated tissue culture plates and non-treated tissue culture plates.

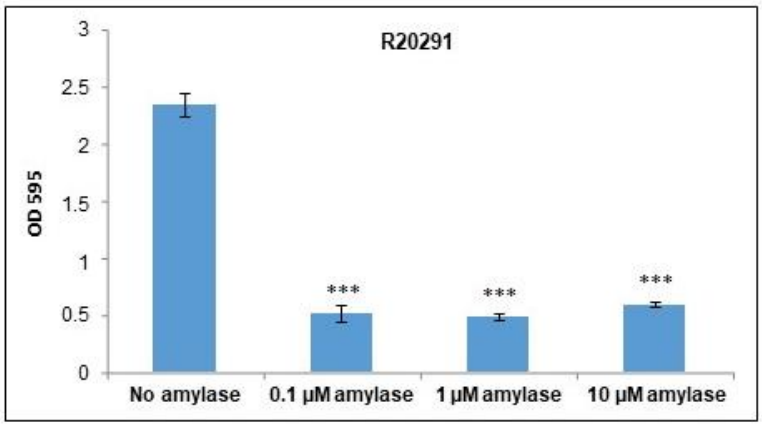
Biomass formation by R20291 is significantly ($P < 0.05$) higher than 630Δ*erm* on all abiotic surfaces. Biomass formation by *spo0A* mutant derivative of strain 630Δ*erm* is significantly ($P < 0.001$) lower than the wild-type strain on all abiotic surfaces. *C. difficile* biomass grows better on treated tissue culture plates compared to glass coverslips and non-treated tissue culture plates. Results are mean counts of three independent experiments. Error bars represent standard deviation. Statistical significance is denoted by * ($P < 0.05$) and *** ($P < 0.001$) as determined by two-way ANOVA.

4.4.2. *C. difficile* biomass formation is significantly decreased in the presence of pancreatic α-amylase.

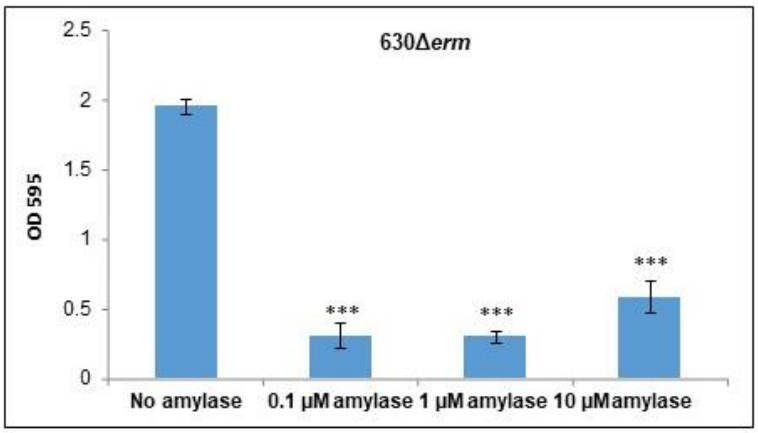
4.4.2.1. Treated tissue culture plates

The ability of *C. difficile* strains (Table 4.1) to form biomass on treated tissue culture plates was assessed in the presence of pancreatic α-amylase. The results showed that all *C. difficile* strains produced highly significantly decreased amounts of biomass ($P < 0.001$, ~ 4-fold) in the presence of all concentrations of pancreatic α-amylase (Figure 4.2).

A)



B)



C)

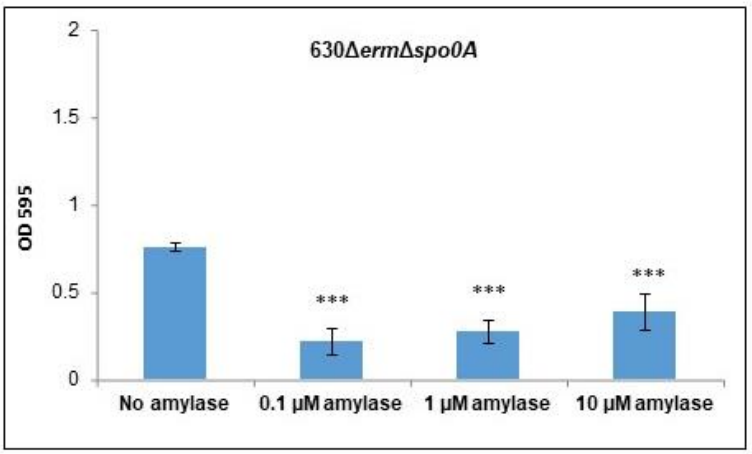


Figure 4.2 Effect of pancreatic α-amylase on biomass formation by *C. difficile* strains A) R20291 B) 630Δ*erm* C) 630Δ*erm*Δ*spo0A* on treated tissue culture plates.

A highly significant decrease in biomass formation was observed in the presence of all different concentrations of amylase. Results are mean counts of three independent experiments. Error bars represent standard deviation. Statistical significance is denoted by *** ($P < 0.001$) as determined by one-way ANOVA.

4.4.2.2. Non-treated tissue culture plates

Biomass formation on non-treated tissue culture plates in the presence of pancreatic α-amylase resulted in significantly decreased biomass quantities ($P < 0.001$). Strain R20291 showed ~ 5-fold decrease in biomass formation in the presence of amylase. Strain 630Δ*erm* showed a ~ 4-fold reduction and strain 630Δ*erm*Δ*spo0A* presented a ~ 2.5-fold reduction when pancreatic α-amylase was added to the culture media. Like α-amylase-supplemented biomass growth on treated tissue culture plates, the significant decrease in biomass formation was observed in the presence of all concentrations of α-amylase tested (Figure 4.3).

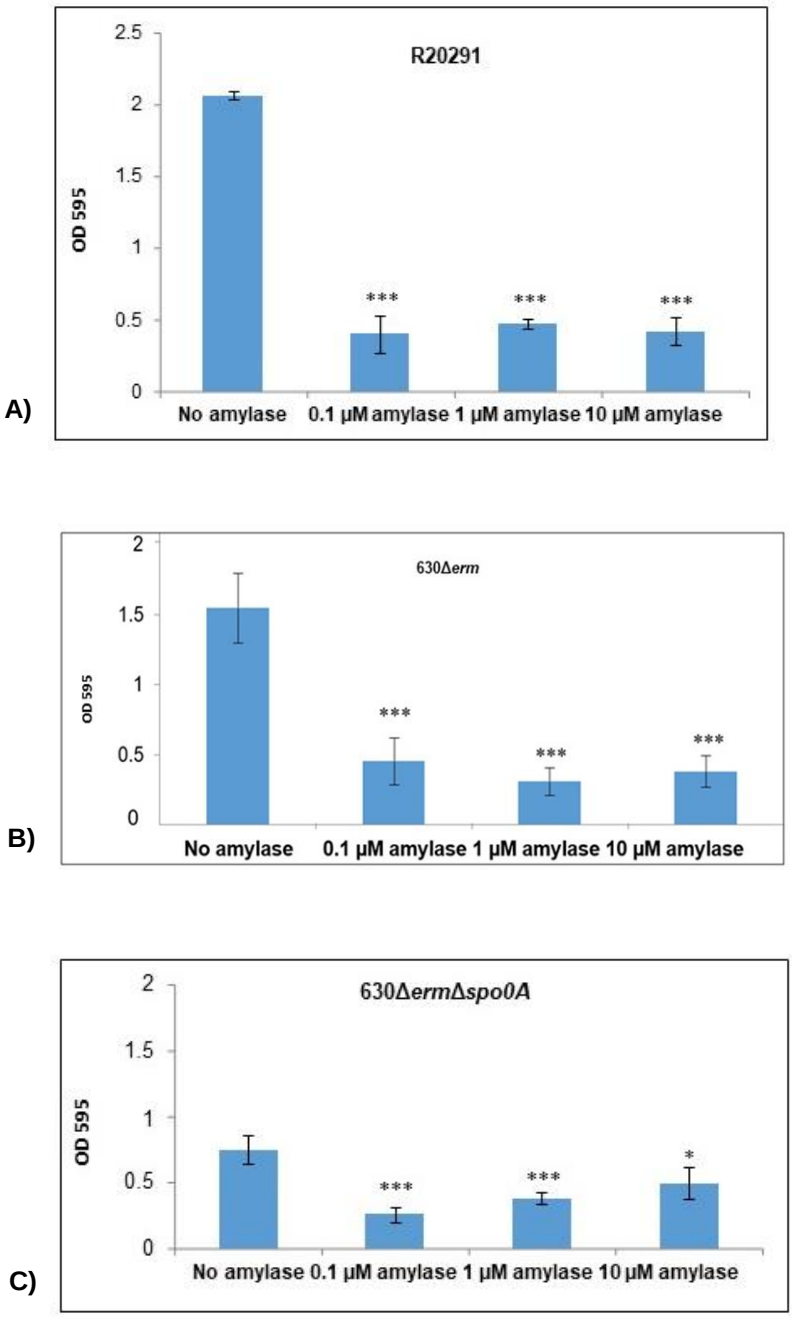


Figure 4.3 Effect of pancreatic α-amylase on biomass formation by *C. difficile* strains A) R20291 B) 630Δ*erm* C) 630Δ*erm*Δ*spo0A* on non-treated tissue culture plates.

A highly significant decrease in biomass formation was observed in the presence of all different concentrations of pancreatic α-amylase. Results are mean counts of three independent experiments. Error bars represent standard deviation. Statistical significance is denoted by * ($P < 0.05$) and *** ($P < 0.001$) as determined by one-way ANOVA.

4.5. Discussion

C. difficile's life cycle is complex (Smits *et al.*, 2016). It begins when a host is contaminated with environmental or hospital spores. Dysbiosis in the gut microbiota leads to spore germination and vegetative cell proliferation (Abt *et al.*, 2016). Adhesion to the epithelial cells and stress-adaptation factors play important roles in colonization and pathogenicity (Poquet *et al.*, 2018). One of the challenges in understanding diseases caused by *C. difficile* is how the bacterium can cause recurrent CDI despite successful treatment of initial infection (Shields *et al.*, 2015). Recurrent CDI can be treated successfully by the second course of the same antibiotics (vancomycin, metronidazole) which was initially applied to treat the first episode of infection. However, up to 65% of patients may have multiple recurrence throughout their lives (Cole & Stahl, 2015).

Cells within biofilms are known to be resistant to antimicrobial agents compared to their planktonic form of growth (Stewart, 2015). It has been hypothesized by Soavelomandroso *et al.*, (2017) and Đapa *et al.*, (2013) that *C. difficile* biofilm formation may play a part in the recurrence of *C. difficile* infection. However, there is little known about the factors triggering *C. difficile* biofilm formation in the human gut (Đapa *et al.*, 2013, Hammond *et al.*, 2014). Determining these factors is important for developing appropriate control measures and a deeper understanding of recurrent gut colonization by *C. difficile*.

Our data show that *C. difficile* cells do not attach particularly well to the glass coverslips. Moreover, epidemic hypervirulent strain R20291 adheres better to the treated and/ or non-treated tissue culture plates and forms more biomass compared to strain 630Δ*erm* and its *spo0A* mutant derivative 630Δ*erm*Δ*spo0A*. It has been

determined by Warny *et al.*, (2005) that strain R20291 produces higher amounts of toxins A and B *in vitro* compared to strain 630. This observation might explain the hypervirulence of strain R20291 and its involvement in the increase and severity of the CDI in North America and Europe between 2000 and 2008 (Lanis *et al.*, 2010). Although more investigations need to be done to explain the colonization pathways of these strains *in vivo,* higher biomass formation by R20291 observed in our study may indicate its better colonization and persistence during the infection and the consequent hypervirulence. In agreement with our results, (Dawson *et al.*, 2012) demonstrated that R20291 can form higher amounts of biofilm compared to 630 ($P < 0.05$) on tissue culture plates, suggesting that there might be a link between severity of the disease caused by this particular strain and its increased biofilm/ biomass formation.

Sporulation and biofilm formation are closely linked in *B. subtilis* by Spo0A, sporulation transcription factor (Fujita & Losick, 2005). In *B. subtilis,* Spo0A regulates one of these three physiological pathways depending on the environmental signals: (i) sporulation, (ii) matrix production (iii) cannibalism, all controlled by different states of Spo0A phosphorylation (Aguilar *et al.*, 2010). In case of low levels of Spo0A phosphorylation, the expression of genes such as exopolysaccharide biosynthesis genes (*epsA-O*) are induced which lead to exopolymeric matrix production and biofilm formation (Hamon & Lazazzera, 2001). High levels of Spo0A phosphorylation lead the bacterium into the irreversible sporulation pathway; however, sporulation itself is not required for biofilm formation (Kearns *et al.*, 2005). The role for Spo0A in *C. difficile* biomass/ biofilm formation was assessed in this chapter and the results showed that the quantity of biomass formed on treated and/ or non-treated tissue culture plates by 630Δ*erm*Δ*spo0A* is significantly reduced compared to the parent strain 630Δ*erm* ($P < 0.001$). Dawson *et al.,* 2012 showed that inactivation of the master regulator Spo0A

using the ClosTron system in strain R20291 visibly decreased the amounts of biofilm quantified by CV assay compared to R20291 wild-type strain and the *spo0A* complement. Besides, the thickness of R20291Δ*spo0A* biofilm measured by confocal microscopy showed 36% reduction after 3 days and 69% reduction after 6 days. We hypothesize that *C. difficile* Spo0A has a similar regulatory function as *B. subtilis* Spo0A that is to switch between numerous pathways such as sporulation, biofilm formation and toxin production (Mackin *et al.*, 2013) as a response to different environmental signals. It has been determined that *C. difficile* sporulation occurs in planktonic growth, but not in biofilms under the same growth conditions which provides further evidence for the Spo0A role in *C. difficile* biofilm formation (Đapa *et al.*, 2013). Furthermore, the *C. difficile spo0A* mutant has been shown to cause infection in mice, but it does not persist in murine gut and does not transfer to other mice efficiently due to lack of sporulation ability, indicating that Spo0A plays an essential role in CDI including persistence (reoccurrence) and transmission (Deakin *et al.*, 2012).

The data presented in this chapter show that *C. difficile* produces significantly decreased amounts of biomass on tissue culture plates in the presence of pancreatic α-amylase. The rate of reduction in biomass formation was similar in all *C. difficile* strains tested ($P < 0.001$) and using different abiotic surfaces with various hydrophobicities did not have any significant impact on the results. Furthermore, the decrease in biomass formation was observed in response to all concentrations of amylase (0.1, 1 and 10 μM) to the same extent, suggesting an inhibitory or perhaps a disruptive effect of pancreatic α-amylase at any concentration on *C. difficile* biomass formation.

The data presented in chapter 3 revealed that exposure to pancreatic α-amylase induced mucoid colony formation in *C. difficile* which was associated with increased

secretion of EPS. Although overexpression of the EPS is usually linked to increased levels of biofilm formation in numerous bacterial species such as *P. aeruginosa* (Jones & Wozniak, 2017), *C. jejuni* (Jowiya *et al.,* 2015) and *Legionella pneumophila* (Wang *et al.,* 2016), the finding from our study indicates that α-amylase-induced EPS overexpression in *C. difficile* strains R20291 and 630Δ*erm* results in a significant reduction and/ or disruption of the biomass.

Antibiofilm activity of α-amylase has been investigated in strains of *S. aureus* (Watters *et al.*, 2016) and up to 97% decrease in biofilm formation by all *S. aureus* strains was found. Scanning electron microscopy confirmed the detachment of EPS and bacterial cells from the surface following the incorporation of α-amylase into the culture medium. A dose-dependent response from *S. aureus* strains to amylase was found; however, increasing the time duration of treatment led to a reduction of concentration required to degrade the biofilm. Therefore, it has been suggested that anti-polysaccharide enzymes such as α-amylase could be a therapeutic agent to be used in clinical settings against biofilm producing pathogens (Watters *et al.*, 2016).

In another study, the antibiofilm activity of α-amylase from *B. subtilis* strain S8-18, against MRSA, *P. aeruginosa* ATCC10145 and *Vibrio cholera* was investigated using CV assay and scanning electron microscopy. The results revealed the inhibitory impact of α-amylase on the initiation of biofilm formation by 73%. In addition to the inhibition, a mature robust biofilm was degraded when exposed to α-amylase. The inhibitory and/or disruptive effect of amylase on biofilms could lead to antibiofilm therapeutic approaches (Kalpana *et al.*, 2012).

Antibiofilm activity of α-amylase is not exclusive to mono-species biofilms. Treatment of dual-species biofilms composed of *S. aureus* and *P. aeruginosa* with α-amylase resulted in more than 90% disruption of the biofilm structure formed on plastic cell

culture coverslips after 48 h. Amylase-dependent biofilm disruption was also noticed when a murine model coinfected with *S. aureus* and *P. aeruginosa* was tested. Similar to our findings in which the lowest concentration of pancreatic α-amylase (0.1 µM) is adequate to disrupt *C. difficile* biomass, the concentration as low as 0.0025% of α-amylase significantly reduced *S. aureus* and *P. aeruginosa* dual-species biofilm formation (Algburi *et al.*, 2017).

One of the predominant components of biofilm EPS matrix is polysaccharide which has various roles including adhesion to the surface, protection against stress and environmental factors, cell-to-cell interactions, and structure. The presence of polysaccharides in the intercellular regions of *C. difficile* biofilms has been confirmed using fluorescently labelled concanavalin A (ConA) and analysis with confocal laser-scanning microscopy (Semenyuk *et al.*, 2015). As a glycoside hydrolase, α-amylase cleaves α-1,4 glycosidic linkage between two or more monosaccharides (Naumoff, 2011). Owing to the fact that polysaccharides are the most abundant macromolecules constituting biofilms, we hypothesize that cleavage of glycosidic bonds that link them together may give rise to EPS degradation, detachment of the bacterial cells from the surfaces and the consequent substantial reduction in the amounts of *C. difficile* biofilms.

Together with the antibiofilm activity, α-amylase may exhibit some surfactant properties to inhibit biofilm formation by preventing initial attachment of cells onto surfaces. It is possible that amylase displays an anti-adhesive activity by reducing surface tension between hydrophobic surface of bacterial cells and the abiotic surface and allowing them to spread across the surface but not aggregate to form a biomass. Surfactant/ biosurfactant compounds have been shown to have the ability to inhibit biofilm formation in pathogens such as *P. putida* 852 and *Rhodococcus erythropolis*

via influencing bacteria/ surface interaction (Feng *et al.*, 2013). In a study of antibiofilm activity of biosurfactants, purified *Lactobacillus* biosurfactant composed of carbohydrate, lipid and protein disrupts the biofilm of *E. coli* and *Staphylococcus saprophyticus* by modifying the cell surface hydrophobicity and preventing bacterial adhesion to the abiotic surface (Morais *et al.*, 2017). Furthermore, a significant disruption or removal of *S. aureus* biofilms developed on polystyrene tissue culture plates using nutrient broth and milk as the growth media has been achieved by Rhamnolipid, a biosurfactant compound produced by *P. aeruginosa* strains (SS *et al.*, 2017).

Physicochemical properties and hydrophobicity of a solid surface as well as interactions between bacterial cells and abiotic surfaces determine the nature of initial adherence at the early stages of biofilm development. In order to optimise biomass formation by *C. difficile* strains, we grew biomass on a variety of abiotic surfaces including polystyrene plates and glass coverslips using the same culture medium. Six-day-old biomass was subjected to the crystal violet staining assay and measurements of absorbance at 595 nm of the dye solution were taken to determine the amount of biomass formed. The data obtained here show that the absorbance values of resolubilized CV stain indicating biomass formation on glass coverslips were notably lower in R20291, 630Δ*erm* and 630Δ*erm*Δs*po0A* after 6 days by approximately 30-fold, 20-fold and 10-fold respectively, compared to values which were obtained from biofilm formation on polystyrene plates (Figure 4.1). It is possible that the significant reduction in attachment to the glass coverslips could result from surface properties such as roughness and gloss.

The effect of surface roughness on biofilm formation has been determined by Mei *et al.*, (2011) who analysed the role of composite resins with varying surface roughness on streptococcal adhesion force. *Streptococcus sanguinis* and *Streptococcus mutans* adhesion to three orthodontic composites, smooth (roughness 20 nm), moderately rough (150 nm) and rough (350 nm) were measured using atomic force microscopy. The results showed that adhesion was increased with increasing surface roughness. Therefore, it has been determined that bacterial adhesion to the surfaces and biofilm formation are directly influenced by surface roughness since gaps, irregularities and holes on the rough surface provide a favourable niche for biofilm producing bacteria (Teughels *et al.*, 2006). Moreover, surface roughness aids bacterial cells to aggregate efficiently and adhere firmly due to increased contacts and prolonged interactions as well as providing protection against environmental stresses. For example, the incidence of dental implant site infections is higher in porous materials compared with glossy and robust ones (Shah *et al.*, 2013).

A modification to the tissue culture surface to make the typically hydrophobic polystyrene more hydrophilic maximises animal cell attachment and makes treated tissue culture plates favourable for eukaryotic cell line growth *in vitro* (Kwasny & Opperman, 2010). Despite the animal cells which have a primarily hydrophilic outer surface, different types of bacteria have hydrophilic or hydrophobic surfaces, but the degree of hydrophobicity can vary based on bacterial species, age, surface structure, growth medium and pH (Katsikogianni & Missirlis, 2004). There is a correlation between bacterial surface hydrophobicity and adhesion, i.e. the more hydrophilic cells adhere strongly to the hydrophilic surfaces, whereas hydrophobic cells tend to attach to hydrophobic abiotic surfaces (Giaouris *et al.*, 2015). However, bacterial communities within the biofilm are heterogenous and it is possible to see both groups

of hydrophilic and hydrophobic cells. Furthermore, bacteria can switch between a hydrophobic and hydrophilic phenotype in response to changes in temperature, molecular component of the surrounding media and growth phase (Rühs *et al.*, 2014, Oliveira *et al.*, 2001). Therefore, due to the hydrophobicity of bacterial cell surface and possible fluctuations between hydrophobic and hydrophilic nature of the biofilm, more biomass formation was observed when treated tissue culture plates were used.

Antibiofilm activity of α-amylase derived from multiple sources such as microbes and the mammalian pancreas has been shown in *V. cholera* (Kalpana *et al.*, 2012) , *S. aureus* (Craigen *et al.*, 2011, Watters *et al.*, 2016, Fleming *et al.*, 2017) and *P. aeruginosa* (Stiefel *et al.*, 2016) where the glycoside hydrolase activity of α-amylase hydrolyses α(1,4) glycosidic linkages of polysaccharides present within the biofilm. The results in this chapter showed a similar role for pancreatic α-amylase in inhibiting and/ or disrupting biofilm formation by *C. difficile*.

Our findings together with other studies suggest that the use of enzymes may be an effective way of eradicating biofilms and a promising strategy to improve treatment of bacterial infection. By targeting macromolecules present in the EPS which encloses and protects the microbes, degrading enzymes facilitate detachment of the cells from the biofilm and their release into the environment as planktonic bacteria. By isolating and purifying these enzymes, clinicians can add them to pre-formed biofilms at high concentrations in order to disrupt the biofilm structure and make the biofilm-associated bacteria more susceptible to the host immune response and antimicrobials (Fleming & Rumbaugh, 2017). Targeted delivery of enzyme therapeutics has been tried for cancer therapy using nanocarriers (NCs) (Dean *et al.*, 2017). A similar approach could be used to deliver antibiofilm enzymes to patients suffering from CDI reoccurrence.

Moreover, the frequency of horizontal gene transfer mechanisms such as conjugation is considerably higher between sessile microorganisms than their planktonic counterparts since donors and recipients are in close contacts within biofilms. Targeting biofilms with degrading enzymes can lead to reduced frequencies of conjugation and antibiotic resistance or virulence genes transfer.

Chapter 5 Investigations into the effects of pancreatic α-amylase and deoxyribonuclease I on horizontal gene transfer in *C. difficile*

5.1. Introduction

Horizontal gene transfer (HGT) plays an important role in microbial evolution and enables microorganisms to adapt and survive in a changing environment (Oliveira *et al.*, 2017). Evidence suggests that HGT can be influenced by changes in bacterial lifestyle, particularly growth temperature, pH, oxygen utilization and nutrient availability (Zeng & Lin, 2017). Moreover, host factors such as metabolites (e.g., bile salts), host defence factors (e.g., antimicrobial peptides), enzymes and chemicals found in the mammalian gastrointestinal (GI) tract (e.g., stress hormones) also influence the HGT between gut bacteria (Fuchsman *et al.*, 2017). The impact of stress factors on HGT between gut bacteria is not limited to the GI tract hormones and/ or metabolites. Other factors such as bacterial SOS response and quorum sensing (QS) system can also influence the HGT frequencies. Incorporation of SOS-inducing agents (e.g., fluoroquinolones and β-lactams) in culture media stimulates the transfer of antibiotic resistance and virulence genes via conjugation (Beaber *et al.*, 2004), natural transformation (Charpentier *et al.*, 2012) and transduction (Kim *et al.*, 2016).

It has been shown that the frequency of transformation in *B. subtilis* is significantly enhanced by stress factors such as starvation and/ or antibiotic exposure. When *B. subtilis* strains BG2036 or BR151 were cultured on a basal defined medium (BDM), transformation occurred at frequencies ranging from 9×10^{-2} to 5.3×10^{-3} transformants/ µg DNA. Addition of tryptophan to the BDM decreased the frequency from 8×10^{-2} to 7×10^{-5} transformants/ µg DNA (Zhang *et al.*, 2018). However, there are conflicting reports showing that the addition of nutrient sources to culture media does not always decrease the transformation frequency.

In other words, the effect of nutrient sources on HGT varies depending on the transferring element, microbial habitat and other physiological parameters such as bacterial population (Hirkala & Germida, 2004). Furthermore, higher transformation frequencies (1000- fold) were obtained when kanamycin, erythromycin and chloramphenicol were added to the BDM agar inoculated with *B. subtilis*, indicating that exposure to antibiotics influences the frequency of HGT (Zhang *et al.*, 2018).

In order to adapt to the constantly changing intestinal environment, *C. difficile* must react to many stresses including exposure to antibiotics and environmental shocks such as heat, pH and oxidative shock. Stress-induced enhanced horizontal gene transfer has only been reported in *C. difficile* strain R20291 (Kirk & Fagan, 2016). The conjugation frequency from *E. coli* to *C. difficile* was increased from 10^{-7} to 10^{-4} transconjugants/ per recipient in response to 15 minutes of 50 °C heat shock before transferring the mating mixture onto selective plates (Kirk & Fagan, 2016).

In general, our understanding of HGT processes is mainly obtained from *in vitro* experiments. There is still a significant knowledge gap regarding *in vivo* HGT occurrence and the role of environmental stress factors affecting the HGT frequencies in GI tract and/ or different ecological niches. A better understanding of these factors and the way they stimulate antibiotic resistance (AR) or virulence gene transfer can be used to develop effective strategies to combat rising AR in pathogenic bacteria.

5.2. Aims and objectives

We hypothesize that the EPS overexpression and its accumulation on the cell surface in response to pancreatic α-amylase affects the frequency of HGT in *C. difficile* since transfer of genetic elements from one bacterium to another via conjugation requires the element to pass through the cell membranes of both donor and recipient, and therefore be influenced by any structural alterations of the cell surface.

Objectives

- To determine the effect of pancreatic α-amylase and DNase I on the conjugal transfer of Tn*5397* from *B. subtilis* to *C. difficile* CD37

- To determine the effect of pancreatic α-amylase and DNase I on the conjugal transfer of conjugative transposon, 023_Tn3 from *C. difficile* strain CD305 (ribotype 023) into *C. difficile* CD37

- To determine the effect of pancreatic α-amylase and DNase I on the transfer of mobilisable plasmid pMTL9301 from *E. coli* strain CA434 into *C. difficile* CD37

5.3. Methods and materials

5.3.1. Strains, plasmids and culture media

The list of bacterial strains and plasmids are shown in table 5.1. *E. coli* strains carrying plasmids were grown in Luria-Bertani (LB) broth and agar from Sigma-Aldrich (Dorset, UK) and Life Technologies (Paisley, UK), respectively at 37 °C. *C. difficile* and *B. subtilis* strains were grown in brain heart infusion (BHI) agar or broth from Oxoid Ltd (Basingstoke, UK), the former supplemented with 5% defibrinated horse blood (E and O Laboratories, Bonnybridge, Scotland, UK) and incubated under anaerobic conditions (80% nitrogen, 10% hydrogen and 10% carbon dioxide) in a Macs-MG-1000-Anaerobic work station (Don Whitley scientific) or aerobically. Media were supplemented when required with antibiotics at the following concentrations: erythromycin 400 µg/ml or 10 µg/ml, rifampicin 25 µg/ml and tetracycline 10 µg/ml. When specified, deoxyribonuclease I (DNase) from bovine pancreas and/ or α-amylase from porcine pancreas was added to the mating mix to a final concentration of 50 µg/ml and 10 µM, respectively. All the antibiotics, pancreatic α-amylase and DNase were obtained from Sigma-Aldrich (Dorset, UK). The storage of all bacterial isolates was made by adding an equal volume of 20% (v/v) of sterile glycerol to the broth overnight culture, resulting in 10% (v/v) glycerol stock. One ml aliquots of the glycerol stocks were kept at -80 °C.

Table 5.1 Bacterial strains and plasmids used in this study.
Tc = Tetracycline, Erm = Erythromycin, Rif = Rifampicin. ^{R} = Resistant, ^{S} = Sensitive

Bacterium, Plasmid	Properties	Reference, source
C. difficile CD37 (ribotype 009)	Tc^{S} Erm^{S} Rif^{R}	Smith *et al.*, (1981)
C. difficile 630Δ*erm* (ribotype 012)	Tc^{R} Erm^{S} Rif^{S}	Hussain *et al.*, (2005)
C. difficile CD305 (ribotype 023)	Isolated from a 74-year-old patient with severe symptoms in 2008	Dr Trevor Lawley, Unpublished, Welcome Sanger Institute
DH5α	F^{-} *recA*1 *end*A1 *hsd*R17 (rk^{-}, mk^{+}) *pho*A *sup*E44 λ^{-} *thi*$^{-}$ 1 *gyr*A96 *rel*A	Gibco BRL
E. coli CA434	HB101 carrying the IncP conjugative plasmid R702	Purdy *et al.*, (2002) Williams *et al.*, (1990)
B. subtilis strain BS6A	*B. subtilis* CU2189:: Tn*5397*, Tc^{R}	Roberts *et al.*, (1999)
pMTL9301	Erythromycin resistance encoding *E. coli-C. difficile* shuttle vector	Purdy *et al.*, (2002)

5.3.2. Chemical transformation

E. coli strain CA434 competent cells preparation and transformation were carried out based on the protocol described in chapter 2 (Section 2.3).

5.3.3. Confirmation of transformation

Twenty transformants were picked from 400 µg/ml erythromycin-supplemented selective LB plates and inoculated into 10 ml LB broth containing 200 µg/ml erythromycin. Cultures were incubated with shaking (200 rpm) for 16 hours at 37 ˚C. Plasmid was extracted from 3 ml of culture using a Mini-prep kit (QIAprep Spin Miniprep Kit, Cat No. 27106) based on the manufacturer's instructions and eluted in molecular biology grade water. Plasmid was assessed by restriction digest and PCR before the correct transformant was selected as a donor and stored at -20 °C or -80 °C for conjugation experiments.

Plasmid pMTL9301 was digested with *Eco*RI. Approximately, 1 µg of plasmid DNA was digested with 1 µl restriction enzyme (10 U) with appropriate 1 X reaction buffer in a final volume of 25 µl for 16 h at 37 °C water bath. Digested DNA was separated on a 1% agarose gel with an appropriate marker. Fragments were visualized on a transilluminator after staining with GelRed Nucleic acid gel stain (Bioline, United Kingdom). Plasmid DNA was amplified using primer pairs (Table 5.2) in a standard PCR reaction containing 2 µl template DNA, 22 µl BioMix^{TM} Red (Bioline, London, UK), 0.5 µl of each primer in a final volume of 50 µl.

PCR conditions are as follows: [Denaturation: 94 °C 4 min] x 1 cycle, [Denaturation: 94 °C 30 sec, Annealing: 50 °C-58 °C 1.5 min, Elongation: 72 °C 1min ~ 1 Kb product] x 25 cycles, [Elongation: 72 °C 5 min] x 1 cycle, Pause, 4 °C. Samples were separated on a 1% agarose gel with an appropriate marker. Bands were visualised on a transilluminator after staining with GelRed. Desired constructs were stored at -20 °C or -80 °C.

Table 5.2 The list of primers used in this study to confirm the presence of pMTL9301 in *E. coli* CA434 transformants.

Primer pair erm(B) F and erm(B) R amplifies 800 bp erythromycin resistance gene carried by pMTL9301 as its selective marker. Primer pair ORIT-1 and ORIT-2 amplifies a sequence of 800 bp within the origin of transfer *oriT*, successful amplification of the *oriT* indicates that the plasmid carries this region which is essential for conjugation. All primers synthesised by Sigma- Aldrich, UK.

Primer	Sequence (5'-3')	Annealing Tm°	Product size
erm(B) F	GTCCCGGGCCTCTTGCGGGATCAAAAG	81.2	800 bp
erm(B) R	GATCCGGACTCATAGAATTATTTCCTC	65.5	
ORIT-1	GTGCCTTGCTCGTATC	54.7	800 bp
ORIT-2	CCTGCTTCGGGGTCATTATAG	64.4	

5.3.4. Mating experiments

C. difficile recipient strain CD37 (Table 5.1) was grown in 20 ml BHI broth using Coring® polystyrene tissue culture flasks with vented caps (VWR, USA) for 18-20 hours under anaerobic conditions at 37 °C. This was used to start a 10 ml culture at an OD_{600} ~ 0.1. This was grown shaking at 50 rpm for 4-6 hours until the OD_{600} was between 0.6 and 0.8 (mid exponential phase). *E. coli* donor strain CA434 (HB101 carrying the conjugative plasmid R702) (Table 5.1) was grown in 10 ml LB broth supplemented with 200 µg/ml erythromycin for 16-18 hours with shaking (200 rpm) at 37 °C. A one-ml aliquot was centrifuged at 14680 x *g* (13000 rpm, Eppendorf centrifuge 5415 D) for 1 minute in an Eppendorf tube and washed twice by repeating the centrifugation step with sterile PBS to remove antibiotics.

B. subtilis donor strain BS6A (Table 5.1) was grown shaking at 200 rpm in 10 ml BHI broth supplemented with tetracycline (5 µg/ml) for 18 hours. Next day, the culture was grown for 3-4 hours in 100 ml BHI broth supplemented with 5 µg/ml tetracycline in a 500 ml flask at 37 °C until mid- exponential phase (OD_{600} ~ 0.45-0.6). Cells were centrifuged at 4000 x *g* for 15 min and washed twice with PBS.

The harvested *E. coli* cells were resuspended in 0.5 ml BHI broth and mixed with 0.5 ml overnight culture of *C. difficile* inside the anaerobic chamber. In some cases, 50 µg/ml deoxyribonuclease I from bovine pancreas (DNase I) (Sigma-Aldrich, UK) and/ or 10 µM pancreatic α-amylase (Sigma-Aldrich, UK) were added to the mating mix. This mating mix was then spotted onto antibiotic free BHI plates and incubated anaerobically for 18 hours. In case of using *B. subtilis* as a donor, 100 µl of mating mix was spread onto 0.45 µm pore size nitrocellulose filters (Merck, UK), placed on BHI plates and incubated anaerobically for 18 hours.

The bacterial growth was harvested by flooding the agar surface with 1 ml pre-reduced BHI broth twice to ensure good recovery of transconjugants. When *B. subtilis* was a donor, the filters were removed from the agar plates and placed in 20 ml tubes containing 1 ml pre-reduced BHI broth and vortexed for 10-20 seconds. 100 µl of the cell suspension was plated onto BHI agar supplemented with erythromycin (10 µg/ml) to select for plasmid uptake, tetracycline (10 µg/ml) to select for Tn*5397* uptake, D-cycloserine (250 µg/ml) and cefoxitin (8 µg/ml) to select against *E. coli* or *B. subtilis*. Plates were incubated anaerobically for 48-72 hours and examined regularly for transconjugants.

Putative transconjugants were picked and re-streaked onto fresh selective plates containing appropriate antibiotics. Control plates were also included in initial experiments containing *E. coli, B. subtilis* or *C. difficile* alone and incubated alongside experimental plates to confirm there were no surviving donor cells or spontaneous mutants.

In case of using *C. difficile* as a donor, cultures of both donor (CD305; Rif^R, Erm^R) (Table 5.1) and recipient (CD37; Rif^R, Erm^S) were grown for 16 h in pre-reduced BHI broth. These were used to start a 10 ml culture of the donor strain and a 50 ml culture of the recipient, both at an OD_{600} ~ 0.1. These were grown shaking at 50 rpm anaerobically. After 4-6 h, when the OD_{600} was between 0.6 and 0.8, the cultures were centrifuged for 10 min at 4,500 x *g* and the pellet was resuspended in 500 ml pre-reduced BHI broth. The two cultures were mixed, DNase (50 µg/ml) was added and 200 µl was spread onto each of four 0.45 µm pore size cellulose nitrate filters (Sartorius, Epsom, UK), on antibiotic free BHI agar. After 24 h the filters were placed into 25 ml tubes and 1 ml BHI broth was added. The tubes were vortexed, and the resulting cell suspension was spread onto selective plates containing rifampicin 25

µg/ml (to select for CD37) and erythromycin 10 µg/ml (to select for RAM). After 72-96 h, the putative transconjugants were counted and sub-cultured onto fresh selective plates. In some cases, 10 µM pancreatic α-amylase was added to the mating mix to investigate the role of amylase on HGT in *C. difficile*.

5.3.5. Confirmation of transfer

PCR was used to confirm the transfer using the appropriate primers (Table 5.3). In some cases, the *tcdB* gene or the region flanking the PaLoc (these confirm that the transconjugants are *C. difficile* strain 630Δ*erm* or CD37, respectively), was amplified and sequenced. Furthermore, the *stpK* gene was also amplified and sent for sequencing to confirm the genuineness of the transconjugants using primers listed in table 5.3. PCR reaction was carried out using MyTaq^{TM} Red Mix (Bioline, UK) as described previously in chapter 2. PCR conditions are as follows: [Denaturation: 94 °C 4 min] x 1 cycle, [Denaturation: 94 °C 30 sec, Annealing: 50 °C-58 °C 1.5 min, Elongation: 72 °C 1 min ~ 1 Kb product] x 25 cycles, [Elongation: 72 °C 5 min] x 1 cycle, Pause, 4 °C. Primers were designed with the aid of DNAMAN (Lynnon, USA) and checked using Sigma oligonucleotides design/ ordering online software (Sigma-Aldrich, UK). Locations of the primers are shown in figure 5.1.

Table 5.3 The list of primers used in this study to confirm the transfer of MGEs into *C. difficile* in the absence and presence of pancreatic α-amylase and/ or DNase.

Primer	Sequence (5'-3')	Annealing Tm°	Product size
tndX F30	CTTACAATGTTAAAACAGCAAG	59.4	1.6 kb
tndX R1100	GTATGAAATTGCGTGAGTAGTGC	62.4	
erm(B) F	GTCCCGGGCCTCTTGCGGGATCAAAAG	81.2	800 bp
erm(B) R	GATCCGGACTCATAGAATTATTTCCTC	65.5	
Lok 3F	GAAAGAAGAACATAATTTACCAG	55.4	750 bp
Lok 1R	CTGCACATCTGTATACATATAACTG	57.0	
tcdB-F	CAATGAACTTGTACTTCGAGATAAG	59.8	400 bp
tcdB-R	CTCACCTCCATAGTTATATCTTATACGG	62.1	
stpK F1900	CATTAATGATGATATAGACTTTACATCAGAAG	62	420 bp
stpK R1120	CAGCCGCAATTACTTTTAATC	60.2	

CD305_02409 F (AS246)	CAGGAAGTACAGGCAATCCTAAAG	61	~ 130 bp
CD305_02409 R (AS247)	CCACTCTGATACATTTTTATTATCACTTGG	61.3	
CD305_02451 F (AS218)	GCGAGGGCGAGGACATTC	60.5	~ 130 bp
CD305_02451 R (AS219)	GTCGCTTGCGGCAAGGTC	60.5	
CD305_0248 F (AS224)	GGACAACATATTTTTGGAACGGTAAAAG	60.7	~ 130 bp
CD305_0248 R (AS225)	CATCATTAAACCCACCACTCAAAC	59.3	

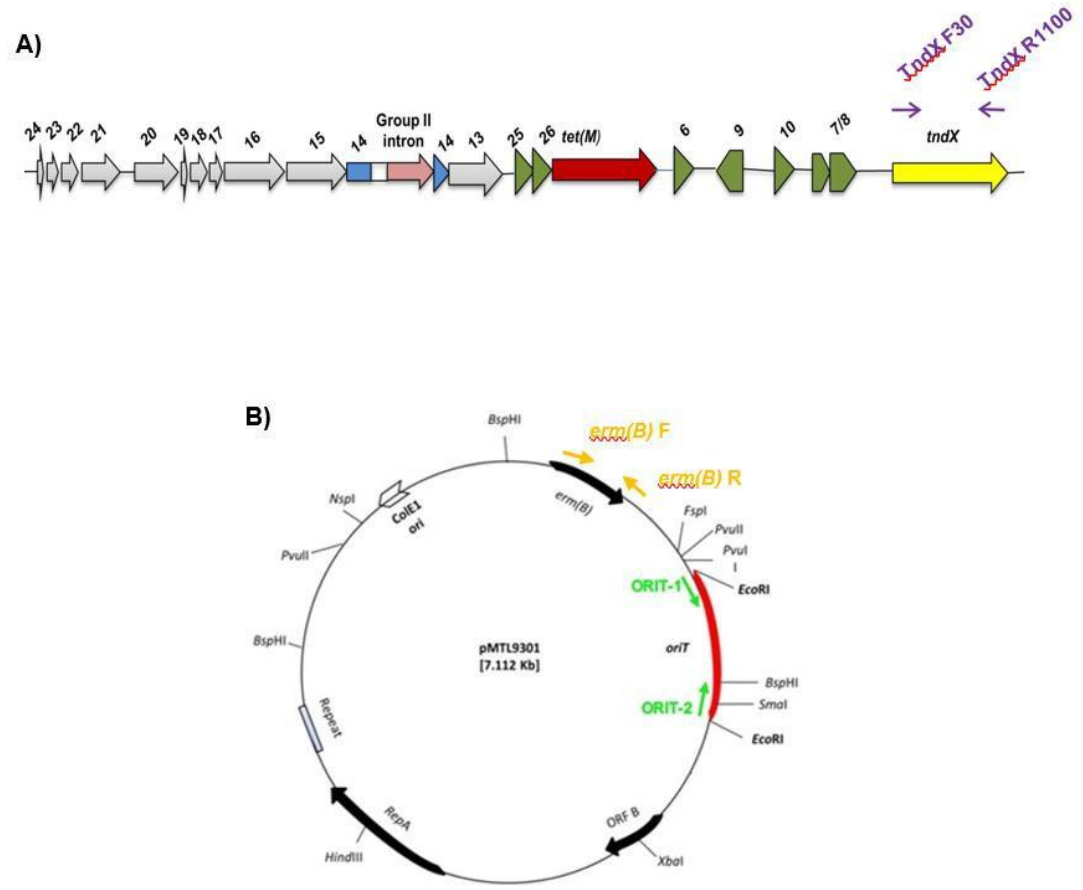


Figure 5.1 Schematic representation of Tn*5397* and plasmid pMTL9301 and the locations of primers used in this study to confirm the transfer.

A) Grey arrows represent the ORFs of the conjugation module. Blue arrow represents the interrupted *orf14.* The pink arrow represents the Group-II intron. The red arrow represents the *tet*(*M*) gene. The green arrows represent the ORFs involved in transcriptional regulation. The yellow arrow represents the serine recombinase (*tndX*). The purple arrows represent the binding site of the primers to amplify the *tndX* gene. B) Schematic representation of pCD6- derived *E. coli- C. difficile* shuttle vector containing erythromycin resistance selective marker (*erm*(B)). Origin of transfer (*oriT*) derived from broad host range IncP conjugative plasmid RK2. ColE1 origin of replication to replicate in *E. coli.* Green arrows represent primer pair to amplify the *oriT* region. Yellow arrows represent primer pair to amplify the erm(B) gene (Adapted from (Purdy *et al.*, 2002)).

5.4. Results

5.4.1. Tn*5397* conjugation occurs at a significantly higher frequency in the presence of pancreatic α-amylase than when amylase is not present.

Conjugal transfer of Tn*5397* was performed using *B. subtilis* strain BS6A (Table 5.1) donor and *C. difficile* CD37 recipient. Transfer of Tn*5397* into CD37 occurred at a frequency of 2.06 x 10^{-6} transconjugants per donor (standard deviation is 1.07 x 10^{-7}) or 4.72 x 10^{-5} transconjugants per recipient (standard deviation is 3.99 x 10^{-6}) in the presence of pancreatic α-amylase, whereas a transfer frequency of 5.02 x 10^{-8} transconjugants per donor (standard deviation is 1.55 X 10^{-8}) or 5.93 x 10^{-7} transconjugants per recipient (standard deviation is 9.68 x 10^{-8}) was detected in the absence of pancreatic α-amylase. Therefore, the frequency of Tn*5397* transfer was significantly increased ($P < 0.05$) when 10 μM pancreatic α-amylase was incorporated into the mating mix (Table 5.4).

Table 5.4 Mating experiment between *B. subtilis* BS6A and *C. difficile* CD37 in the absence and presence of pancreatic α-amylase.

[a] SD Standard deviation

Experiment. #	1		2		3	
Treatment	Amylase	No amylase	Amylase	No amylase	Amylase	No amylase
Total number of BS6A cells (CFU/ml)	3×10^8	5×10^8	2×10^8	2×10^8	2×10^8	8×10^8
Total number of CD37 cells (CFU/ml)	1×10^7	2×10^7	1×10^7	4×10^7	4×10^7	5×10^7
Conjugation frequency (per recipient, SD) [a]	$5.51 \pm 2.2 \times 10^{-5}$	$7 \pm 1.04 \times 10^{-7}$	$4.25 \pm 2.4 \times 10^{-5}$	$4.25 \pm 2 \times 10^{-7}$	$4.3 \pm 2.5 \times 10^{-5}$	$6.8 \pm 1.6 \times 10^{-7}$
Conjugation frequency (per donor, ± SD) [a]	$1.84 \pm 0.9 \times 10^{-6}$	$2.83 \pm 0.52 \times 10^{-8}$	$2.12 \pm 0.6 \times 10^{-6}$	$8 \pm 1.3 \times 10^{-8}$	$2.1 \pm 0.43 \times 10^{-6}$	$4.25 \pm 1.4 \times 10^{-8}$

5.4.2. Tn*5397* transfer into *C. difficile* is not affected by deoxyribonuclease I (DNase I)

The effect of DNase (50 µg/ml) alone and in combination with pancreatic α-amylase on Tn*5397* transfer was investigated using *B. subtilis* strain BS6A as donor (Table 5.1) and *C. difficile* CD37 recipient. DNase resistance is an indicator of conjugation and no change in the frequency of conjugation was expected when DNase was added to the mating mix.

A significant increase ($P < 0.05$) in Tn*5397* transfer has already been demonstrated when pancreatic α-amylase was incorporated into the mating mix (Table 5.4). Similarly, a significant increase was observed in the presence of DNase and α-amylase combination (Table 5.5). The frequency was not affected by DNase alone proving that Tn*5397* transfer is by conjugation and DNA is always protected from the degrading activity of DNase (this is the first formal proof that Tn*5397* transfers via conjugation).

Table 5.5 The effect of DNase alone and in combination with pancreatic α-amylase on Tn*5397* frequency of transfer.

[a]SD, standard deviation

	Treatments			
	None	Amylase (10 µM)	DNase (50 µg/ml)	Amylase + DNase
Number of donors (CFU/ml)	2×10^8	2×10^8	2×10^8	2×10^8
Number of recipients (CFU/ml)	4×10^7	3×10^7	6×10^7	3×10^7
Frequency of conjugation/ (donor, ± SD) [a]	$1.5 \pm 0.23 \times 10^{-7}$	$1.7 \pm 0.7 \times 10^{-6}$	$1.5 \pm 0.55 \times 10^{-7}$	$1.4 \pm 0.92 \times 10^{-6}$
Frequency of conjugation/ (recipient, ± SD) [a]	$7.2 \pm 1.2 \times 10^{-7}$	$1.1 \pm 0.4 \times 10^{-5}$	$4.6 \pm 2.6 \times 10^{-7}$	$1.2 \pm 0.8 \times 10^{-5}$

5.4.3. Conjugative transposon 023_Tn3 transfers from toxigenic strain CD305 into CD37, and the transfer is not affected by pancreatic α-amylase and/ or DNase

Unpublished work by Shaw *et al.*, (2018) has shown that the CD305 (Table 5.1) genome harbours a 136.4 kb insertion consisting of 103 predicted coding sequences (CD305_02397-02499) inserted within the CD630_CTn2 locus (Figure 5.2). Three serine recombinases are distributed along this region (CD305_02395, CD305_02439, CD305_02469), suggesting that this island is potentially composed of three transposable elements named as 023_Tn1, 023_Tn2 and 023_Tn3.

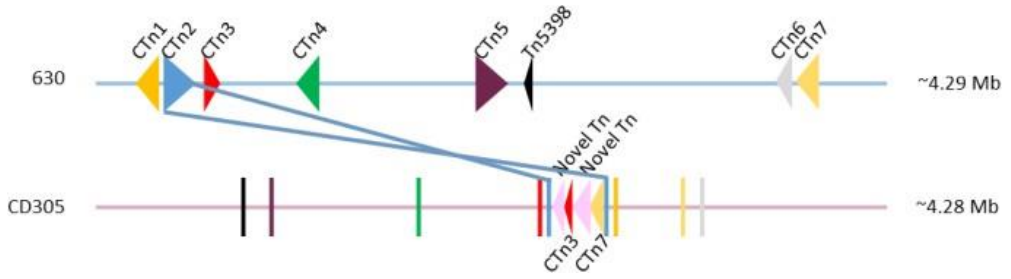


Figure 5.2 The location of CD305 novel transposons.

(Retrieved from (Shaw *et al.*, 2018)). Whole genome analysis of *C. difficile* CD305 revealed a 136.4 kb region of putative transposable elements harbouring 103 ORFs (CD305_02397-02499) inserted within the 630_CTn2 locus. This insertion is partially matched to half of 630_CTn7 and part of CTn3. The presence of three serine recombinases suggests that this region is composed of at least three transposable elements.

These putative transposons were marked with a ClosTron to provide a selectable marker, so that transfer from CD305 to other strains of *C. difficile* can be detected (Shaw *et al.*, 2018). In this study, filter mating was performed between CD305 donor and CD37 recipient in the absence and presence of pancreatic α-amylase and DNase. Results showed that only 023_T3 was transferred at a frequency of 10^{-7} transconjugants per donor or recipient and the presence of DNase or pancreatic α-amylase had no effect on the transfer. PCR was performed using primers listed in table 5.3 based on the protocol described in chapter 2.

Table 5.6 Frequency of 023_Tn3 transfer per donor or recipient.
[a]SD, standard deviation. [b]Amy, pancreatic α- amylase

Donor	Recipient	Frequency of conjugation/ donor, SD[a]				Frequency of conjugation/ recipient, SD[a]			
		+ DNase	No DNase	+ [b]Amy	No [b]Amy	+ DNase	No DNase	+ [b]Amy	No [b]Amy
CD305 (clone 1)	CD37	1.42 ± 0.8 x 10^{-7}	4.53 ± 0.9 x 10^{-7}	5 ± 2.3 x 10^{-7}	5.6 ± 2.3 x 10^{-7}	5.4 ± 0.5 x 10^{-7}	6.6 ± 1.8 x 10^{-7}	4 ± 0.7 x 10^{-7}	4.8 ± 0.7 x 10^{-7}
CD305 (clone 2)	CD37	5.5 ± 1.2 x 10^{-7}	2.4 ± 0.8 x 10^{-7}	3.5 ± 1.6 x 10^{-7}	1.4 ± 0.8 x 10^{-7}	6 ± 0.3 x 10^{-7}	2.8 ± 0.4 x 10^{-7}	3 ± 1.2 x10^{-7}	3.7 ± 1.2 x 10^{-7}

5.4.4. Transfer of pMTL9301 is not affected by pancreatic α-amylase

E. coli strain CA434 (Table 5.1) was mated with *C. difficile* CD37 in the absence and presence of pancreatic α-amylase. The frequency of conjugation was 9.75 x 10^{-7} transconjugants per recipient (standard deviation is 1.41 x 10^{-7}) and 3 x 10^{-8} transconjugants per donor (standard deviation is 3.75 x 10^{-9}) in the absence of amylase. When pancreatic α-amylase was present in the mating mixture, the conjugation frequency was 8.6 x 10^{-7} transconjugants per recipient (standard deviation is 5.73 x 10^{-8}) and 4.12 x 10^{-8} transconjugants per donor (standard deviation is 2.28 x 10^{-9}). Results showed that the transfer of pMTL9301 was not affected by pancreatic α-amylase (Table 5.7). PCR was performed to confirm the transfer using primers listed in table 5.3.

Table 5.7 Mating experiment between *E. coli* CA434 containing pMTL9301 and *C. difficile* CD37 in the absence and presence of pancreatic α-amylase.

[a] SD, Standard deviation

Experiment. #	1		2		3	
Treatment	Amylase	No amylase	Amylase	No amylase	Amylase	No amylase
Total number of CA434 (CFU/ml)	3.8×10^{9}	4.78×10^{9}	3.2×10^{9}	4.12×10^{9}	3.88×10^{9}	4.18×10^{9}
Total number of CD37 (CFU/ml)	1.8×10^{8}	1.52×10^{8}	1.63×10^{8}	1.71×10^{8}	1.85×10^{8}	1.78×10^{8}
Conjugation frequency (per recipient, ± SD) [a]	$9.7 \pm 1.4 \times 10^{-7}$	$1 \pm 0.2 \times 10^{-6}$	$8.2 \pm 1.2 \times 10^{-7}$	$8.8 \pm 1.3 \times 10^{-7}$	$7.8 \pm 0.8 \times 10^{-7}$	$7.8 \pm 1.4 \times 10^{-7}$
Conjugation frequency (per donor, ± SD) [a]	$4.5 \pm 1.2 \times 10^{-8}$	$3.9 \pm 1.1 \times 10^{-8}$	$4.12 \pm 0.7 \times 10^{-8}$	$4.6 \pm 1.1 \times 10^{-8}$	$3.7 \pm 0.6 \times 10^{-8}$	$3.3 \pm 0.8 \times 10^{-8}$

5.4.5. DNase drastically reduces pMTL9301 frequency of transfer from *E. coli* strain CA434 into *C. difficile* CD37

E. coli strain CA434 (Table 5.1) and *C. difficile* CD37 were mated under three different conditions: (i) pancreatic α-amylase, (ii) DNase, (iii) pancreatic α-amylase and DNase. It has already been shown that unlike Tn*5397*, pMTL9301 frequency of transfer is not affected by pancreatic α-amylase (Table 5.7). Here, the effect of DNase and DNase/ α-amylase combination on pMTL9301 transfer was investigated. Results showed that the addition of 50 µg/ml DNase to the mating mixture significantly decreased the frequency of transfer from 10^{-7} to 10^{-9} transconjugants per donor, but it did not completely abolish it ($P < 0.001$) (Table 5.8). The decrease in the frequency of transfer was also observed in the presence of DNase and α-amylase combination. PCR was performed to confirm the transfer using primers listed in table 5.3.

Table 5.8 The effect of DNase alone and in combination with pancreatic α-amylase on pMTL9301 transfer into *C. difficile*. [a]SD, standard deviation

	Treatments			
	None	Amylase	DNase	Amylase + DNase
Number of donors (CFU/ ml)	3.3×10^{8}	2.8×10^{8}	4×10^{8}	4.3×10^{8}
Number of recipients (CFU/ ml)	5.3×10^{7}	6×10^{7}	4.8×10^{7}	4.5×10^{7}
Frequency of transfer/ (donor, SD) [a]	$5.75 \pm 1.03 \times 10^{-7}$	$6.56 \pm 1.1 \times 10^{-7}$	$5 \pm 2.2 \times 10^{-9}$	$5 \pm 2.2 \times 10^{-9}$
Frequency of transfer / (recipient, SD) [a]	$3.58 \pm 1.1 \times 10^{-6}$	$3 \pm 0.9 \times 10^{-6}$	$4 \pm 1.06 \times 10^{-8}$	$4 \pm 1.06 \times 10^{-8}$

5.5. Discussion

The effect of 10 µM pancreatic α-amylase and 50 µg/ml deoxyribonuclease I (DNase) on the conjugal transfer of Tn*5397* and pMTL9301 from *B. subtilis* and *E. coli* donors, respectively into *C. difficile* CD37 recipient was investigated in this chapter. Tn*5397* transferred following filter mating at a frequency of $5 \pm 1.22 \times 10^{-8}$ transconjugants per donor. The frequency of transfer was significantly ($P < 0.05$) increased to $2.06 \pm 1.07 \times 10^{-6}$ transconjugants per donor when filter mating was performed in the presence of pancreatic α-amylase, but no difference was observed when 50 µg/ ml DNase I was added to the mating mix.

Subinhibitory concentrations of antibiotics induce various stress responses such as increased frequency of antibiotic resistance and/ or virulence genes transfers amongst bacterial species (Ubeda *et al.*, 2005). Furthermore, subinhibitory concentrations of biocides induce the conjugal transfer of Tn*916*-like elements and any antibiotic resistance gene they carry (Seier-Petersen *et al.*, 2014). The effect of ethanol, hydrogen peroxide, chlorhexidine digluconate and sodium hypochlorite on the conjugative transfer of Tn*916* between *B. subtilis* strains were analysed and results showed that ethanol significantly ($P < 0.05$) increased the transfer frequency of Tn*916* by 5 orders of magnitude. In contrast, hydrogen peroxide, chlorhexidine digluconate and sodium hypochlorite did not have any effect on the transfer frequency (Seier-Petersen *et al.*, 2014). It has been hypothesized that ethanol does not only stimulate the transfer of Tn*916*, but it can also affect the conjugal transfer of many other members of theTn*916*-like family including Tn*5397* and subsequently the dissemination of their antibiotic resistance genes (Seier-Petersen *et al.*, 2014).

Transcription of the *tet*(*M*) gene in Tn*916* leads to the transcription of genes involved in the conjugation and recombination of the element. Transcription of *tet*(*M*) is regulated by a tetracycline-dependent transcriptional attenuation mechanism controlled by the levels of charged tRNA molecules inside the cells (Su *et al.*, 1992). Therefore, it is suggested that any stress that the cell encounters which results in the accumulation of charged tRNA is also likely to cause an increase in the transcription of *tet*(*M*) and downstream genes and possibly an increase in transfer (Roberts & Mullany, 2009).

It is possible that a similar interaction is taking place when pancreatic α-amylase is added to the mating mix. In the mixture of *B. subtilis* BS6A and *C. difficile* CD37, an amylase-induced stress response occurs to promote Tn*5397* transfer since pancreatic α-amylase treatment significantly increased the conjugation frequency of Tn*5397* (Table 5.4). Amylase-induced stress response in *C. difficile* has already been reported in chapter 3 where mucoid colony formation and EPS overexpression was observed in the presence of 10 µM pancreatic α-amylase.

However, amylase-induced stress response was not observed when pMTL9301 was transferred from *E. coli* owing to fact that this shuttle vector is a lab construct, designed to carry foreign genes into *C. difficile* and possibly does not contain regulatory genes required to sense the presence of an environmental stress factor.

Overall, the data obtained from this chapter clearly highlights that the incorporation of pancreatic α-amylase into the mating mixture significantly increases the frequency of Tn*5397* transfer into *C. difficile* by two orders of magnitude. Even though there are differences between the regulatory regions of Tn*5397* and Tn*916* and the knowledge about the regulation of the Tn*916*/Tn*1545* family is almost completely limited to Tn*916*,

the expression of tetracycline resistance gene is still inducible in Tn*5397*, as determined by comparative growth curves analysis and reverse transcriptase PCR (Roberts, 2001). Thus, the stress-induced increase in the horizontal transfer of MGEs is a possible concern which can lead to the dissemination of conjugative elements harbouring antibiotic resistance and/ or virulence genes amongst different bacterial species and give rise to antibiotic resistance crisis.

In this chapter, we also showed the occurrence of putative novel transposon 023_Tn3 transfer from toxigenic strain CD305 into *C. difficile* CD37 at a frequency of 10^{-7} transconjugants per donor confirming that this region is a transferable element. The transfer was shown to be DNase-resistant indicating that this novel transposon transfers via conjugation. Results also showed that the frequency of transfer was not influenced by the presence of pancreatic α-amylase. Further investigation is required to explain why Tn*5397* frequency of transfer was influenced by the α-amylase, whereas 023_Tn3, another CTn present in *C. difficile* strain CD305 was not.

In contrast to pancreatic α-amylase, the addition of DNase significantly ($P < 0.001$) reduced the transfer frequency of pMTL9301 by two orders of magnitude which was an unexpected observation since conjugation is expected to be DNase resistant. The effect of DNase on HGT in *C. difficile* was first reported by (Wasels *et al.*, 2015). These workers performed filter mating experiments to evaluate the role of DNase on transfer frequencies of Tn*5398* from *C. difficile* 630 and Tn*6215* from *C. difficile* F17 into CD37 and CD13. Addition of DNase to the mating mixture lowered the frequency of Tn*5398* transfer into CD13 but not into CD37. Moreover, transfer of Tn*6215* from F17 was not detected in the presence of DNase when only CD13 was used as a recipient.

In this experiment, cell-to-cell contact was required, and free DNA did not result in the transfer of Tn*6215*. These workers suggested that a possible transformation-like mechanism occurs in *C. difficile* which is not like conventional transformation since the transfer was not completely abolished by DNase and more importantly, it required the presence of live donor cells (Wasels *et al.*, 2015).

A donor-dependent transformation-like mechanism has been reported in *B. subtilis*. In this process, plasmid pAPR8-1, an *E. coli- B. subtilis* non-conjugative non-mobilisable shuttle vector harbouring selective markers for both organisms has been used to transform *B. subtilis*. Antibiotic resistant transformants appeared on selective plates and plasmid encoded protease activity was only expressible in *B. subtilis*. The results showed that DNase-sensitive transformation did occur in this experiment but did not behave like conventional transformation. Unlike well-known transformation mechanisms, the presence of donor was crucial as no transfer occurred when naked plasmid was used. Furthermore, the addition of DNase lowered the transformation frequency but did not stop it. This finding is similar to our observation when pMTL9301 transfer frequency was noticeably decreased but not completely abolished in the presence of DNase. This will be discussed at greater length in the next chapter.

A DNase-sensitive cell-to-cell transformation-like mechanism has also been shown by (Etchuuya *et al.*, 2011) whose results seem to be partly related to the results of this chapter. They reported that non-conjugative plasmid pHSG299 (a pUC-like high-copy cloning vector that lacks the *tra*, *mob*, and *nic* regions) transfers from plasmid harbouring *E. coli* donor strains including DH5α, MG1655, CAG18439, HB101 and MC4100 into plasmid-free *E. coli* recipient at frequencies between 10^{-4} and 10^{-6} per donor (depending on the donor and recipient combinations). Addition of DNase significantly decreased ($P < 0.05$) plasmid transfer frequency but did not completely

stop it. A DNase-sensitive transformation-like mechanism of plasmid transfer was also observed when heat-killed donors were used indicating that the mechanism of transfer was a type of transformation. However, when purified plasmid DNA was used in the same culture system, no transformants appeared on the selective plates with any combinations of strains tested indicating that the presence of donor was required for transfer which was then termed as cell-to-cell transformation. This observation did not fit into the conventional definition of transformation since the absence of donor strains did not result in any transfer. Further experiments by the same group showed that *E. coli* strain CAG18439 was able to release a peptide signalling molecule to promote cell-to-cell transformation and keep the donor and recipient in proximity (Etchuuya *et al.*, 2011). It is possible that *E. coli* strain CA434 which was used as a donor in our study secretes a signalling molecule to stimulate the CD37 recipient to take up plasmid DNA in a DNase-sensitive transformation-like manner. Cell-free CA434 filtrate will be subjected to gene transfer experiments to further investigate the role of donor cells (Chapter 6).

Based on our observations, the response of HGT to the external stress factors varies depending on the mobile element itself and the nature of stress-inducing molecule. More investigations are needed to understand these variations and the molecular basis behind the increased frequency of Tn*5397* transfer from *B. subtilis* and/ or increased excision from the chromosome in the presence of pancreatic α-amylase. Moreover, more investigations are required to find out why the transfer of pMTL9301 was sensitive to DNase even though conjugation is known to be resistant to DNase.

Chapter 6 Investigation of DNase-sensitive plasmid transfer into *Clostridium difficile*

6.1. Introduction

The remarkable genome plasticity in prokaryotes is mediated by horizontal gene transfer (HGT) which is one of the key factors in the success of microorganisms to evolve and adapt to the changing environment. HGT is an extremely advantageous feature of microbial life; however, it contributes to one of the biggest threats to global health that is the development of antimicrobial resistance (AMR). There are three main mechanisms of HGT, transformation in which naked DNA is taken up by the recipient organism, transduction in which bacteriophages are responsible for the transfer of genetic materials from a donor to a recipient and conjugation in which the conjugation apparatus (encoded by specialized genetic elements) mediates the transfer of conjugative or mobilisable elements from a donor to a recipient (Oliveira *et al.*, 2017).

Conjugative transfer of mobile DNA elements is usually mediated by Type IV secretion systems (T4SSs) which transport single-stranded DNA and protein substrates across prokaryotic cell envelopes generally by a mechanism requiring direct contact with a recipient cell. Conjugative T4SSs are encoded on conjugative plasmids, integrative and conjugative elements also known as ICEs or conjugative transposons (Reviewed in Grohmann *et al.*, 2018). One of the best-studied T4SSs is encoded by the broad-host-range plasmid RK2, a 60 kb self-transmissible plasmid belonging to the incompatibility group IncP. Conjugative transfer of the plasmid requires a *cis*-acting origin of transfer (*oriT*) and several trans-acting mating pair formation (Mpf) complex proteins encoded by genes located on regions Tra1 and Tra2 (Motallebi-Veshareh *et al.*, 1992). DNA sequence analysis showed that the RK2 *oriT* is composed of a 112 bp region containing a 19 bp imperfect inverted repeat sequence and a 20 bp nick region (Figure 6.1) (Guiney & Yakobson, 1983).

Figure 6.1 Schematic representation of the *oriT* region on plasmid RK2

(information for this figure retrieved from (Guiney & Yakobson, 1983))

A) Schematic representation of multidrug resistant plasmid RK2 (60 Kb) encoding conjugative T4SS, B) 112 bp core *oriT* region containing the 19 bp imperfect inverted repeat (IR) (sequence in green) required for hairpin structure formation to be recognized and nicked by the relaxase. The sequence also contains an imperfect direct repeat (sequence in pink).

Briefly, DNA relaxase (one of the key enzymes to initiate the conjugation) catalyses cleavage at the unique *nic* site within the *oriT* in a strand and site-specific manner leading to the formation of a relaxosome (Furste *et al.*, 1989). The genes involved in the relaxosome formation are from the Tra1 region and named as follows: *traH, traI, traJ* and *traK* (Furste *et al.*, 1989). Genes *traL, traM, traF* are also located on the Tra1 region and involved in the translocation of the DNA across the cell membrane (Grahn *et al.*, 2000). The *traG* gene also located on the Tra1 region connects the relaxosome and the mating pair formation (mpf) complex. Ten genes from the Tra2 region encode essential components of the mpf complex are *trbB, trbC, trbD, trbE, trbF, trbG, trbH, trbI, trbJ*, and *trbL* which have all been determined by constructing knockout mutations in each gene of the Tra2 region (Figure 6.2) (Lessl *et al.*, 1993, Haase *et al.*, 1995).

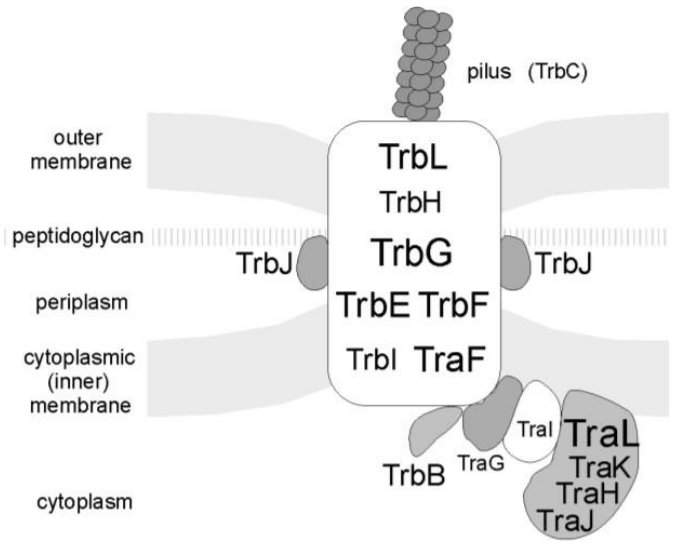


Figure 6.2 Schematic representation of the membrane-spanning and membrane-associated DNA transfer apparatus in plasmid RK2.

(Retrieved from (Grahn *et al.*, 2000))**.** TraI (relaxase), TraH, TraJ and TraK form the relaxosome. TraG connects the ralaxosome and the Mpf complex. TrbB, TrbC, TrbE, TrbF, TrbG, TrbH, TrbL, TrbJ and TrbI from the Tra2 region and TraF from the Tra1 region are involved in the mating pair formation.

There are a number of other conjugation systems that do not fit the conventional well-studied T4SS-mediated transfer from donor to recipient. In the genus *Streptomyces*, (hyphae-producing, Gram-positive filamentous bacteria) a unique conjugation system exists in which only a single plasmid-encoded protein, TraB, is sufficient to translocate a double stranded DNA into a recipient (Thoma & Muth, 2012). There is a resemblance between the pore-forming TraB and the chromosome segregator protein FtsK, suggesting a similar but not identical function for the TraB during conjugation (Vogelmann *et al.*, 2011). During chromosomal segregation, the DNA is already aligned at the closing septum. FtsK can easily translocate it to the daughter cell without the need for a pore in the septum (Gordon & Wright, 2000). However, TraB has to translocate the DNA through intact cell envelopes of the donor and the recipient, and therefore it needs a fusion protein which is normally found in the hyphal tips of mycelium and responsible for fusion of peptidoglycan layers of donor and recipient (Reuther *et al.*, 2006). Sequence analysis of the *Streptomyces lividans* plasmid pIJ101 (The first conjugative plasmid from a Gram-positive bacterium to be fully sequenced (Kendall & Cohen, 1988)) revealed that beside the TraB, specific 8 bp repeats present within the plasmid *clt* locus (**c**is-acting-**l**ocus of **t**ransfer ~ 50 bp) is also required to initiate conjugation by forming a recognizable loop structure (Pettis & Cohen, 1994).

Despite the existence of unconventional conjugation systems which do not require a conjugative relaxase and a T4SS complex, in all conjugation systems DNA is protected within the cells, and therefore the process is resistant to deoxyribonuclease (DNase). In contrast to conjugation in transformation, the recipient takes up free DNA from the environment and incorporates it into its genome via homologous recombination. In the case of plasmids, if it contains a recognizable origin of replication, it initiates replicating by using the host cell's DNA replication machinery resulting in plasmid maintenance.

Bacteria that are capable of natural transformation encode proteins mediating this process which is typically sensitive to DNase (Blokesch, 2016).

C. difficile has been shown to have a highly mobile and mosaic genome. A large proportion (11%) of the genome is composed of mobile genetic elements mainly in the form of integrative conjugative elements (ICEs) (discussed further in chapter 1) (Sebaihia *et al.*, 2006). These elements (CTn*1*, CTn*2*, Tn*5397*, CTn*4*, CTn*5*, CTn*7* and non-conjugative transposon Tn*5398*) are capable of transfer between *C. difficile* strains and in some cases (Tn*5397*, Tn*5398*) to other bacterial species such as *B. subtilis* and *Enterococcus* spp. (Mullany *et al.*, 1990, Mullany *et al.*, 1995, Jasni *et al.*, 2010, Brouwer *et al.*, 2011). In addition to the ICEs, the PaLoc is also capable of transfer from toxigenic to nontoxigenic strains. The transfer is DNase resistant, and therefore it is presumably mediated by conjugation (Brouwer *et al.*, 2013).

One of the reasons that gene transfer in *C. difficile* was investigated was to develop genetic tools to study this important pathogen. Conjugative transposons were the first genetic elements used to genetically manipulate the *C. difficile* genome. Initially, the conjugative transposon Tn*916* was used for gene cloning. Genes were introduced into Tn*916* in *B. subtilis* and transferred from this host to *C. difficile* (Mullany *et al.*, 1994). Subsequently, the use of replication-proficient plasmids was investigated to introduce small shuttle plasmids such as pMTL9301 from *E. coli* donor to *C. difficile* (Purdy *et al.*, 2002). pMTL9301 contains *E. coli* and *C. difficile* origins of replication and an *oriT* derived from the broad host range plasmid R702 (Tra^+, Mob^+, conjugative plasmid from RK2/ RP4 family). The means of transfer had been assumed to be by conjugation since the mobilisation of pMTL9301 resulted from the *oriT* recognition by the T4SS encoded by R702, but it was never proven by mutagenesis. Therefore, a DNase sensitivity test had never been performed to formally prove the occurrence of

conjugation as the means of pMTL9301 transfer into *C. difficile*. Only one research article has been published demonstrating the effect of DNase on HGT in *C. difficile* (Wasels et al., 2015). Filter mating experiments were performed to evaluate the role of DNase on transfer frequencies of Tn*5398* from *C. difficile* 630 and Tn*6215* from *C. difficile* F17 into CD37 and CD13. Addition of DNase to the mating mixture lowered the frequency of Tn*5398* transfer into CD13 but not into CD37. Moreover, transfer of Tn*6215* from F17 was not detected in the presence of DNase when only CD13 was used as a recipient. In this experiment, cell-to-cell contact was required, and free DNA did not result in the transfer of Tn*6215*. These workers suggested that a possible transformation-like mechanism occurs in *C. difficile* which is not like conventional transformation since the transfer was not completely abolished by DNase. In contrast to Tn*6215* and Tn*5398* conjugation-like transfer into *C. difficile*, the transfer of Tn*5397* and Tn*916* from *B. subtilis* donors has never been tested for DNase sensitivity.

6.2. Aims and objectives

In chapter 5, we observed a notable decrease in the frequency of conjugation (from 10^{-7} to 10^{-9} per donor) when pMTL9301 was transferred from *E. coli* strain CA434 into *C. difficile* CD37 in the presence of 50 µg/ ml DNase. This chapter focuses on exploring the reason behind this significant reduction ($P < 0.001$) and finding out if a 'novel' mechanism of DNA transfer exists in *C. difficile*. We hypothesize that pMTL9301 is capable of transfer both by a DNase sensitive transformation-like mechanism and a DNase resistant conventional conjugation.

Objectives

- To investigate direct transformation of *C. difficile* strain CD37 by purified non-conjugative, mobilisable plasmid pMTL9301
- To investigate the effect of plasmid-free CA434 donor cells, CA434 donor filtrate (supernatant) and heat-killed plasmid-free CA434 on DNA transfer into CD37.
- To determine the effect of *oriT* deletion on plasmid transfer into *C. difficile* strains in the absence and presence of DNase
- To determine the presence of competence genes in CD37 and 630Δ*erm* and their role in DNase-sensitive plasmid transfer into *C. difficile*
- To inactivate potential competence genes in the *C. difficile* genome using ClosTron mutagenesis and perform mating experiments between *C. difficile* mutant recipients and *E. coli* donors

- To investigate the presence of T4SS in *E. coli* strain HB101 (CA434 parental strain)

- To determine the effect of DNase onTn*5397* and Tn*916* transfer from *B. subtilis* to *C. difficile*

6.3. Methods and materials

6.3.1. Strains, plasmids and culture media

The list of bacterial strains and plasmids are shown in table 6.1. *E. coli* strains carrying plasmids were grown in Luria-Bertani (LB) broth and agar from Sigma-Aldrich (Dorset, UK) and Life Technologies (Paisley, UK), respectively at 37°C. *C. difficile* and *B. subtilis* strains were grown in brain heart infusion (BHI) agar or broth from Oxoid Ltd (Basingstoke, UK), the former supplemented with 5% defibrinated horse blood (E and O Laboratories, Bonnybridge, Scotland, UK) and incubated in an anaerobic atmosphere (80% nitrogen, 10% hydrogen and 10% carbon dioxide) in Macs-MG-1000-Anaerobic work station (Don Whitley scientific) or aerobically. Media were supplemented when required with antibiotics at the following concentrations: erythromycin 400 µg/ml or 10 µg/ml, rifampicin 25 µg/ml and tetracycline 10 µg/ml. When specified, deoxyribonuclease I (DNase) from bovine pancreas was added to the mating mix to a final concentration of 50 µg/ml. All antibiotics and DNase enzyme were obtained from Sigma-Aldrich (Dorset, UK). All restriction enzymes were obtained from New England Bio (Hitchin, UK). The storage of all bacterial isolates was made by adding an equal volume of 20% (v/v) of sterile glycerol to the broth overnight culture, resulting in 10% (v/v) glycerol stock. One ml aliquots of the glycerol stocks were kept at -80°C.

Table 6.1 Bacterial strains and plasmids used in this study.

Organisms, Plasmids	Characteristics, Resistance Markers	Reference, source
C. difficile 630Δ*erm* (ribotype 012)	Tc^r Erm^s Rif^s	Hussain *et al.*, (2005)
C. difficile CD37 (ribotype 009)	Tc^s Erm^s Rif^r	Smith *et al.*, (1981)
DH5α	F^- *rec*A1 *end*A1 *hsd*R17 (rk^-, mk^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A	Gibco BRL
E. coli HB101	Hybrid of *E. coli* K12 and *E. coli* B, parent of CA434	Boyer & Roulland-Dussoix, (1969), Obtained from Promega, USA
E. coli CA434	HB101 carrying the IncP conjugative plasmid R702	Williams *et al.*, (1990), Purdy *et al.*, (2002)
B. subtilis strain BS6A	*B. subtilis* CU2189:: Tn*5397*, Tc^r	Roberts *et al.*, (1999)
B. subtilis strain BS34A	*B. subtilis* CU2189:: Tn*916*,containing a single copy of Tn*916*	Roberts *et al.*, (2003)
pRPF185	*E. coli-C. difficile* shuttle vector with tetracycline-inducible promoter; P_{tet}::*gusA cat* CD6oriV RP4oriT-traJ pMB1oriV	Fagan & Fairweather, (2014)
pMTL9301	Erythromycin resistance encoding *E. coli-C. difficile* shuttle vector	Purdy *et al.*, (2002)

pMTL9301Δ*oriT*	pMTL9301 with 800 bp *Eco*RI fragment containing *oriT* deleted	This study

6.3.2. Direct transformation of *C. difficile* CD37 with pMTL9301

6.3.2.1. Transformation assay with purified plasmid DNA in liquid culture

Plasmid DNA purification was carried out based on the protocol discussed in chapter 2. DNA concentration and purity were determined by ultraviolet (UV) absorbance and an A260/A280 ratio of 1.9 was obtained, indicating a high degree of purity for DNA. Restriction digest and gel electrophoresis were performed for assessing DNA quality. 200 µl of exponential phase (OD_{600} ~ 0.8) BHI culture of *C. difficile* strain CD37 overnight culture was mixed with four different concentrations of purified plasmid DNA (5, 10, 15 and 20 µg/ml) inside the anaerobic chamber. Strain CD37 and plasmid mixture were spotted on pre-reduced antibiotic free BHI plates and incubated anaerobically for 18 h. The overnight growth was harvested by 1 ml pre-reduced BHI broth and used to inoculate selective plates containing 10 µg/ml erythromycin. Viable colony counts were also determined before and after adding the plasmid DNA to check the viability of the recipient cells throughout the experiment.

6.3.2.2. Transformation assay with purified plasmid in the presence of CA434

2 x 10 ml LB broth containing 200 µg/ml erythromycin were inoculated with a single colony of CA434 (test) and CA434 (pMTL9301) (positive control). The inocula were grown at 37 °C with shaking (200 rpm) for 16 h and centrifuged at 4000 x *g* for 15 min. The pellet was mixed with 200 µl of exponential phase (OD_{600} ~ 0.8) CD37 overnight culture inside the anaerobic chamber. Four different concentrations of purified plasmid

DNA (5, 10, 15 and 20 µg/ml) were added to the CD37 and CA434 (test) mixture. All reactions were mixed gently by pipetting, spotted onto antibiotic free BHI plates and incubated anaerobically for 18 h. The growth was harvested in 1 ml pre-reduced BHI broth and used to inoculate selective plates containing 10 µg/ml erythromycin and *C. difficile* selective supplement. The number of *C. difficile* and *E. coli* cells was determined before and after adding the plasmid DNA.

6.3.2.3. Transformation using cell-free filtrate (supernatant)

The overnight culture of CA434 in exponential phase was centrifuged at 4000 x *g* for 15 min, and the liquid of the supernatant was collected for further study. The cell-free filtrate was obtained by filtering the LB culture through a sterile 0.22 µM- pore membrane filters (Merck, UK). The cells were harvested by centrifugation at 14680 x *g* (13000 rpm, Eppendorf centrifuge 5415 D) in an Eppendorf tube. The cell-free supernatant was then mixed with 1 ml *C. difficile* CD37 overnight culture (OD_{600} ~ 0.8). Four microgram purified plasmid DNA was added to the CD37 and donor filtrate mixture and mixed gently by pipetting. The reaction was then spotted onto antibiotic-free BHI plates for 18 h and then transferred onto erythromycin-supplemented BHI agar plates. Viable colony count of recipient cells was determined before and after adding the plasmid DNA.

6.3.2.4. Transformation using heat-killed donor cells

CA434 (pMTL9301) overnight culture was washed twice by 1 ml phosphate buffered saline (PBS) to remove erythromycin prior to killing the cells by application of heat at 70 °C for 30 min. This procedure resulted in no viable cells as determined by plate counts. Heat-killed cells were used in liquid transformation experiments as described above. Briefly, 1 ml of heat-killed donor was mixed with 1 ml *C. difficile* strain CD37 (OD_{600} ~ 0.8). Four microgram plasmid DNA was added to the heat-killed donor and recipient mixture and spotted onto antibiotic free plates. The 18 h-growth was then harvested by 1 ml BHI broth and spread onto selective plates supplemented with appropriate antibiotics.

6.3.3. Construction of pMTL9301Δ*oriT*

6.3.3.1. Restriction endonuclease reaction and ligation

Plasmid pMTL9301 (Figure 6.3) was digested with *Eco*RI (NEB, Hitchin, UK) to delete the *oriT* region (800 bp *Eco*RI fragment containing *oriT*). The standard digestion reaction was prepared in a 10 µl total volume, containing 1 µl restriction enzymes (20 U), 1 µl 10 X digestion buffer, 1 µg plasmid DNA sample and topped up with molecular biology grade water. The reaction was incubated at 37 °C for at least 1 h for complete digestion. The digest was separated on a 1% agarose gel and the appropriate band (800 bp) extracted using a gel extraction kit (QIAquick Gel Extraction KIT, cat No. 28704) and eluted in 50 µl elution buffer (EB). The reaction was then purified using the PCR clean- up kit (Qiagen, Manchester, UK) and eluted in 30 µl molecular biology grade water. The protocols used for the gel extraction and PCR purification were mentioned in chapter 2.

For ligation, 2 µl of T4 DNA ligase, 1X T4 DNA ligase buffer (in a total volume of 40 µl) and 4 µl molecular biology grade water were added to the 30 µl purified DNA (the whole content from the gel extraction and DNA purification) and incubated approximately for 18 h at 15 °C-20 °C to generate pMTL9301Δ*oriT* (DNA ligation was discussed in chapter 2). The ligation reaction was gently mixed and kept on ice to be transformed and stored (Figure 6.4).

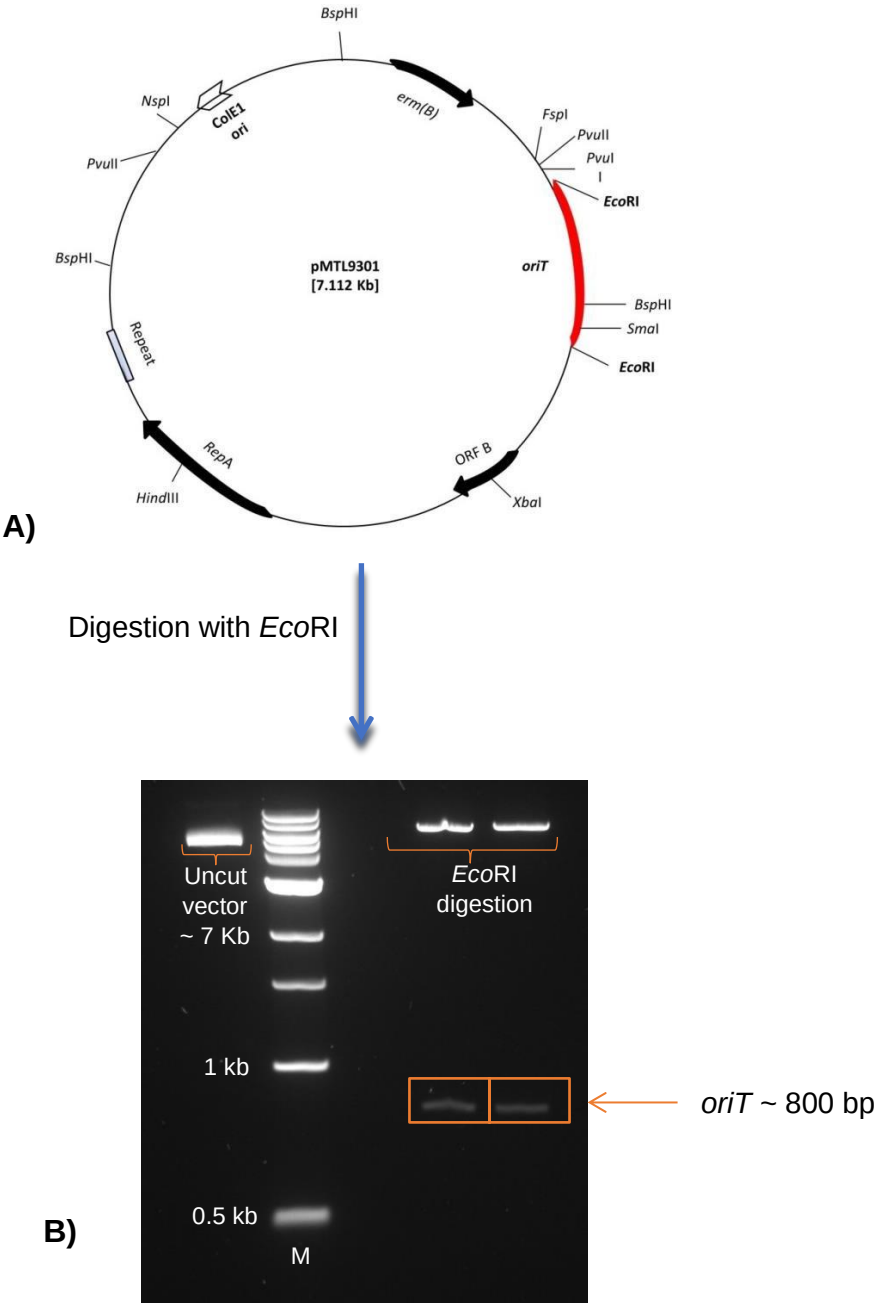


Figure 6.3 Schematic representation of *E. coli-C. difficile* shuttle plasmid pMTL9301 showing the protocol for deleting *oriT*.

A) Schematic representation of pMTL9301 (Purdy *et al.*, 2002). The *oriT* region between the *Eco*RI sites is shown in red. B) The *Eco*RI digestion products from pMTL9301 were separated on 1% agarose gel. Lane M, 1 Kb molecular ladder (NEB)

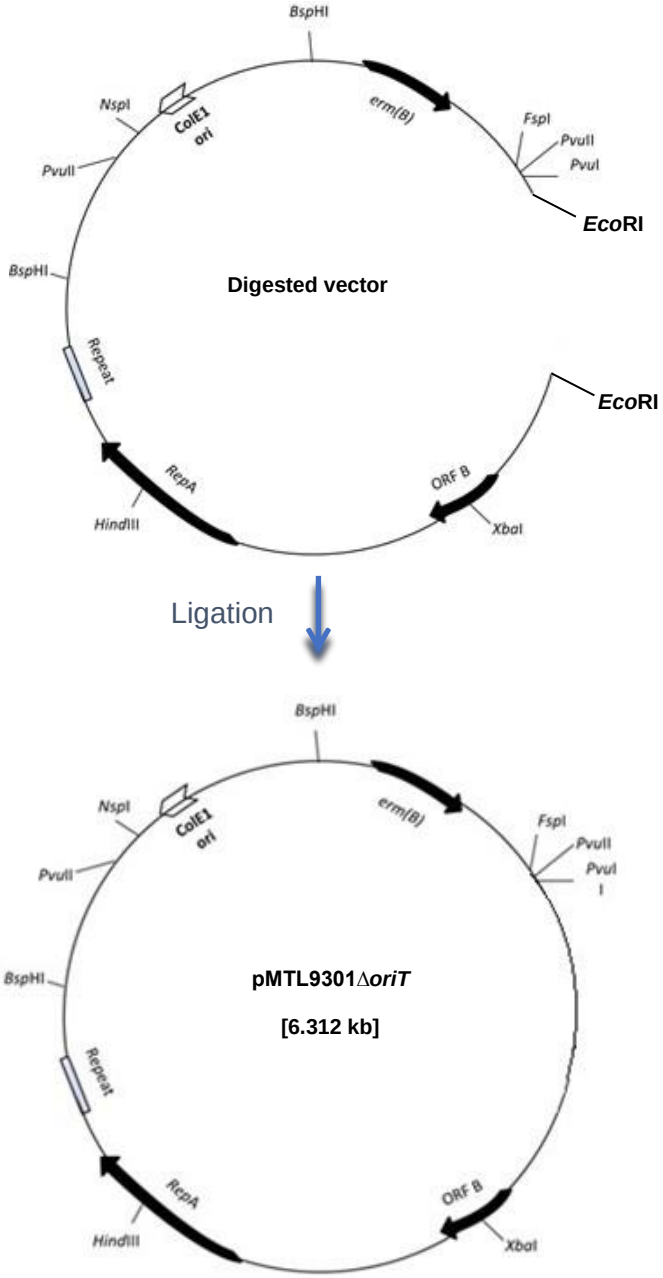


Figure 6.4 Schematic cartoon of the generation of pMTL9301Δ*oriT.*

Plasmid pMTL9301 was digested with restriction enzyme *Eco*RI to allow removal of the 800 bp fragment containing the *oriT*. The digested plasmid lacking the *oriT* was ligated with T4 DNA ligase to create pMTL9301Δ*oriT.* The plasmid contains important features including: erythromycin resistance gene *erm*(B), ColE1 for replication in *E. coli,* RepA (replication region derived from naturally occurring plasmid pCD6 isolated from *C. difficile* strain CD6) for replication in *C. difficile.*

6.3.3.2. Chemical transformation

Generation of CA434 competent cells: Bacteria were grown in 100 ml LB broth in 250-500 ml conical flasks at 37 °C with shaking to reach the OD_{600} ~ 0.6-1.0. Cultures were chilled on ice for 30 minutes and centrifuged at 4000 x *g* for 15 minutes. Bacterial pellets were gently resuspended in pre-chilled sterilized water and centrifuged at 4000 x *g* for 15 minutes. Pellets were resuspended in pre-chilled sterilized 10% glycerol solution and centrifuged at 4000 x *g* for 15 minutes and then resuspended in pre-chilled sterilized 10% glycerol solution. Bacteria were divided into 50 µl aliquots and immediately frozen on a dry ice and ethanol bath prior to storage at -80 °C.

Competent cells of *E. coli* strain CA434 together with commercially prepared competent cells of *E. coli* strain HB101 (Promega, USA) were transformed with wild type plasmid pMTL9301 or ligation mixtures pMTL9301Δ*oriT*. Briefly plasmid or ligation mixtures were added to 50 µl of *E. coli* competent cells which were thawed on ice. Bacteria were incubated on ice for 45-60 minutes before being subjected to a heat shock at 42 °C for 45 seconds. Cells were returned to ice for 2 minutes before adding 800 µl pre-warmed super optimal broth with catabolite repression (SOC) medium. Cultures were incubated at 37 °C with shaking (200 rpm) for 1 h in 1.5 ml tubes. Bacteria were spread onto pre-warmed LB agar plates containing appropriate antibiotic (400 µg/ml erythromycin) before incubation at 37 °C for 16-18 h. Overnight cultures supplemented with 200 µg/ml erythromycin were prepared from a single colony and used to prepare glycerol stocks. Stocks were kept at -80 °C to be used for mating experiments.

6.3.4. Confirmation of construct

Twenty *E. coli* strain CA434 transformants were picked from 400 µg/ml erythromycin-supplemented selective LB plates and inoculated into 10 ml LB broth containing 200 µg/ml erythromycin. Cultures were incubated with shaking (200 rpm) for 16 h at 37 °C. Plasmid was extracted from 3 ml of culture using a Mini-prep kit (QIAprep Spin Miniprep Kit, cat No. 27106) based on manufacturers instruction and eluted in molH_2O. Plasmids were assessed by restriction digest and PCR before the correct construct was selected and stored at -20 °C or -80 °C. Plasmids pMTL9301 and pMTL9301Δ*oriT* were digested with *Eco*RI. Approximately, 1 µg of wild type or *oriT*-deleted plasmids were digested with 1 µl restriction enzyme (10 U) with appropriate 1 X reaction buffer in a final volume of 25 µl for 16 h at 37 °C water bath. Digested DNA was separated on a 1% agarose gel with an appropriate marker; fragments were visualized on a transilluminator after staining with GelRed Nucleic acid gel stain (Bioline, United Kingdom).

Plasmid DNA was amplified using primer pairs (Table 6.2) in a standard PCR reaction containing 2 µl template, 22 µl BioMix^{TM} Red (Bioline, London, UK), 0.5 µl of each primer in a final volume of 50 µl. PCR conditions [Denaturation: 94 °C 4 min] x 1 cycle, [Denaturation: 94 °C 30 sec, Annealing: 50 °C-58 °C 1.5 min, Elongation: 72 °C 1 min~1 Kb product] x 25 cycles, [Elongation: 72 °C 5 min] x 1 cycle, Pause, 4 °C. Samples were separated on a 1% agarose gel with an appropriate marker; bands were visualised on a transilluminator after staining with GelRed. Desired constructs were stored at -20 °C or -80 °C.

Table 6.2 The list of primers used for analysis of the construction of pMTL9301Δ*oriT.*

Primer pair erm(B) F and erm(B) R amplify erythromycin resistance gene carrying by pMTL9301 plasmid. ORIT-1 and ORIT-2 primer pair amplifies a sequence of 800 bp within *oriT*. oriT (F1050) and oriT (R2210) primer pair amplify the region flanking the *oriT* and in pMTL9301Δ*oriT* will produce a PCR product of 300 bp. All primers synthesised by Sigma-Aldrich, UK.

Primer	Sequence (5'-3')	Annealing Tm°	Product size
erm(B) F	GTCCCGGGCCTCTTGCGGGATCAAAAG	81.2	800 bp
erm(B) R	GATCCGGACTCATAGAATTATTTCCTC	65.5	
ORIT-1	GTGCCTTGCTCGTATC	54.7	800 bp
ORIT-2	CCTGCTTCGGGGTCATTATAG	64.4	
oriT (F1050)	GGAAAGTTACACGTTACTAAAGGG	60.7	300 bp
oriT (R2210)	GTACTCTCTCTCTTATATATACAAATGG	55.9	

6.3.5. Mating experiments

C. difficile recipient strains CD37 and 630Δ*erm* were grown in 20 ml BHI broth using Coring® polystyrene tissue culture flasks with vented caps (VWR, USA) for 18-20 h in anaerobic condition at 37 °C. These were used to start a 10 ml culture at an OD_{600} ~ 0.1. These were grown shaking at 50 rpm for 4-6 h until the OD_{600} was between 0.6 and 0.8 (mid exponential phase). *E. coli* donor strains CA434 (HB101 carrying the conjugative plasmid R702) and HB101 (Parent strain of CA434) were grown in 10 ml LB broth supplemented with 200 µg/ml erythromycin for 16-18 h with shaking (200 rpm) at 37 °C. A one ml aliquot was centrifuged at 14680 x *g* (13000 rpm, Eppendorf centrifuge 5415 D) for 1 minute in an Eppendorf tube and washed twice by repeating the centrifugation step with sterile PBS to remove antibiotics.

When using *B. subtilis* as a donor, strains BS6A and BS34A (Table 6.1) were grown shaking at 200 rpm in 10 ml BHI broth supplemented with tetracycline (5 µg/ml) for 18 h. Next day, the culture was grown for 3-4 h in 100 ml BHI broth supplemented with 5 µg/ml tetracycline in 500 ml flask at 37 °C until mid- exponential phase (OD_{600} ~ 0.45-0.6). Cells were centrifuged at 4000 x *g* for 15 min and washed twice with PBS.

The harvested *E. coli* cells were resuspended in 0.5 ml BHI broth and mixed with 0.5 ml overnight culture of *C. difficile* inside the anaerobic chamber. In some cases, 50 µg/ml deoxyribonuclease I from bovine pancreas (DNase) (Sigma-Aldrich, UK) was added to the mating mix. This was then spotted onto antibiotic free BHI plates and incubated anaerobically for 18 h. When using *B. subtilis* as a donor, 100 µl of mating mix was spread on 0.45 µm pore size nitrocellulose filters (Merck, UK) on BHI plates and incubated anaerobically for 18 h.

Bacteria was harvested by flooding the agar surface with 1 ml pre-reduced BHI broth twice to ensure good recovery of transconjugants.

When *B. subtilis* was donor, the filters were removed from the agar plates and placed in 20 ml tubes containing 1 ml pre-reduced BHI broth and vortexed for 10-20 seconds. 100 µl of the cell suspension was plated onto BHI agar supplemented with erythromycin (10 µg/ml) to select for plasmids, tetracycline (10 µg/ml) to select for Tn*5397* or Tn*916*, D-cycloserine (250 µg/ml) and cefoxitin (8 µg/ml) to select against *E. coli* or *B. subtilis*. Plates were incubated anaerobically for 48-72 h and examined regularly for transconjugants. Putative transconjugants were picked and re-streaked onto fresh selective plates containing appropriate antibiotics. Control plates were also included in initial experiments containing *E. coli, B. subtilis* or *C. difficile* alone and incubated alongside experimental plates to confirm there were no surviving donor cells or spontaneous *C. difficile* mutants.

Table 6.3 The list of primers used for the analysis of transconjugants/ transformants.

Locations of the primers are shown in figures 6.5, 6.6 and 6.7. Primers synthesized by Sigma-Aldrich, UK.

Primer	Sequence (5'-3')	Annealing Tm°	Product size
oriT (F1050)	GGAAAGTTACACGTTACTAAAGGG	81.2	300 bp
oriT (R2210)	GTACTCTCTCTCTTATATATACAAATGG	65.5	
Lok 3F	GAAAGAAGAACATAATTTACCAG	55.4	750 bp
Lok 1R	CTGCACATCTGTATACATATAACTG	57.0	
tcdB-F	CAATGAACTTGTACTTCGAGATAAG	59.8	400 bp
tcdB-R	CTCACCTCCATAGTTATATCTTATACGG	62.1	
tet M (R *sma*I)	GACCCGGGACTAAGTTATTTTATTGAAC	65.9	1.2 Kb
tet M (F *sma*I)	GTCCCGGGAGTATATCGACCAGCAGACC	76.2	
tndX F30	CTTACAATGTTAAAACAGCAAG	59.4	800 bp
tndX R1100	GTATGAAATTGCGTGAGTAGTGC	62.4	
stpK F1900	CATTAATGATGATATAGACTTTACATCAGAAG	62	420 bp
stpK R1120	CAGCCGCAATTACTTTTAATC	60.2	

6.3.6. Confirmation of transfer

PCR was used to confirm the presence of plasmids or transconjugants using the appropriate primers. In some cases, the *tcdB* gene or the region flanking the PaLoc (these confirm that the transconjugants are *C. difficile* strain 630Δ*erm* or strain CD37, respectively) was amplified and sequenced. Furthermore, the *stpK* gene was also amplified and sequenced to confirm the genuineness of the transconjugants using primers listed in table 6.3. Both strains contain the *stpK* gene; however, there are SNPs and INDELs that differ in this gene between CD37 and 630Δ*erm* allowing spontaneous mutants of the donor to be distinguished from genuine transconjugants. PCR reaction was carried out using MyTaq^{TM} Red Mix (Bioline, UK) as described previously in chapter 2. PCR conditions are as follows: [Denaturation: 94 °C 4 min] x 1 cycle, [Denaturation: 94 °C 30 sec, Annealing: 50 °C-58 °C 1.5 min, Elongation: 72 °C 1 min~1 Kb product] x 25 cycles, [Elongation: 72 °C 5 min] x 1 cycle, Pause, 4 °C. Primers were designed with the aid of DNAMAN (Lynnon, USA) and checked using Sigma oligonucleotides design/ordering online software (Sigma-Aldrich, UK). Location of primers is shown in figures 6.5, 6.6 and 6.7.

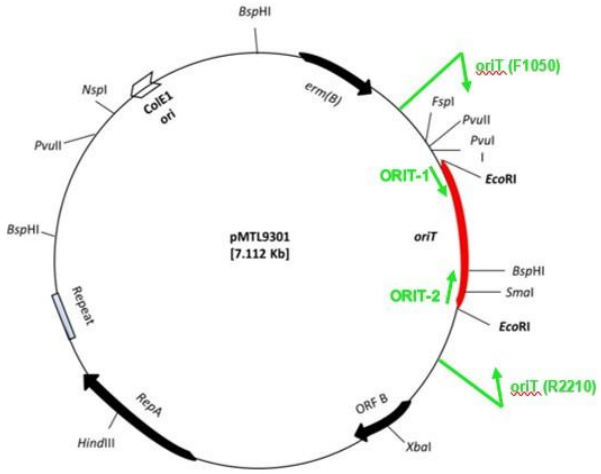
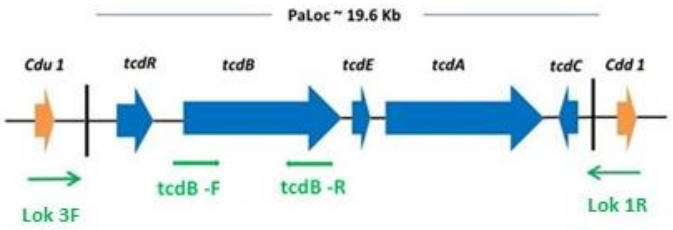


Figure 6.5 Location of primers on pMTL9301.

oriT (F1050) and oriT (R2210) primer pair amplifies a 300 bp region flanking the *oriT* in pMTL9301Δ*oriT* and <1 Kb region in pMTL9301. Primer pair ORIT-1 and ORIT-2 amplifies an 800 bp region within the *oriT* in pMTL9301 but does not have a binding site in pMTL9301Δ*oriT*.

A) PaLoc region in toxigenic strain 630



B) PaLoc region in non-toxigenic strain CD37

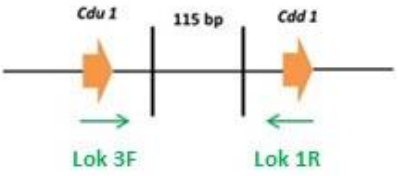


Figure 6.6 Location of primers on pathogenicity locus (PaLoc).

A) Lok 3F and Lok 1R primer pair amplifies region flanking pathogenicity locus in toxigenic strain 630Δ*erm*. No amplification was expected in toxigenic strains as the amplicon is over 20 Kb, too large to be amplified under current PCR conditions. tcdB-F and tcdB-R primer pair amplifies the *tcdB* gene specific to toxigenic strains. B) In non-toxigenic *C. difficile* strain CD37, a product of 750 bp is amplified as the PaLoc is replaced by a 115 bp non-coding region in this strain (Braun *et al.*, 1996).

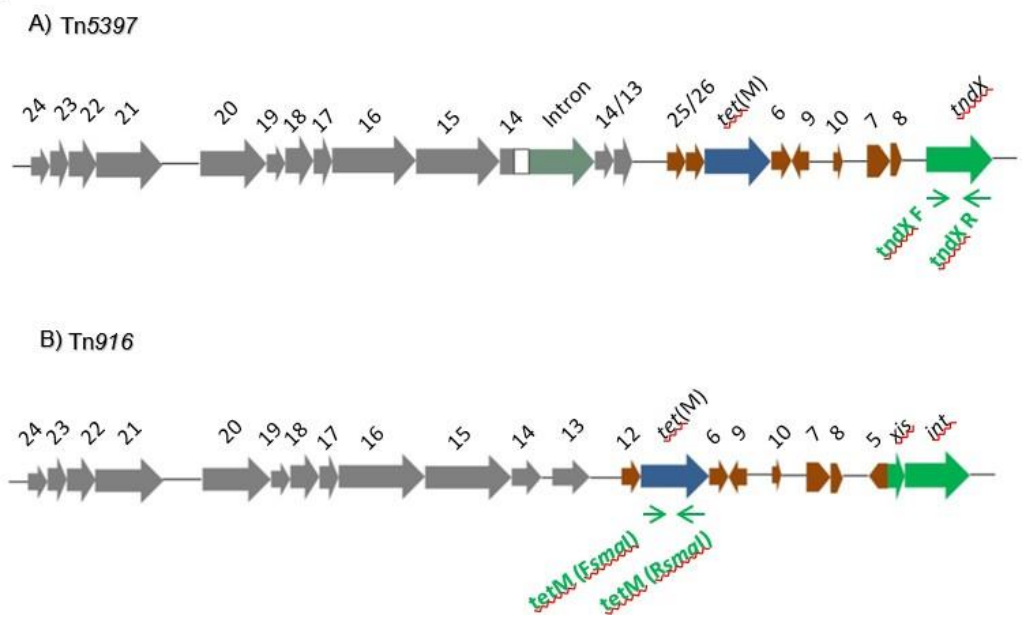


Figure 6.7 Location of primer pairs onTn*5397* and Tn*916*.

A) tndX F and tndX R primer pair amplifies a region of 700 bp within the *tndX* gene. B) tetM (F*sma*I) and tetM (R *sma*I) amplify a region of 1.8 Kb within the *tet(*M) gene.

6.3.7. Plasmid pMTL9301Δ*oriT* fate in *C. difficile*

In order to make sure that plasmids pMTL9301 and pMTL9301Δ*oriT* remained structurally stable during transfer into *C. difficile,* two millilitres of CD37 (pMTL9301) or CD37 (pMTL9301Δ*oriT*) overnight cultures grown in 10 ml pre-reduced BHI broth with 5 µg/ml erythromycin for 16-18 h at 37 °C were subjected to centrifugation at 14689 x *g* (13000 rpm Eppendorf centrifuge 5415 D) for 2 minutes. Bacteria were digested with 5 µg/ml lytic enzyme solution and 20 µg/ ml lysozyme for 30 minutes at 37 °C. Plasmids were extracted using a Mini-prep kit and eluted in 30 µl molecular biology grade water. Ten microliters of the eluates were transformed into DH5α competent cells (Table 1) (see chapter 2 for the protocol). Transformants were plated onto LB agar with 400 µg/ml erythromycin and incubated at 37 °C for 16 h. Five to ten colonies were selected and inoculated into 5 ml LB broth containing 200 µl/ml erythromycin, followed by an overnight incubation at 37 °C with shaking at 200 rpm. Plasmid was extracted from 3 ml of culture using the Mini-prep kit and eluted in 30 µl molecular biology grade water. Plasmid DNA from CD37 (pMTL9301) and CD37 (pMTL9301Δ*oriT*) was also isolated by making whole genome DNA preparation (Puregene Yeast/Bact.KitB, Qiagen, UK) (see chapter 2 for the protocol), then using this to transform *E. coli* strain DH5α competent cells. Plasmids were subsequently isolated from this strain as described above. Isolated plasmids were subjected to digestion with *Eco*RI for 18 h at 37 °C water bath. The digest was separated on a 1% agarose gel with an appropriate marker. PCR was performed on pMTL9301, pMTL9301Δ*oriT* before transfer and pMTL9301Δ*oriT* after transfer. Four microliters of plasmid templates were mixed with 1 µl primers oriT (F1050) and oriT (R2210) (Table 6.3), 50 µl MyTaq^{TM} Red Mix and 44 µl water to reach the total volume of 100 µl reaction.

PCR was carried out under the following conditions: [Denaturation: 94 °C 4 min] x 1 cycle, [Denaturation: 94 °C 30 sec, Annealing: 50 °C 1.5 min, Elongation: 72 °C 1 min] x 25 cycles [Elongation: 72 °C 5 min] x1, pause 4 °C. Two microliters of the PCR reaction was used for gel electrophoresis and a 300 bp band representing region flanking the *oriT* in pMTL9301Δ*oriT* was observed. A second PCR was performed on the whole content of PCR reaction (100 µl) to amplify the *oriT* flanking region in pMTL9301Δ*oriT* (before and after transfer). PCR product was extracted and sequenced at (GENEWIZ, Essex, UK) to determine whether the *oriT* flanking region remained intact before and after pMTL9301Δ*oriT* transfer.

6.3.8. Bioinformatics Search for putative competence genes in *C. difficile*

6.3.8.1. Search for competence genes in *C. difficile* 630

Whole genome sequencing of *B. subtilis* reveals a number of putative competence (*com*) genes involved in transformation pathways for which the biological activities have been shown (Hamoen *et al.*, 2003). The sequences of the known functional *com* genes deposited in GenBank were used here to search for competence genes in *C. difficile*. The presence of the *com* genes in *C. difficile* was predicted based on DNA sequence homology alignment algorithms such as BLASTx and BLASTn (Altschul *et al.*, 1990) using *C. difficile* 630 whole genome annotated sequence as a reference (query) (Sebaihia *et al.*, 2006). To predict the biological function of a *com* gene more accurately another approach was to search with the amino acid sequence of the predicted protein using protein prediction tool BLASTp (Altschul *et al.*, 1990).

However, sequences that are identified with the highest E-values do not necessarily have the most relevant predicted biological function. High E-values in BLAST searches may be caused by a common ancestry of the proteins or structural similarities rather than similar biological function (Tian & Skolnick, 2003). The hits of the search determining the chance that a homologue with a similar predicted function as the query were analysed using NCBI Open Reading Frame finder (Wheeler *et al.*, 2005) and the SMART tool that can recognise conserved protein domains from various databases. The presence of conserved domains in an amino acid sequence can help to support the predicted function of the protein.

6.3.8.2. Search for the *com* genes in CD37

PCRs were carried out using primers to amplify regions within the *comEA*, *cinA*, *ftsK* and *CDftsK* genes (Wasels *et al.*, 2015) using *B. subtilis* and 630Δ*erm* as positive controls. Primers were designed manually, checked for specificity in NCBI/ Primer-BLAST online software and synthesized by Sigma-Aldrich, UK. Primers are listed in table 6.4. PCR reactions were carried out using MyTaq™ Red Mix (Bioline, UK) as described previously under the following condition: [94 °C 4 min] x 1 cycle, [94 °C 30 sec, 55 °C 1.5 min, 72 °C 1 min] x 25 cycles [72 °C 5 min] x1, hold 4 °C. Annealing temperatures and extension times were adjusted according to the primer set and expected product length (Table 6.4). PCR products containing amplified regions of the *comEA*, *cinA* and *ftsK* genes were purified using QIAquick PCR Purification Kit (QIAGEN, UK) and sequenced at GENEWIZ (Essex, UK).

Table 6.4 The list of primers used in this study to detect competence genes in *C. difficile*.

Primer pairs to detect (i) the *comEA, cinA, ftsK* and CD*ftsK* genes in *C. difficile* strains CD37 and 630Δ*erm*, (ii) the *cat (P)* gene harboring by pRPF185 in *E. coli* donors are shown in this table. Primers were designed by Sigma-Aldrich.

Primers	Sequence (5'-3')	Annealing Tm^{o}	Product size
comEA F	GTGGAGCAGTAAATAGACCTGGA	63.2	328 bp
comEA R	CCAACCCCAGGTAGAGAATCC	65.6	
cinA F	AAGTCCTTTAGCAACGCCCT	63.8	842 bp
cinA R	TTGGGCCAACTGGTGATGAT	67.6	
ftsK F	ATTGAAGCCCCAATTCCTGGT	67.5	1 kb
ftsK R	GAGGCAGATGCTTGACCAC	63.6	
CD ftsK F	GCTGGTTGTCGGACTGTTTC	64.7	770 bp
CD ftsk R	AACGGTCAATGCAGGAAAGC	66.2	
Cat P (F)	CCTGCCACTCATCGCAGT	58.5	190 bp
Cat P (R)	CCACCGTTGATATATCCC	60	

6.3.9. ClosTron mutagenesis

Intron target site and Intron redirection sequence were determined using the intron targeting and design tool as described by (Heap *et al.*, 2009). The intron redirection sequence was ordered to be cloned into vector pMTL007C-E5 (Figure 6.8) (DNA 2.0, Menlo Park, USA). ClosTron mutants were constructed as described by Heap *et al.,* (2009) through DNA 2.0 program and the mutants were selected on BHI agar plates containing *C. difficile* selective supplement and 15µg/ ml thiamphenicol, as pMTL007 contains the *catP* gene conferring resistance to chloramphenicol (Cm) and thiamphenicol (Tm). PCR was used to determine if the ErmRAM had spliced out and to determine if the intron had inserted into the desired target site (Figure 6.9). Primers were designed manually and synthesized by Sigma-Aldrich (Table 6.5).

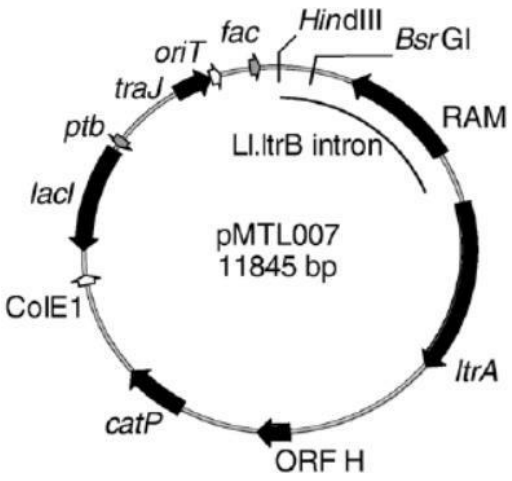


Figure 6.8 Schematic representation of *Clostridium-E. coli* modular plasmid pMTL007.

(Retrieved from (Heap *et al.*, 2007))**.** This plasmid contains ColE1 to replicate in *E. coli*. pCD6-derived origin to replicate in *C. difficile*. *traJ* and *oriT* regions derived from plasmid RK2 to facilitate conjugative transfer into *C. difficile*. Group II intron (derived from *Lactobacillus lactis*) containing *erm (B)* which itself is interrupted by group I intron. Only after a successful site-specific integration, the group I intron splices out and the *erm (B)* gene results in an intact Erm(B) conferring resistance to MLS antibiotics. Such a selective marker is called Retrotransposition-Activated Marker (RAM).

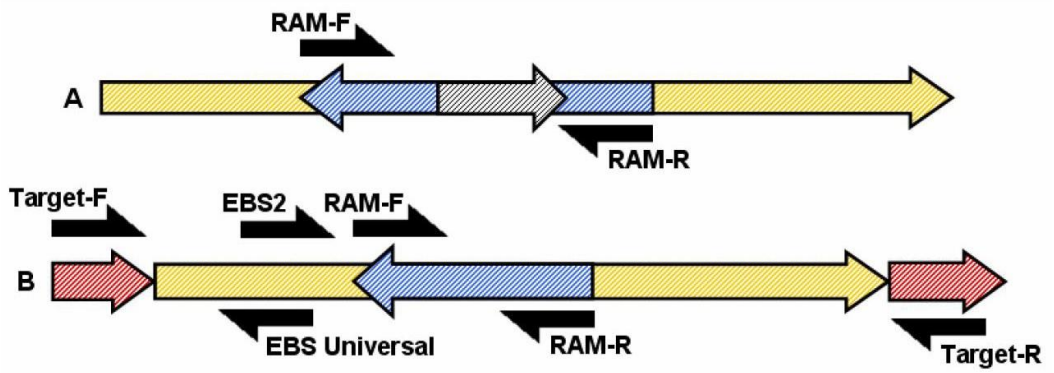


Figure 6.9 PCR screening for intron integration.

The intron on the plasmid is represented by the upper yellow arrow. The intron integrated in the target site is shown by the lower yellow arrow. The red arrows show the interrupted target site. The blue arrows represent the ErmRAM, which in the upper schematic is interrupted by the group I intron, shown as a grey arrow. PCR primers are represented by black half-arrows (Table 6.5). [Reproduced from the protocol for Clostridial Gene Knockout using pMTL007 (The Minton Laboratory UoN, 2006)].

Plasmids harbouring ClosTron-based mutation in the *comEA*, *cinA* and *ftsK* genes were recovered from GFC filters according the manufacturer's instruction (DNA 2.0, USA). Recovered plasmids were run on 1% agarose gel to make sure they were not degraded. The concentration of plasmids was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, UK). Plasmids were transformed into *E. coli* strain DH5α commercially prepared competent cells and stored at -80 °C.

Gene transfer experiments were performed as described by Heap *et al.,* (2009). Briefly, ClosTron mutants were transformed into chemically competent CA434 donor cells. Transformants appeared on LB plates supplemented with 25 µg/ml chloramphenicol. PCR was performed to ensure successful transformation. Transformants were grown for 18 h in LB broth containing 12.5 µg/ ml chloramphenicol, washed with sterile PBS and the harvested pellet was mixed with CD37 or 630Δ*erm* recipient (0D600 ~ 0.8).

Serial dilutions of the growth harvested from BHI mating plates were set up to determine the cell counts of donor and recipient cells. Donor cells containing the ClosTron insertion were selected on chloramphenicol (25 µg/ml) supplemented LB plates. *C. difficile* CD37 recipient cells were selected on rifampicin (25 µg/ml) supplemented BHI plates. *C. difficile* 630Δ*erm* recipient cells were selected on tetracycline (10 µg/ml) supplemented BHI plates. CD37 transconjugants were selected on thiamphenicol (15 µg/ml) and rifampicin (25 µg/ml). 630Δ*erm* transconjugants were selected on thiamphenicol (15 µg/ml) and tetracycline (10 µg/ml). Identification of the transconjugants was confirmed by two sets of PCRs, one using a primer pair F + R to amplify the wild type gene with the RAM insert to give a band of 4 Kb and the other using a primer pair F + (exon binding) EBS universal to amplify the wild type gene just upstream of the *erm*RAM to give a band of 0.4 Kb (Table 6.5).

To investigate the role of the *com* genes in DNase-sensitive conjugation-like mechanism in *C. difficile*, pRPF185 (Table 6.1,) was transformed into chemically competent CA434 cells and commercially prepared competent HB101 cells (Table 6.1), alongside two control transformations using pMTL9301 and pUC19. Plasmids were extracted using QIAprep SpinMiniprep Kit (QIAGEN, UK) and digested with *Sma*I and *Bam*HI restriction enzymes to allow assessment of the plasmid quality. Two bands were expected in both cases, pMTL9301 produced fragments of the expected size (1700 bp and 5412 bp), and pRPF185 produced fragments of the expected size (1866 bp and 7185 bp). Conjugation was performed as described by Heap *et al.,* 2009. Briefly, *E. coli* strains CA434 and HB101 were used as donor strains for pRPF185. CD37, CD37Δ*comEA*::ErmRAM, CD37Δ*cinA*::ErmRAM, 630Δ*erm*, 630Δ*erm*Δ*comEA*::ErmRAM and 630Δ*erm*Δ*cinA*::ErmRAM were used as recipients. DNase (50 µg/ml) was added to the mating mix when required.

Table 6.5 The list of primers used for ClosTron mutagenesis.

Target (F) and (R) primer pairs were designed manually specific for each target gene, checked by DNAMAN and synthesized by Sigma-Aldrich, UK. EBS universal primer is common to all PCRs for intron targeting. The EBS primer was designed by Heap *et al.,* (2009) and synthesized by Sigma-Aldrich. Locations of the primers are shown in figure 6.9.

Primers	Sequence (5'-3')	Annealing Tm^{o}
cinA closT (F)	CAGGTGGTCTTGGGCCAACTGGTG	76.1
cinA closT (R)	GCAACTGTTGGATTAGTCTGCTC	63.9
comEA closT (F)	CGTTTATAAATCGCTTATTTTCAAAGATG	64.9
comEA closT (R)	GATAGATTGACTCCATTTAAATCTGC	61.8
ftsk closT (F)	GGTATAGCAGGTGGATGGTTAATAAG	63.3
ftsk closT (R)	CCTCATTAACTGGCTCTGCTAC	61.7
EBS universal	CGAAATTAGAAACTTGCGTTCAGTAAAC	60

6.3.10. Investigation of the potential influence of the transfer genes on plasmid RK2 on DNase sensitive *oriT*-independent plasmid transfer into *C. difficile*

Bioinformatic search for transfer genes in *E. coli* strain HB101

The sequencing of the Birmingham IncP-alpha plasmid RK2 (GeneBank Accession number: BN000925) revealed a number of putative *tra* and *trb* genes involved in conjugation (Pansegrau *et al.*, 1994). All these annotated transfer genes in the sequence of RK2 were studied in *E. coli* strain RR1 (HB101 RecA^{+}) (Jeong *et al.*, 2017) at the nucleotide level using BLASTn and amino acid level using BLASTp (Altschul *et al.*, 1990).

Another approach to verify the presence of the *tra* and *trb* genes in the HB101 genome is to perform standard PCR with gene specific primers. All the *tra* and *trb* genes were subjected to PCR using primers listed in table 6.6. Primers were designed manually specifically to amplify regions inside the target genes, checked with NCBI/ Primer-BLAST and synthesized by Sigma-Aldrich, UK. PCR conditions were as follows: [94 °C 4 min] x 1 cycle, [94 °C 30 sec, 55 °C 1.5 min, 72 °C 1 min] x 25 cycles [72 °C 5 min] x 1 cycle, hold 4 °C. Samples were separated on a 1% agarose gel with an appropriate marker. Bands were visualized on a transilluminator after staining with GelRed.

Table 6.6 The list of primers used for detecting the *tra* and *trb* genes in *E. coli* strain HB101 (parent strain of CA434).

Primers	Sequence (5'-3')	Annealing Tm°	Product size
traL F	GCGCAGTACAAGATGGACAA	63.9	237 bp
traL R	GCTGATGAGGTAATGCGACA	63.9	
traK F	CTCGTCACCATTTGGGAGCA	68.5	405 bp
traK R	CCTTTTTGTCCGGTGTTGGGG	68.4	
traF F	CACCAAAAGCATTCCGGTCG	69.6	388 bp
traF R	ATTGACAGGCCCGAAGTAGC	65.3	
traG F	CAGAACCTTGCCACCCTGAT	66.3	799 bp
traG R	TCGTGGTGCCTGTCAGTTTT	65.7	
traM F	TGAAGAGCTGATCCGGGAGA	67.4	265 bp
traM R	GGCGCTGTCCTTCATTACCT	65.3	
traI F	GCGGAAACGGCTTCATCTTC	68	917 bp
traI R	TCCGTCATTTCGTTCGGGTT	68.6	
traJ F	GGTGTACTGCCTTCCAGACG	64.7	133 bp
traJ R	GACGTGCTCATAGTCCACGA	64	
trbA F	CAACATCCTTCGTCTGCTCG	65.9	274 bp
trbA R	CGGCAAAACCACACTGATGC	68.8	
trbB F	CAGGCGATTATAGAAACGGT	60.5	762 bp

trbB R	TTACAGGGTTTTGGTGATGT	59.3	
trbC F	GAATCGCGGCATTTTGTTC	65.4	240 bp
trbC R	ATCAGGGTTCGGAAGAAGG	63	
trbD F	AACCGAGAAAACCTGTTCAT	60	270 bp
trbD R	GTATTGCTTCCCTTGGCTAT	59.6	
trbE F	CTCATCAACACGATGAACCT	60.2	689 bp
trbE R	TTCATCAGCTCTTCGATCTC	59.7	
trbF F	GGTGACGCCGGACGTAGCTT	70.6	247 bp
trbF R	GCCTTTCACCACGCCTTGCC	73.1	
trbG F	CAAGTCAACTCGATCAACCT	59.3	531 bp
trbG R	AATCGTATCGACGATGTAGC	59.2	
trbH F	CCGCCTACAACCAGACCATT	66	308 bp
trbH R	CATGACGGTCAGGCGATACA	67.1	
trbI F	GCGAAGATCAAATGGCACCG	69.8	455 bp
trbI R	CTTGATCGCCTCTTCCAGCA	67.8	
trbJ F	GAGGAATACCGGACGCAGTT	65.2	298 bp
trbJ R	CGTTGGCCTTTTTCTGCGAT	68.2	
trbL F	TGGAGTGATGCTGATCGTCG	67.6	679 bp
trbL R	CCTGTTGTTGCTTGCGGAAA	68.2	
trbM F	AGCTGGGACGACCAGTTCTA	63.8	215 bp

trbM R	CCATAGCGACCGTACTGCTG	65.6	
trbN F	TCGCCATTCGGGAAAAGGAG	70.3	249 bp
trbN R	TGCGCGAGTGATAGTTAGCG	66.3	
trbP F	TTCATTGCGTTGGGTGGAGT	67.7	386 bp
trbP R	CAAAGAGCGGCAAGATGCAG	68.2	

6.4. Results

6.4.1. DNase sensitive plasmid transfer into *C. difficile*

Results shown in the previous chapter revealed that the frequency of pMTL9301 transfer from *E. coli* strain CA434 into *C. difficile* CD37 was significantly decreased when DNase was present in the mating mixture (from 10^{-7} to 10^{-9} per donor). To gain a deeper insight into these observations the following experiments were performed, all under the same conditions.

6.4.1.1. Using purified plasmid DNA in liquid culture of *C. difficile* did not result in transformation

Results obtained from chapter 5 implied that pMTL9301 might be transferring from *E. coli* to *C. difficile* by transformation. To investigate this further, 5, 10, 15 and 20 µg/ml of pMTL9301 was added to CD37 overnight culture (OD_{600} ~ 0.8). No erythromycin resistant (Erm^R) transformants were obtained when DNA was used to treat 10^8 CFU/ml CD37 cells (Table 6.7).

6.4.1.2. Transfer of free plasmid DNA into CD37 did not occur in the presence of plasmid- free CA434

Purified plasmid DNA in various concentrations (5, 10, 15 and 20 µg/ml) was added to the mixture of CD37 recipient and plasmid-free CA434. As shown in table 6.7, no Erm^R transformants was detected after 24-48 h. This indicates that the presence of donor cells harbouring the plasmid is required for its transfer into CD37 and the combination

of plasmid-free CA434 and free plasmid is not sufficient for transfer. This experiment was performed under the same conditions as when pMTL9301-harbouring CA434 was used.

6.4.1.3. Using heat-killed donor or donor culture supernatant/ filtrate did not result in plasmid transfer into CD37

To examine whether a donor-secreted component in LB broth stimulates the plasmid transfer into CD37, we performed the following two experiments: (i) Exposure of the donor culture to heat (70 °C) for 30 minutes, (ii) Passing the donor overnight culture through 0.22 µm-pore membrane filters to have a cell-free supernatant/ filtrate. These experiments did not result in the formation of Erm^R transformants on selective plates (Table 6.7). Therefore, the presence of live donors harbouring the plasmid is required for the DNase sensitive plasmid transfer into *C. difficile.*

Table 6.7 Direct transformation of *C. difficile* CD37 with free plasmid pMTL9301.
Erm^R: erythromycin resistant.

Effect of media conditioned with *E. coli* and/or plasmid	Recipient	Viable count of recipient (CFU/ ml)	Number of transformants (Erm^R colonies on selective plates)
Purified plasmid	CD37	$1.67 \pm 1.44 \times 10^8$	None
Plasmid-free CA434 + Plasmid	CD37	$1.15 \pm 1.17 \times 10^8$	None
Heat-killed donor + 4 µg purified plasmid	CD37	$1.2 \pm 1.28 \times 10^8$	None
Donor filtrate + 4 µg purified plasmid	CD37	$1.64 \pm 1.58 \times 10^8$	None

6.4.1.4. Plasmid transfer into *C. difficile* requires close contact between live donor cells and recipients, and DNase treatment only reduces the transfer frequency of pMTL9301 into CD37 but has no effect on the transfer frequency to *C. difficile* 630Δ*erm*

The effect of DNase on plasmid transfer into *C. difficile* was investigated using strain 630Δ*erm* as well as CD37 to determine whether the DNase-sensitive transformation-like mechanism is a recipient-dependent phenomenon. Plasmid pMTL9301 was transferred from *E. coli* strain CA434 to *C. difficile* CD37 at a frequency of around 3.26×10^{-5} transconjugants per donor and to *C. difficile* 630Δ*erm* at a frequency of around 3×10^{-5} transconjugants per donor. Incorporation of DNase into the mating mix prior to plating onto selective agar resulted in the frequency of transfer decreasing by 5 orders of magnitude to around 10^{-10} transconjugants per donor for CD37 but no change in transfer frequency was observed when 630Δ*erm* was used as the recipient (Table 6.8) (Figure 6.12).

6.4.2. A complete *oriT* is not required for plasmid transfer into *C. difficile*

6.4.2.1. pMTL9301∆*oriT* construction and validation

In order to determine if *oriT* is required for the transfer of plasmid pMTL9301 DNA into *C. difficile*, the *oriT* and *nic* sites from pMTL9301 were deleted (for methods see section 6.3.3). Transformants were detected by PCR (Figures 6.10) and sequencing (Figure 6.11).

Figure 6.10 PCR amplification to confirm the *oriT* deletion.

Lanes 1, 2 and 3 are PCR amplification of the *oriT* using primer pairs ORIT-1 and ORIT-2, no band suggests the successful deletion of the *oriT.* Lanes 4, 5 and 6 are PCR amplification of the region flanking the *oriT* using primers oriT(F1050) and oriT(R2210), in pMTL9301Δ*oriT* it shows a product approximately 300 bp. Lane M is 1 Kb molecular ladder.

Figure 6.11 Sequencing results of the 800 bp region deleted from pMTL9301 using *Eco*RI restriction enzyme in order to construct pMTL9301Δ*oriT*.

The sequence of the *oriT* is highlighted in green (~112 bp). The inverted repeat sequence (IR~19bp) within the *nic* site (recognized by the conjugative apparatus of plasmid R702) is in purple and underlined. The sequencing result shows that the *oriT* is successfully removed from the plasmid. No obvious homologs to the *oriT* or the IR sequence were found elsewhere on the plasmid backbone.

6.4.2.2. Deletion of the *oriT* from pMTL9301 does not abolish transfer from *E. coli* to *C. difficile* but does abolish transfer to strain CD37 in the presence of DNase

The fact that pMTL9301 transfer to CD37 is drastically reduced in the presence of DNase (chapter 5) indicates that as well as transferring by conjugation, the plasmid may also be entering *C. difficile* by a transformation-like mechanism. To investigate this further, we deleted the *oriT* region from pMTL9301 to generate pMTL9301Δ*oriT* (Figure 6.4). This plasmid transferred from *E. coli* strain CA434 in the absence of DNase to CD37 and 630Δ*erm* at frequency of approximately 10^{-10} per donor, a much lower frequency than observed with pMTL9301 (Table 6.8). No transfer (the detection limit was $<10^{-10}$ transconjugants per donor or recipient) was observed to CD37 when DNase was included in the medium, although transfer to 630Δ*erm* was still observed (Table 6.8). That pMTL9301 and pMTL9301Δ*oriT* were transferred to *C. difficile* CD37 and 630Δ*erm* was confirmed by preparing plasmids from representative transconjugants and demonstrating that they contain either intact *oriT* (pMTL9301) or had this region deleted in the case of transconjugants containing pMTL9301Δ*oriT* (Figure 6.12). Transfer of pMTL9301Δ*oriT* into *C. difficile* was verified by DNA sequencing and restriction mapping (Figure 6.13). That the transconjugants were genuine *C. difficile* strains and not *E. coli* spontaneous mutants were confirmed by PCR and sequencing (as described in the methods). Furthermore, the transconjugants could not grow aerobically indicating that they are not *E. coli* spontaneous mutants. Plasmid fate was also investigated by extracting pMTL9301Δ*oriT* from *C. difficile* transconjugants/ transformants and PCR amplification (Figure 6.14).

6.4.2.3. Deletion of the *oriT* from pMTL9301 does not abolish transfer from *E. coli* strain HB101 to *C. difficile* 630Δ*erm* but does abolish transfer to CD37 in the absence of DNase.

According to Purdy *et al.,* (2002), CA434 was made by transferring the conjugative plasmid R702 into HB101 (Table 6.1). Therefore, in order to investigate the role of R702 in the transfer of pMTL9301 and pMTL9301Δ*oriT,* HB101 was used as a donor strain. Plasmid pMTL9301 transferred from this host to 630Δ*erm* at a frequency of around 10^{-9} per recipient and 10^{-10} per donor and at a frequency of 10^{-9} per recipient and 10^{-10} per donor to CD37 (Figure 6.12) (Table 6.8). When HB101 containing pMTL9301Δ*oriT* was used as the donor and 630Δ*erm* the recipient, transconjugants appeared at a similar frequency, but no transconjugants were observed when CD37 was the recipient (Figure 6.12). When DNase was incorporated in the media, no transconjugants were obtained in any of the above combinations of recipients and donors (Table 6.8). The detection limit in these experiments was $<10^{-10}$ transconjugants per donor or recipient. That transconjugants/ transformants were genuine was confirmed as described above and in the methods section.

Strains		Frequency of transfer/ donor		Frequency of transfer/ recipient	
Donor	Recipient	No DNase	DNase (50 µg/ml)	No DNase	DNase (50 µg/ml)
CA434 (pMTL9301)	CD37	$3.26 \pm 1.24 \times 10^{-5}$	$3.67 \pm 1.41 \times 10^{-10}$ (a)	$3.32 \pm 4.71 \times 10^{-6}$	$7.53 \pm 4.71 \times 10^{-10}$ (a)
CA434 (pMTL9301 Δ*oriT*)	CD37	$4.68 \pm 1.24 \times 10^{-10}$ (b)	ND	$6.65 \pm 3.48 \times 10^{-10}$ (b)	ND
CA434 (pMTL9301)	630Δ*erm*	$3 \pm 0.47 \times 10^{-5}$	$4.5 \pm 0.47 \times 10^{-5}$	$1.62 \pm 0.04 \times 10^{-5}$	$1.8 \pm 0.04 \times 10^{-5}$
CA434 (pMTL9301 Δ*oriT*)	630Δ*erm*	$1.98 \pm 1.13 \times 10^{-10}$ (c)	$1.67 \pm 1.13 \times 10^{-10}$ (c)	$4.6 \pm 1.5 \times 10^{-9}$ (c)	$6.2 \pm 1.5 \times 10^{-9}$ (c)
HB101 (pMTL9301)	CD37	$4.3 \pm 2 \times 10^{-10}$ (d)	ND	$5.67 \pm 2.85 \times 10^{-9}$ (d)	ND

HB101 (pMTL9301 Δ*oriT*)	CD37	ND	ND	ND	ND
HB101 (pMTL9301)	630Δ*erm*	$4.67 \pm 1.1 \times 10^{-10}$ (e)	ND	$4.09 \pm 1.01 \times 10^{-9}$ (e)	ND
HB101 (pMTL9301 Δ*oriT*)	630Δ*erm*	$2.08 \pm (0) \times 10^{-10}$ (f)	ND	$2.3 \pm (0) \times 10^{-9}$ (f)	ND

Table 6.8 Frequency of pMTL9301 and pMTL9301Δ*oriT* transfer into C. difficile (±SD).

SD, data were expressed as standard deviation (SD) based on at least three independent experiments. ND, no transconjugants/ transformants detected after mating, i.e. a conjugation frequency below the detection limit ($<10^{-10}$).

Each experiment was repeated at least three times on separate occasions. All colonies obtained were verified using PCR (as described in the Methods and Results section) to confirm that they were indeed the correct strain harbouring the expected plasmid. [a]) *C. difficile* strain CD37 (pMTL9301) transconjugants/ transformants arose at a frequency of 10^{-10} had 9–16 colonies per mating. [b]) *C. difficile* strain CD37 (pMTL9301Δ*oriT*) transconjugants/ transformants arose at a frequency of 10^{-10} had 1–3 colonies per mating. [c]) *C. difficile* strain 630Δ*erm* (pMTL9301Δ*oriT*) transconjugants/ transformants arose at a frequency of 10^{-10} to 10^{-9} had 3–12 colonies per mating. [d]) *C. difficile* strain CD37 (pMTL9301Δ*oriT*) transconjugants/ transformants arose at a frequency of 10^{-10} to 10^{-9} had 1–3 colonies per mating. [e]) *C. difficile* strain 630Δ*erm* (pMTL9301) transconjugants/ transformants arose at a frequency of 10^{-10} to 10^{-9} had 1–3 colonies per mating. [f]) *C. difficile* strain 630Δ*erm* (pMTL9301Δ*oriT*) transconjugants/ transformants arose at a frequency of 10^{-10} to 10^{-9} had 1–3 colonies per mating.

6.4.3. Analysis of transconjugants/ transformants

Erm^R transconjugants/ transformants were sub-cultured onto fresh selective plates and incubated both aerobically and anaerobically. Incubation under aerobic condition did not result in the appearance of colonies indicating that Erm^R colonies were not spontaneous *E. coli* mutants. Anaerobically-grown colonies were picked randomly and subjected to PCR to determine plasmid transfer into *C. difficile* (Figure 6.12).

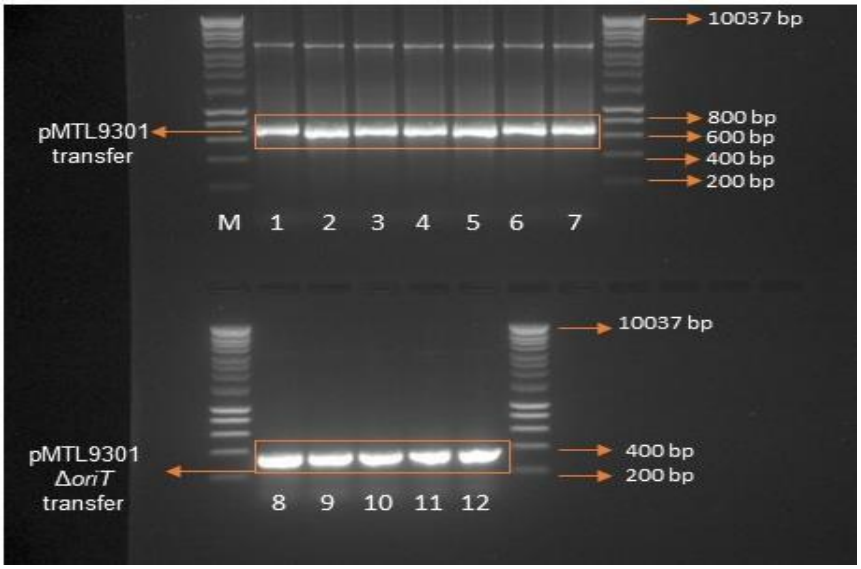


Figure 6.12 PCR analysis of transconjugants/ transformant.

Amplification of ~800 bp region within *oriT*/*mob* region in *C. difficile* containing pMTL9301: Lanes 2-7 (DNA was amplified with primers ORIT-1 and ORIT-2) and ~300 bp region flanking the deleted *oriT*/*mob* region in *C. difficile* containing pMTL9301Δ*oriT*: Lanes 9-12 (DNA was amplified with primers oriT (F1050) and oriT (R2210). Template DNA in each lane is as follows: (1): purified pMTL9301, (2): CA434 (pMTL9301) x CD37 (+DNase), (3): CA434 (pMTL9301) x CD37 (No DNase), (4): CA434 (pMTL9301) x 630Δ*erm* (+DNase), (5): CA434 (pMTL9301) x 630Δ*erm* (No DNase), (6): HB101 (pMTL9301) x CD37 (No DNase), (7): HB101 (pMTL9301) x 630Δ*erm* (No DNase), (8): Purified pMTL9301Δ*oriT*, (9): CA434 (pMTL9301Δ*oriT*) x CD37 (No DNase), (10): CA434 (pMTL9301Δ*oriT*) x 630Δ*erm* (+DNase), (11): CA434 (pMTL9301Δ*oriT*) x 630Δ*erm* (No DNase), (12): HB101 (pMTL9301Δ*oriT*) x 630Δ*erm* (No DNase). M= 1 Kb-plus molecular ladder.

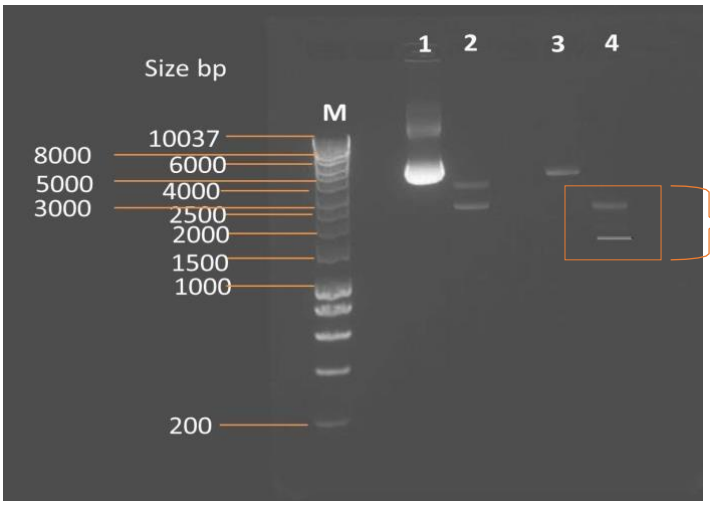


Figure 6.13 *Hind*III and *Xba*I digestion of the plasmids pMTL9301 and pMTL9301Δ*oriT* extracted from putative transconjugants/ transformants.

Lane 1: Uncut pMTL9301. Lane 2: pMTL9301 digested with *Hind*III and *Xba*I resulted in two bands (~ 4000 bp and ~ 3000 bp). Lane 3: Uncut pMTL9301Δ*oriT*, Lane 4: pMTL9301Δ*oriT* digested with *Hind*III and *Xba*I resulted in two bands (~ 3000 bp and ~ 2500 bp)

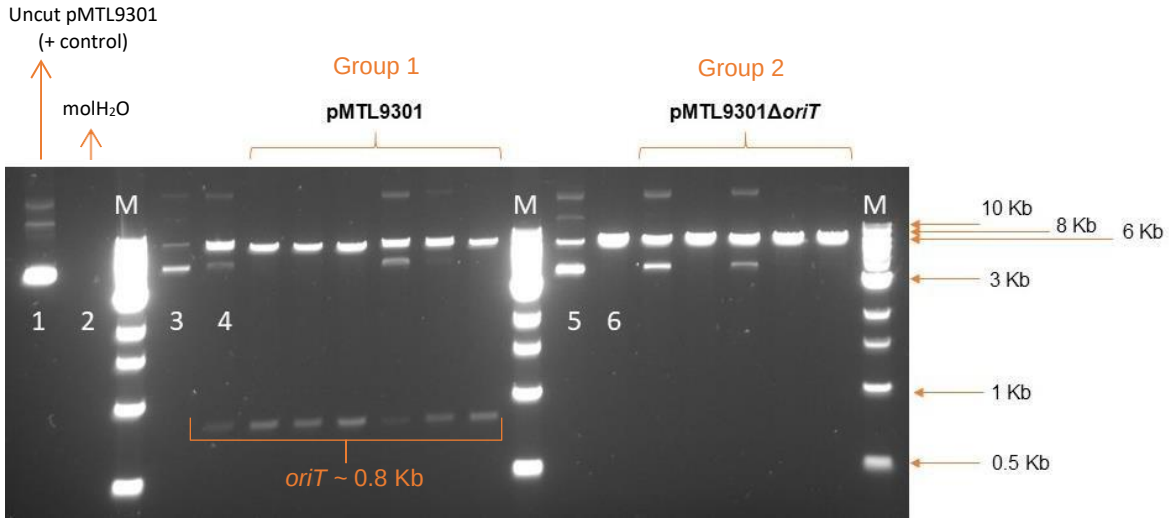


Figure 6.14 *Eco*RI digestions of pMTL9301 and pMTL9301Δ*oriT* shows that plasmid remained structurally stable before and after transfer.

Lane 3: uncut pMTL9301 before transfer. Lane 4: cut pMTL9301 before transfer. Group 1: digested pMTL9301 extracted from *C. difficile* transconjugants/ transformants (800 bp *oriT* is isolated from pMTL9301). Lane 5: uncut pMTL9301Δ*oriT* before transfer. Lane 6: cut pMTL9301Δ*oriT* before transfer. Group 2: digested pMTL9301Δ*oriT* extracted from *C. difficile* transconjugants/ transformants (the 800 bp *Eco*RI fragment bearing *oriT* was removed during plasmid construction (see section 6.4.2.1)). M= 1 Kb ladder. Digestion patterns did not show any difference before and after transfer.

6.4.4. Transfer of Tn*5397* and Tn*916* from *Bacillus subtilis* is not affected by DNase treatment

In order to test if the DNase sensitive transfer is a more general phenomenon, we examined the transfer of Tn*5397* and Tn*916* both carrying tetracycline resistance gene from *B. subtilis* to *C. difficile* CD37. Tn*916* and Tn*5397* containing transconjugants were obtained at a frequency of around 1×10^{-7} and 1×10^{-8} transconjugants per donor, respectively very similar to previously reported transfer frequencies for these elements (Mullany *et al.*, 1990, Mullany *et al.*, 1994). This was the same in both the presence and absence of DNase (data not shown). DNase-resistant conjugative transfer of Tn*5397* and Tn*916* was confirmed by PCR using primers summarised in table 6.3. The location of primers is illustrated in figure 6.7.

6.4.5. Investigation of the potential influence of the *com* genes on DNase-sensitive plasmid transfer into *C. difficile*

6.4.5.1. Bioinformatic Search for putative competence genes in *C. difficile*

In order to further investigate the DNase sensitive *oriT*-independent plasmid transfer into *C. difficile*, we decided to look for possible competence (*com*) genes, potentially involved in the transformation-like mechanism observed. Analysing the whole genome sequence of *C. difficile* 630 (Genebank AM180355) (Sebaihia *et al.*, 2006) using DNA sequence homology alignment algorithms such as BLASTx and BLASTn showed that three putative competence genes including *comEA*, *cinA* and *ftsK* in *B. subtilis* genome are present in CD630 with 99%, 100% and 99% similarity, respectively. BLASTp was also used with translated coding sequences of these genes against *B. subtilis* reference sequence for searching protein databases. The *comEA*-encoded ComEA competence protein, *cinA*-encoded competence damage-inducible protein A and *ftsK*-encoded DNA Ftsk/SpoIII translocase proteins are all candidate proteins for DNA translocation in *C. difficile*. A search for the presence of other *com* genes (Hamoen *et al.*, 2003) in *C. difficile* was also carried out using BLASTn and BLASTp; however, no homologues were found for these (Table 6.9).

Therefore, the *comEA*, *cinA*, and *ftsk* genes with the following locus tags: CD630_24970, CD630_14090 and CD630_13240, respectively were chosen for further investigations to look for potential competence proteins mediating the DNase sensitive *oriT*-independent plasmid transfer into *C. difficile*.

Coding sequences (CDS) of these putative genes were predicted using NCBI Open Reading Frame (ORF) finder and the length of each gene was as follows: *comEA* (705 bp), *cinA* (1257 bp) and *ftsK* (2412 bp). Primers were designed to amplify each gene (Table 6.4) and PCR was carried out to confirm their presence in *C. difficile* strains CD37 and 630Δ*erm* (Figure 6.15). Sequencing of the PCR products confirmed that these genes are present.

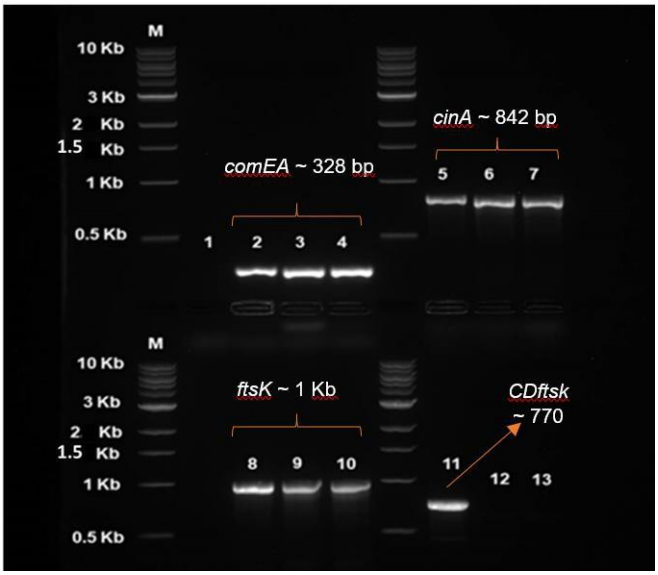


Figure 6.15 PCR amplification of the *comEA*, *cinA* and *ftsK* genes in *C. difficile*.

Lane 1: molecular biology grade water. Lane 2: *comEA* in 630Δ*erm*. Lane 3 and 4: *comEA* in CD37. Lane 5: *cinA* in 630Δ*erm*. Lane 6 and 7: *cinA* in CD37. Lane 8: *ftsK* in 630Δ*erm.* Lane 9 and 10: *ftsK* in CD37. Lane 11: *CDftsK* in 630Δ*erm.* Lane 12 and 13: *CDftsK* not found in CD37. Lane M: 1 Kb molecular ladder.

Table 6.9 Bioinformatic search for putative competence genes in strain 630.

Presence of *B. subtilis* competence related genes was investigated in CD630 using DNA sequence homology alignment algorithms such as BLASTp and BLASTn (the *comEA*, *cinA* and *ftsK* genes, presumably involved in DNA translocation processes in *C. difficile* are not shown in this table as they have been discussed above)

Gene	BLASTn	BLASTp	Description
comK	No similarity	No similarity	Competence transcription factor, ComK activated proteins that directly function in DNA uptake.
comS	No similarity	No similarity	Transporter protein, Regulation of competence
comG	No similarity	No similarity	Regulation of competence
comC	No similarity	type 4 prepilin peptide: 11% identity	type 4 prepilin-like proteins leader peptide-processing enzyme
comEB	No similarity	No similarity	Required for protein binding
comER	No similarity	No similarity	Transmembrane protein
comP	No similarity	1) 26% identity to sensor histidine kinase Clostridiales, *2)* 28% identity to sensor histidine kinase in *C. difficile* 3) 46% identity to 2-iminoacetate synthase ThiH [Clostridiales]	Sensor histidine kinase
comA	No similarity	33% identity to aminopeptidase P family protein [Clostridiales]	transcriptional regulatory protein

comQ	No similarity	No similarity	Competence regulatory protein
comB/yitC	No similarity	No similarity	2-phosphosulfolactate phosphatase
secA	No similarity	1) 74% identity to an uncharacterised protein in CD630 YccF 2) 61% identity to type I methionyl aminopeptidase [Clostridiales] 3) 51% identity to protein translocase subunit SecA 1 [Clostridiales] 4) 57% identity to ATP-dependent helicase [Clostridiales]	Cell membrane associated subunit of type II secretion system, protein translocase
comI	No similarity	100% identity to putative type IV prepilin peptidase [*Clostridioides difficile* 630]	Late competence inhibitor gene
comJ	Not Found	No similarity	Competence specific protein
yjbF	Not Found	No similarity	Competence-induced protein CoiA
comEC	No similarity	1) 22% identity to a membrane protein in *C. difficile* 2) 28% identity to MBL fold protein 3) 32% identity to MBL fold hydrolase in Clostridia	Competence protein

6.4.5.2. Mating experiment using *C. difficile* ClosTron-based mutants as recipients in the absence and presence of DNase

Successful retargeting of the intron was only achieved in the *comEA* and *cinA* genes. The ClosTron-based inactivation of the *ftsK* gene was unsuccessful despite four independent experiments. Genetic organisations of the target genes and intron targeting site are shown in figure 6.16. Since the Group II intron (used for ClosTron mutagenesis) and pMTL9301 both contain the *erm*(B) gene as a selective marker, chloramphenicol-resistant plasmid pRPF185 (Figure 6.17) (Table 6.1) was chosen as the shuttle vector to assess the role of *C. difficile* competence genes on plasmid transfer. *E. coli* strains CA434 and HB101 were both used as donor strains as using HB101 allows the role of R702 on pRPF185 transfer to be studied. Transconjugants were screened by PCR to confirm the transfer. Our results show that inactivation of the *comEA* or *cinA* has no effects on plasmid DNA transfer into *C. difficile*. Plasmid pRPF185 transfers from CA434 donor into *C. difficile* mutant strains at approximately the same frequency as into wild-type strains (10^{-4} transconjugants per donor). Moreover, incorporation of DNase into the mating mix prior to plating onto selective agar, did not impact the frequency of transfer. When *E. coli* strain HB101 was used as the donor, the plasmid transfer was not detectable (detection limit < 10^{-10}) (Table 6.10).

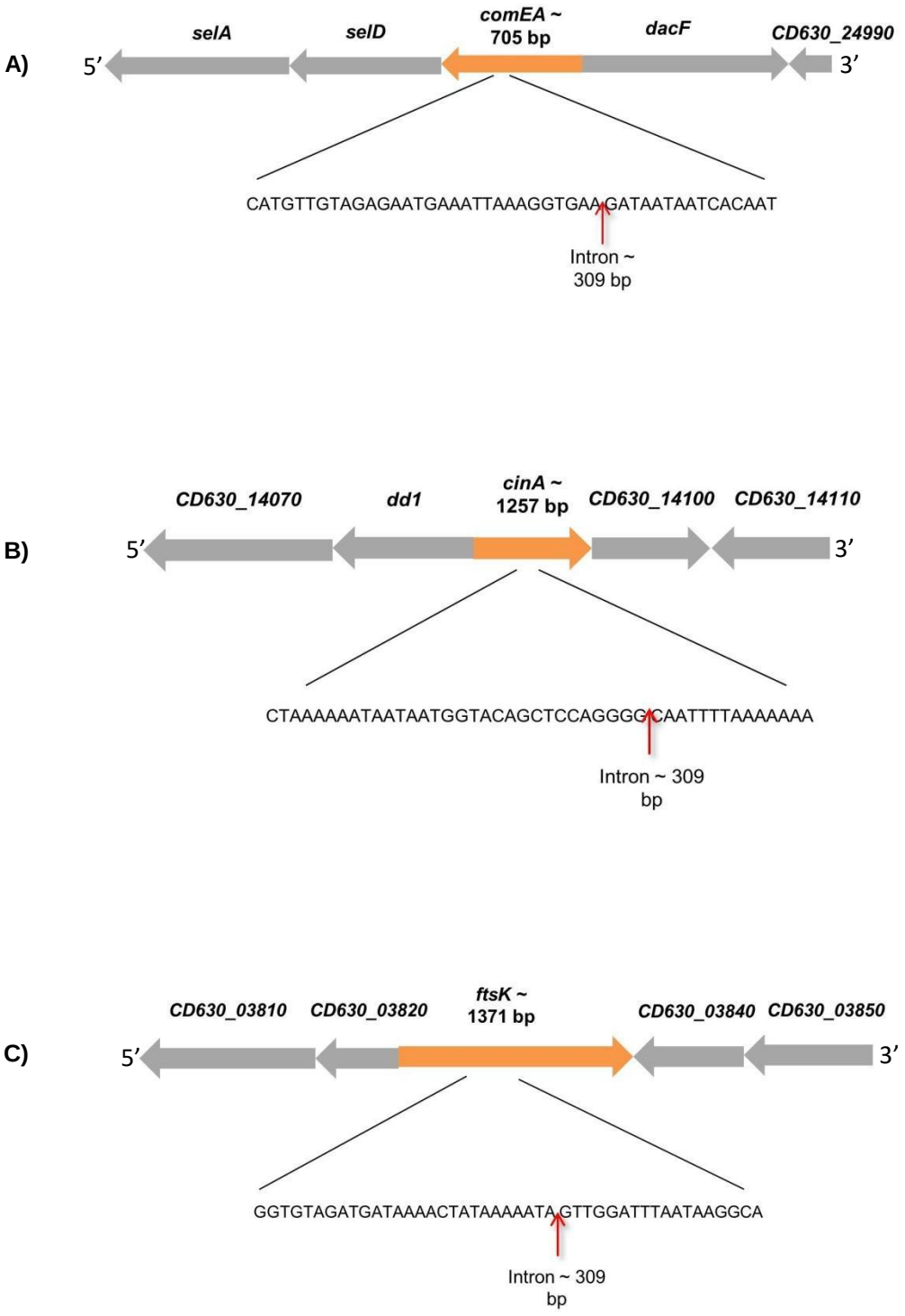


Figure 6.16 Genetic organisations of the target genes and intron targeting site.

ORFs that were ClosTron insertion targets are shown in orange, the integration site of the ClosTron constructs are indicated by red arrows. ORFs upstream and downstream of the target sites are shown in grey. **A)** ClosTron mutagenesis in the *comEA* gene. **B)** ClosTron mutagenesis in the *cinA* gene. **C)** ClosTron mutagenesis in the *ftsK* gene

Table 6.10 Frequency of pRPF185 and pMTL9301 (control) transfer into *C. difficile.*
±SD, standard deviation. ND, no transconjugants/ transformants

Donor	recipient	Frequency of transfer per donor	
		DNase (50 µg/ml)	No DNase
CA434 (pRPF185)	CD37	$6.25 \pm 1.1 \times 10^{-4}$	$7.5 \pm 1.1 \times 10^{-4}$
CA434 (pRPF185)	CD37::Δ*comEA*::ErmRAM	$7.3 \pm 2.5 \times 10^{-4}$	$6 \pm 2.3 \times 10^{-4}$
CA434 (pRPF185)	CD37::Δ*cinA*::ErmRAM	$5.8 \pm 1.8 \pm \times 10^{-4}$	$6.1 \pm 1.8 \times 10^{-4}$
CA434 (pRPF185)	630Δ*erm*	$3.7 \pm 1.2 \times 10^{-4}$	$4.3 \pm 1.4 \times 10^{-4}$
CA434 (pRPF185)	630Δ*erm*::Δ*comEA*::ErmRAM	$6.6 \pm 1.5 \times 10^{-4}$	$6.35 \pm 1.8 \times 10^{-4}$
CA434 (pRPF185)	630Δ*erm*:: Δ*cinA*::ErmRAM	$4.6 \pm 2.2 \times 10^{-4}$	$5.1 \pm 2.1 \times 10^{-4}$
HB101 (pRPF185)	CD37	ND	ND
HB101 (pRPF185)	CD37::Δ*comEA*::ErmRAM	ND	ND
HB101 (pRPF185)	CD37::Δ*cinA*::ErmRAM	ND	ND

HB101 (pRPF185)	630Δ*erm*	ND	ND
HB101 (pRPF185)	630Δ*erm*::Δ*comEA*::ErmR AM	ND	ND
HB101 (pRPF185)	630Δ*erm*:: Δ*cinA*::ErmRAM	ND	ND
CA434 (pMTL9301)	CD37	$2.64 \pm 2.5 \times 10^{-10}$	$3.3 \pm 4.2 \times 10^{-6}$
CA434 (pMTL9301)	630Δ*erm*	$3.8 \pm 2.8 \times 10^{-5}$	$1.7 \pm 0.8 \times 10^{-5}$
HB101 (pMTL9301)	CD37	$4 \pm 3 \times 10^{-10}$	$2.8 \pm 3.1 \times 10^{-10}$
HB101 (pMTL9301)	630Δ*erm*	$4.65 \pm 1.2 \times 10^{-10}$	$4.08 \pm 1.01 \times 10^{-10}$

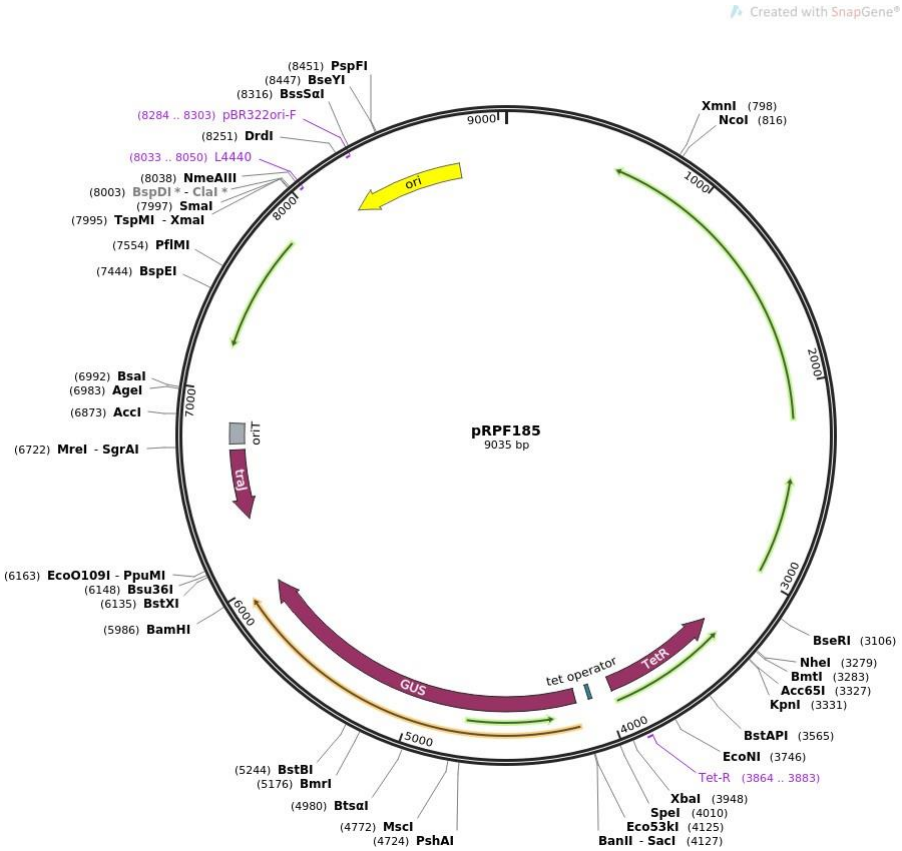


Figure 6.17 Schematic representation of plasmid pRPF185

(Adapted from (Fagan & Fairweather, 2011). *E. coli- C. difficile* shuttle vector with tetracycline-inducible promoter; P_{tet} ::*gusA,* Chloramphenicol resistance gene *catP*, *C. difficile* pCD6-derived origin of replication *oriV*, plasmid RP4-derived origin of transfer *oriT*, plasmid pMB1-derived *oriV* to replicate in *E. coli.*

6.4.6. Investigation of the potential influence of plasmid RK2 transfer genes on pMTL9301Δ*oriT* transfer into *C. difficile*

6.4.6.1. Bioinformatic search for the presence of *tra* and *trb* genes in *E. coli* strain RR1 (HB101 RecA$^+$) genome

An essential feature for conjugation is the presence of conjugation apparatus typically encoded by Type IV secretion system (described in chapter 1) in the donor cell to mediate the DNA transfer. In order to determine if the *tra* and *trb* genes of plasmid RK2 are present in HB101 the published sequence (Jeong *et al.*, 2017) was searched (Table 6.11). HB101 lacks almost the whole transfer (*tra*) operon of the conjugative plasmid RK2, but contains the *traX, traL, traK, traF, traH, traG, traM, traI* and *traJ* genes with 100% homology (except for the *traF* which has 18% homology) both at nucleotide and amino acid level. TraJ and TraI are responsible for the *oriT* region recognition and are crucial for the initiation of conjugation and relaxosome formation. TraM is also required for nicking double stranded DNA and initiating conjugation by signalling the relaxase that a stable mating pair is formed, and transfer can begin (Table 6.11).

None of the *trb* genes (except for the *trbA*) which is required for the mpf complex was present in strain RR1 (HB101 RecA$^+$). Therefore, according to the NCBI BLAST search results presented in table 6.11, there might be parts of the type IV secretion system present in *E. coli* RR1 (HB101 RecA$^+$) to facilitate the transfer of plasmid pMTL9301 into *C. difficile* strains, but some genes such as the *trb* genes (except for the *trbA*) and *traA* gene which have previously been shown to be essential for conjugation are missing.

Table 6.11 Bioinformatics search for transfer genes in *E. coli* strain HB101.

Transfer gene	BLASTn	BLASTp	Function
traA	No significant similarity	No significant similarity	Encodes the precursor of the pilus subunit prepilin
traQ	No significant similarity	No significant similarity	TraA maturation
traX	-Suicide vector pTEC (100%) -*E. coli* plasmid RP4 (100%)	Unnamed protein product (plasmid) (100%)	-TraA maturation -translational coupling protein (Very short product: 13 aa)
traL	*E. coli* plasmid RP4 (100%)	TraL (plasmid) (100%)	Pilus assembly
trak	-Suicide vector pTEC (100%) -*E. coli* plasmid (100%)	No significant similarity	-Putative helicase -DNA binding protein -*oriT* binding protein -Pilus assembly
traB	No significant similarity	No significant similarity	Pilus assembly
traV	Not found in database	Not found in database	Pilus assembly
traC	No significant similarity	No significant similarity	-DNA primase and ssDNA binding protein -Pilus assembly
traF	*E. coli* plasmid RP4 (18%)	No significant similarity	Mating pair formation
traH	*E. coli* plasmid RP4 (100%)	TraH (plasmid) (100%)	Relaxosome stabilisation

traG	*E. coli* plasmid RP4 (100%)	TraG (plasmid) (100%)	Putative DNA transporter during the conjugation
traM	*E. coli* plasmid RP4 (100%)	TraM (plasmid) (100%)	- Directly required for nicking dsDNA - Signals that a stable mating pair has formed, and transfer can begin
traD	No significant similarity	No significant similarity	Unknown
traI	*E. coli* plasmid RP4 (100%)	TraI (plasmid) (100%)	-TraI DNA relaxase -Has a central role in conjugation= Relaxase -Remains covalently linked to the 5' end of the nicked DNA during transfer and involved in relaxosome formation
traJ	Suicide vector pTEC (100%)	TraJ (plasmid)	*oriT* recognising protein
trbA	*E. coli* plasmid RP4 (100%), Query cover: 11%	No significant similarity	Global regulator encoded in the Tra2 region, it represses expression of transfer genes (*traJ, traK, traG, trfA, trbB*). TrbA is a repressor protein.
trbB	No significant similarity	No significant similarity	Mating pair formation, TrbB belongs to a family of bacterial surface/ membrane associated structures such as pili and transport system.

trbC	No significant similarity	No significant similarity	Mating pair formation
trbD	No significant similarity	No significant similarity	Mating pair formation
trbE	No significant similarity	No significant similarity	Mating pair formation
trbF	No significant similarity	No significant similarity	Mating pair formation, essential for transfer between *E. coli* strains
trbG	No significant similarity	No significant similarity	Mating pair formation
trbH	No significant similarity	No significant similarity	Mating pair formation
trbI	No significant similarity	No significant similarity	Mating pair formation
trbJ	No significant similarity	No significant similarity	Mating pair formation and entry exclusion
trbK	No significant similarity	No significant similarity	TrbK entry exclusion protein
trbL	No significant similarity	No significant similarity	-Mating pair formation -Putative topoisomerase
trbM	No significant similarity	No significant similarity	Mating pair formation
trbN	No significant similarity	No significant similarity	Mating pair formation
trbO	No significant similarity	No significant similarity	Not known
trbP	No significant similarity	No significant similarity	possible role in pilus assembly

6.4.6.2. PCR amplification of the transfer genes in *E. coli* RR1 (HB101 RecA$^+$)

To confirm that the HB101 strain used in thesis experiment contained a partial *tra* operon, PCR was performed using primers summarised in table 6.6. *E. coli* strain CA434 (HB101::RK2) and DH5α (F^-) were used as positive and negative controls, respectively. PCR was carried out on the genes which showed similarity to the reference sequence, RK2 (Table 6.11). These were *traG, traI, traJ, traF, traL, traM, traK* and *trbA*. Despite the bioinformatic results showing a partial presence of the *tra* operon in HB101, no *tra* (Figure 6.18 and 6.19) and *trb* genes (Figure 6.19) was amplified in HB101. Multiple PCR reactions were carried out using amended annealing temperatures and/or number of cycles to improve the PCR reaction. It is possible that these genes are still present but there was a PCR failure.

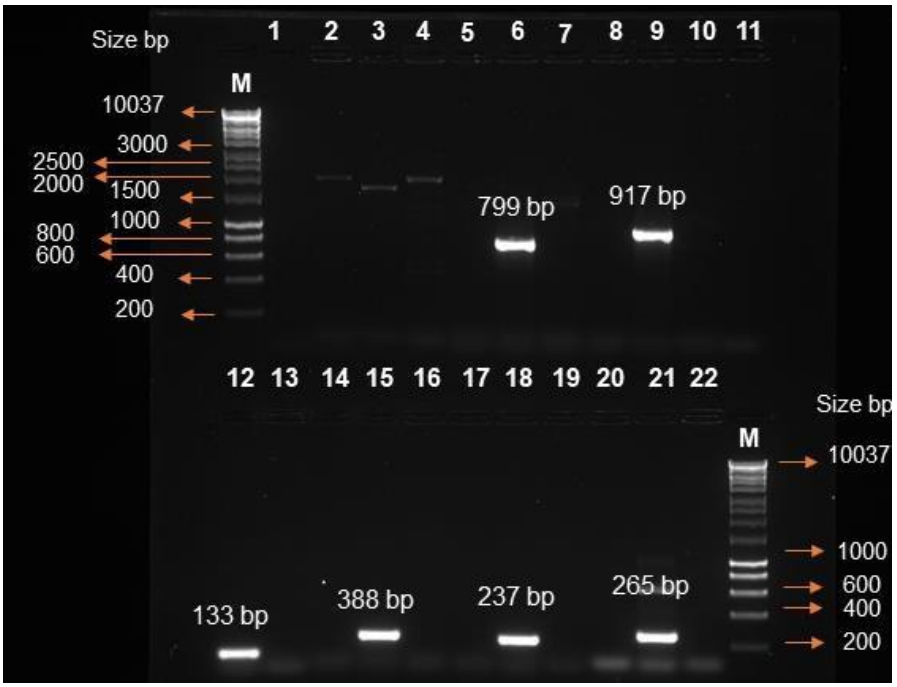


Figure 6.18 PCR amplification of the *tra* genes in *E. coli* strain HB101.

Lane 1: molecular biology grade water. Lanes 2, 3 and 4 are nonspecific amplification of the *traK* gene. PCR for this gene was repeated and results are shown in figure 6.21. Lane 5: *traG* in DH5α (-control), Lane 6: *traG* in CA434 (+control), Lane7: *traG* in HB101. Lane 8: *traI* in DH5α (-control), Lane 9: *traI* in CA434 (+control), Lane 10: *traI* in HB101. Lane 11: *traJ* in DH5α (-control), Lane 12: *traJ* in CA434 (+control), Lane 13: *traJ* in HB101. Lane 14: *traF* in DH5α (-control), Lane 15: *traF* in CA434 (+control), Lane 16: *traF* in HB101. Lane 17: *traL* in DH5α (-control), Lane 18: *traL* in CA434 (+control), Lane 19: *traL* in HB101. Lane 20: *traM* in DH5α (-control), Lane 21: *traM* in CA434 (+control), Lane 22: *traM* in HB101. Lane M: hyper molecular ladder. Sizes of the fragments are shown in the picture.

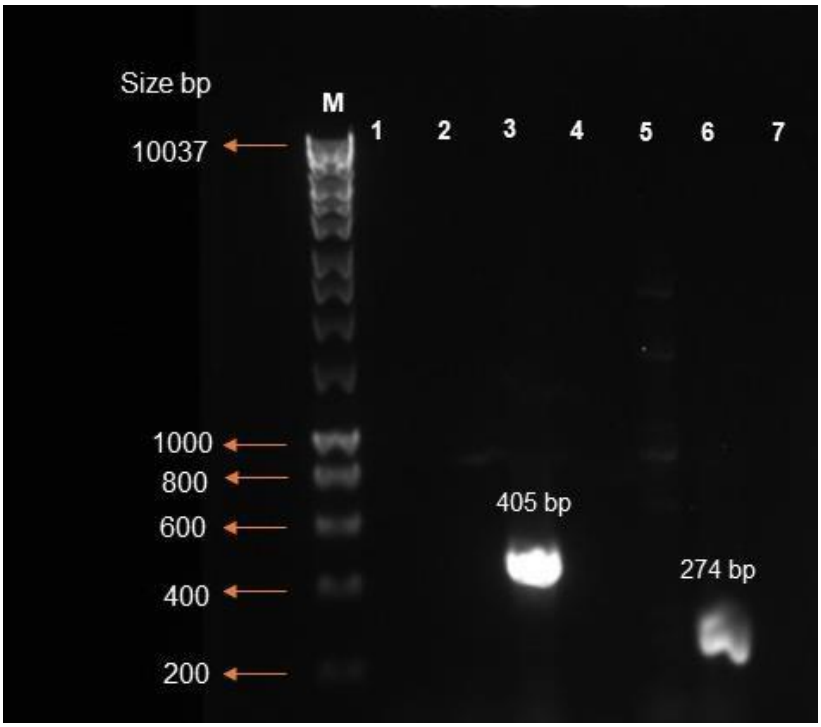


Figure 6.19 PCR amplification of the *tra*K and *trbA* in *E. coli* strain HB101.

Lane 1: molecular biology grade water. Lane 2: *traK* in DH5α (-control), Lane 3, *traK* in CA434 (+control), Lane 4, *traK* in HB101. Lane 5: *trbA* in DH5α (-control), Lane 6: *trbA* in CA434 (+control), Lane 7: *trbA* in HB101. Lane M: hyper molecular ladder. Sizes of the fragments are shown in the picture.

Table 6.12 Summary of the results. (**-**): No detectable transfer (below the detection limit= 10^{-10}). (**+++**): transfer (10^{-4}, 10^{-5}). (**+**): 10-times decrease in tramsfer frequency (10^{-10})

Donor	**Recipient**	**Plasmid**	**Variation in plasmid transfer frequency per donor under different conditions**	
			With DNase	**Without DNase**
CA434	CD37	pMTL9301	**+**	**+++**
CA434	CD37	pMTL9301Δ*oriT*	**-**	**+**
CA434	630Δ*erm*	pMTL9301	**+++**	**+++**
CA434	630Δ*erm*	pMTL9301Δ*oriT*	**+**	**+**
HB101	CD37	pMTL9301	**-**	**+**
HB101	CD37	pMTL9301Δ*oriT*	**-**	**-**
HB101	630Δ*erm*	pMTL9301	**-**	**+**
HB101	630Δ*erm*	pMTL9301Δ*oriT*	**-**	**+**
CA434	CD37	pRPF185	**+++**	**+++**
CA434	CD37::Δ*comEA*::ErmRAM	pRPF185	**+++**	**+++**
CA434	CD37::Δ*cinA*::ErmRAM	pRPF185	**+++**	**+++**
CA434	630Δ*erm*	pRPF185	**+++**	**+++**
CA434	630Δ*erm*::Δ*comEA*::ErmRAM	pRPF185	**+++**	**+++**
CA434	630Δ*erm*::Δ*cinA*::ErmRAM	pRPF185	**+++**	**+++**
HB101	CD37	pRPF185	**-**	**-**
HB101	CD37::Δ*comEA*::ErmRAM	pRPF185	**-**	**-**
HB101	CD37::Δ*cinA*::ErmRAM	pRPF185	**-**	**-**
HB101	630Δ*erm*	pRPF185	**-**	**-**
HB101	630Δ*erm*::Δ*comEA*::ErmRAM	pRPF185	**-**	**-**
HB101	630Δ*erm*::Δ*comEA*::ErmRAM	pRPF185	**-**	**-**

6.5. Discussion

So far, introduction of plasmid DNA into *C. difficile* has only been possible through conjugative transfer from a suitable donor bacterium. *E. coli* strain CA434 can be used to deliver plasmids to *C. difficile* by classical mating experiments (Purdy *et al.*, 2002). However, formal proof that the primary means of transfer is conjugation, i.e. DNase resistant transfer, has never been reported. Several key features are required for a successful plasmid transfer including a replicon for replication in *E. coli*, a replicon for replication in *C. difficile*, a selective marker, transfer proteins encoded by plasmid R702 and an origin of transfer (*oriT*) in the shuttle vector to be recognized and mobilised by R702 conjugative apparatus (Purdy *et al.*, 2002).

The results presented in this chapter and the observations from the previous chapter indicate that *C. difficile* can take up plasmid DNA by at least two mechanisms, a DNase resistant conjugation-like mechanism and a DNase sensitive mechanism. However, the DNase sensitive mechanism differs from most previously described transformation mechanisms since naked DNA alone is not sufficient to transform *C. difficile* and the presence of live donor cells containing the plasmid DNA is necessary.

Based on our results, it seems that in plate mixtures of *E. coli* CA434 and *C. difficile* CD37, a DNase sensitive mechanism and a conventional DNase resistant conjugation-like mechanism occur simultaneously. However, the frequencies of transfer are considerably different, as DNase treatment significantly reduces the frequency from 10^{-5}/ donor to 10^{-10}/ donor but does not completely abolish plasmid transfer. Furthermore, deletion of *oriT* reduces plasmid transfer frequencies in the absence of DNase but does not stop it. Low frequency transfer of pMTL9301Δ*oriT* to CD37 was completely sensitive to DNase as no transconjugant/ transformant appeared on

selective plates after 72 hours. A similar DNase sensitive mechanism has been observed when *C. difficile* donors containing Tn*6194* were mixed with *C. difficile* recipient strains CD37 and CD13 (Wasels *et al.*, 2015). In these experiments, the presence of live donor cells and cell-to-cell contacts between donors and recipients were essential for the transfer (Wasels *et al.*, 2015). Whether or not the transfer into *C. difficile* recipient still occurs without a recognisable *oriT* was not investigated in this study.

The results presented in this chapter show that the *oriT/ mob* region is not required for transfer of pMTL9301Δ*oriT* from *E. coli* CA434 into *C. difficile* 630Δ*erm* and the transfer occurs at the frequency of 10^{-10} per donor both in the absence and presence of DNase. It has previously been shown that plasmids pC194, pBS42 and pHP13, all lacking an obvious *oriT/ mob* region can still be mobilised by an integrative and conjugative element ICE*Bs1* at high frequencies (~ 0.07%-3% plasmid containing transconjugants/ donor) from *B. subtilis* donor (Lee *et al.*, 2012). These workers have hypothesized that the plasmid replicative relaxase is required for plasmid transfer and it may function both in replication and transfer. In this process, the coupling protein encoded by ICE*Bs1* binds to the replicative relaxosome encoded by the element and guides it towards the ICE conjugation machinery (Lee *et al.*, 2012). It is possible that the conjugative plasmid R702 present in strain CA434 recruits the replicative relaxosome encoded by pMTL9301Δ*oriT* and mediates a similar interaction to guide this plasmid towards the conjugation apparatus. This finding shows that the possible interaction between conjugative elements with replicative relaxase as well as conjugative relaxase might lead to the dissemination of antibiotic resistance and other genes.

In this chapter, it has also been confirmed that transfer of Tn*5397* and Tn*916* from *B. subtilis* into *C. difficile* is completely resistant to DNase since the addition of DNase did not affect the frequencies of conjugation. What's more, it has previously been shown that transfer of the PaLoc from *C. difficile* toxigenic strains into nontoxigenic strains is also DNase resistant suggesting that PaLoc transfers via a conjugation-like mechanism (Brouwer *et al.*, 2013).

In order to study the effects of putative competence proteins encoded by *C. difficile*, ClosTron mutants were made in the *cinA* and *comEA* genes. Although several attempts were made to inactivate the *ftsK* gene, no mutant was generated. The mutant strains were used as recipients of pRPF185 (Cm^r, Tm^r) mobilisable plasmid from *E. coli* donors CA434 and HB101. Experiments were carried out in the absence and presence of DNase. It has been observed that the competence genes in *C. difficile* may be employed in the transformation-like mechanism observed in this work, as the transfer of pRPF185 into *C. difficile* mutants was not stopped. The frequency of pRPF185 transfer into *C. difficile* was higher than pMTL9301 (Table 6.10), further demonstrating that acquisition of genetic elements by *C. difficile* can be influenced by the genetic element itself as well as donor and recipient. Furthermore, It is possible that unlike pMTL9301, pRPF185 transfer was resistant to DNase. Plasmid pRPF185 contains the *traJ* gene encoding an *oriT*-recognizing protein essential for initiation of conjugation (Figure 6.17). It is likely that the presence of this gene led to the higher frequency of pRPF185 transfer compared to pMTL9301 (Table 6.10).

Work by (Wang *et al.*, 2007) has shown that a non-conjugative plasmid can be transferred from *E. coli* strain HB101 into *B. subtilis* via a DNase sensitive mechanism, but the transfer was not abolished by DNase. They hypothesized that DNA was protected from degrading activity of DNase by solid agar. A similar observation was

obtained throughout our study, in which plasmid transfer occurred from HB101 donor into *C. difficile* recipient. In addition to the hypothesis proposed by Wang *et al.,* (2007), it is possible that the partial *tra* operon in HB101 (Table 6.11) mediates the formation of a mating bridge that is accessible by DNase.

In order to further investigate the DNase sensitive *oriT*-independent plasmid transfer into *C. difficile*, *E. coli* strain HB101 was used as a donor strain to determine if R702 is required for the transfer process. Our results show that both pMTL9301and pMTL9301Δ*oriT* transfer from *E. coli* HB101 donor into CD37 and 630Δ*erm* at a lower frequency (~ 10^{-10} per donor) compared with when strain CA434 was used as a donor (~ 10^{-5} per donor). Owing to the fact that plasmid transfer from HB101 is completely DNase sensitive and does not require an obvious *oriT* (there is no difference in the frequency of transfer of pMTL9301 and pMTL9301Δ*oriT*), it indicates that a transformation-like mechanism may being employed by *C. difficile* to take up plasmids from HB101 donor cells. Moreover, the fact that the transfer frequency is much lower from HB101 than from CA434 indicates that there might be more than one mechanism in *C. difficile* to acquire plasmid DNA from the donor cells, but one is functioning more efficiently than the other.

A BLAST search of the HB101 genome sequence (https://www.ncbi.nlm.nih.gov/nuccore/CP011113) (Jeong *et al.*, 2017) against RK2 sequence (https://www.ncbi.nlm.nih.gov/nuccore/BN000925.1) revealed that this strain contains some *tra* genes; however, PCR results revealed that none of these genes including TraF-encoding gene (responsible for mating pair formation), TraM-encoding gene (responsible for sending signals to the relaxase to initiate the conjugation) and TraJ-encoding gene (responsible for dsDNA relaxation), all essential for conjugation are present in this strain (Figure 6.17).

Moreover, none of the *trb* genes was found in the HB101 genome (Figure 6.18). The *trb* genes are known to be involved in mating pair formation and mating bridge construction (Zatyka & Thomas, 1998). It is possible that the *tra* genes in HB101 mediate the formation of a mating pair which is accessible by DNase and not effectively protecting the transferring plasmid DNA. Thus, the mating bridge formed by HB101 is more porous than that made by RK2 since HB101 lacks part of the *tra* operon.

An alternative is that the DNase sensitive *oriT*-independent plasmid transfer into *C. difficile* is via a transformation-like mechanism which requires close contact between donor and recipient. For example, horizontal gene transfer in *Thermus thermophilus* does not fit into the conventional definition of transformation and/or conjugation. In this species, a large DNA fragment can be transferred through a novel mechanism which requires the presence of both donor and recipient and needs genes from the natural transformation machinery since mutation in some of these genes stops DNA transfer in a DNase resistant *oriT* independent manner. It has been suggested that donor cell pushes the DNA from the donor in a conjugation-like mechanism and recipient pulls the DNA inside the cell using components of the natural transformation system (Blesa *et al.*, 2015). It is possible that a similar system is present in *C. difficile* in which the *E. coli* donor is secreting DNA or the lysed sub-population of *E. coli* releases DNA into the environment. The secreted DNA is subsequently pulled by the *C. difficile* competence system in a DNase sensitive manner.

Etchuuya *et al.*, (2011) have reported a similar system of gene transfer between *E. coli* strains in which free DNA was not sufficient for transformation and the presence of live donor cells was required. This system was shown to be DNase sensitive and termed ‘cell-to-cell’ transformation. These workers determined that a polypeptide was released from the donor cells to promote cell-to-cell transformation by acting as a

pheromone-like component of the transformation mechanism. Another similar system has been observed in the transfer of pAPR8-1 (Ap^r, Km^r); a non-conjugative shuttle vector from *E. coli* to *B. subtilis* on solid agar. This system was shown to be DNase sensitive and required close contact between donor and recipient. It was also shown that adding *E. coli* donor without plasmid, *E. coli* supernatant liquid and cell-free filtrate into a mixture of recipient and free plasmid led to a 10-fold increase in the transformation frequency. However, when diluted donor culture was used, transformation was reduced or abolished. Therefore, it was hypothesized that the *E. coli* donor had some stimulating effect on enhancing the transformation ability of *B. subtilis* (Wang *et al.*, 2007).

The results presented in this chapter highlight the extraordinary ability of *C. difficile* to receive new DNA from the environment. The surprising observation that it can take up plasmid DNA from an unrelated donor such as *E. coli* strain HB101 with an incomplete conjugation apparatus and only some of the *tra* genes or without a *cis*-acting *oriT* (although *oriV*-encoded relaxase may be able to act as a conjugative relaxase) indicate that *C. difficile* has a remarkable capability to acquire almost any DNA sequence. Factors such as the genetic element itself and the type of donor and/or recipient strain influence the gene transfer process in *C. difficile*; presumably the only restricting factors are the ability of incoming DNA to replicate or be incorporated into the *C. difficile* genome.

Chapter 7 General discussion and future work

A ‘novel cell-to-cell transformation-like’ mechanism in *C. difficile* has been discovered in this study. The results generated here have a considerable impact on the understanding of *C. difficile* biology, both at the biochemical and molecular levels.

The data presented in this thesis show that *C. difficile* can detect the presence of pancreatic α-amylase and respond by secretion of EPS. This response resulted in a change in colony morphology to a mucoid phenotype through the secretion of EPS which includes the secretion of proteins and carbohydrates. Bacteria such as *Burkholderia cepacia* (Bartholdson *et al.*, 2008), *Enterococcus faecalis* (Bottone *et al.*, 1998), *Streptococcus pneumoniae* (Allegrucci & Sauer, 2007) and *Pseudomonas aeruginosa* (Friedl *et al.*, 1992), all have the ability to express mucoidy through secretion of an EPS. These EPS can consist of polysaccharides, extracellular proteins, DNA or lipids and in some cases, they can be a combination of some or even all of these molecules (Costerton *et al.*, 1999).

With the discovery that pancreatic α-amylase-induced EPS in *C. difficile* consists of proteins and carbohydrates, it would be advantageous to assess whether other macromolecules such as lipids and DNA are also increased in quantity. This, in turn, would help to characterise the link between major metabolic pathways in this organism and the presence of environmental stress factors.

Furthermore, the molecular mechanism behind the recognition of pancreatic α-amylase and elucidating the signal transduction pathway need to be investigated. This could be done through screening a *C. difficile* transposon mutant library on BHI agar supplemented with and without pancreatic α-amylase to check for colonies not showing mucoidy in response to α-amylase. A library could be constructed using a *mariner* transposon-based system which has a recognition sequence of TA and would

provide a good degree of genome coverage; it has previously been demonstrated to produce random *in vivo* transposon mutagenesis of *C. difficile* R20291 (Cartman & Minton, 2010, Dembek *et al.*, 2015).

Secretion of EPS is a prerequisite to biofilm formation and has been observed in a number of enteric bacteria such as *Helicobacter pylori* (Stark *et al.*, 1999), *Salmonella typhimurium* (Ledeboer & Jones, 2005) and *Campylobacter jejuni* (Jowiya *et al.*, 2015). However, our results demonstrate that *C. difficile* pre-exposed to α-amylase and thus expressing EPS exhibits significantly decreased biofilm formation on abiotic surfaces. These results could be explained by enzymatic activity of α-amylase to hydrolyse glycosidic linkages of the monosaccharides present in the EPS. Anti-biofilm activity of amylase has been reported in a number of bacteria such as *Bacillus subtilis* (Kalpana *et al.*, 2012), *Bacillus cereus* (Vaikundamoorthy *et al.*, 2018) and *Klebsiella pneumoniae* (Mohamed *et al.*, 2018).

An alternative possibility is that α-amylase acts as a biosurfactant that reduces surface tension between individual molecules of the biofilm at the surface and inhibits the initial attachment stage of *C. difficile* biofilm formation. The latter hypothesis could be tested by drop collapse test (Jain *et al.*, 1991), oil spreading assay (Morikawa *et al.*, 2000), emulsification assay (Rosenberg *et al.*, 1979) and surface tension measurement using a tensiometer. Since the bacteria within the biofilms are less susceptible to antimicrobials, the anti-biofilm feature of α-amylase (either as a biosurfactant or polysaccharide degrading enzyme) would lead to the possibility of incorporating such enzymes into treatment of *C. difficile* infection to prevent the biofilm formation on the surface of epithelial cells.

Transfer of genetic elements from one bacterium to another via conjugation requires the element to pass through the cell membranes of both donor and recipient, and therefore it will be influenced by any structural changes of the cell surface. It was hypothesised that α-amylase-induced overexpression of exopolymers may have effects on horizontal gene transfer in *C. difficile*. This was tested by a set of mating experiments to introduce Tn*5397* or pMTL9301 into *C. difficile* CD37 in the absence and presence of pancreatic α-amylase. By also adding DNase I to the mating mixtures, we could eliminate transformation.

The frequency of Tn*5397* transfer was shown to be increased by two orders of magnitude in the presence of α-amylase. Stress-induced increase in conjugal transfer of Tn*916* has been observed in the presence of ethanol, tetracycline and non-tetracycline ribosome targeting antibiotics (Seier-Petersen *et al.*, 2014, Scornec *et al.*, 2017). It is possible that any stress that the cell encounters which results in the accumulation of charged tRNA is also likely to cause an increase in the transcription of *tet*(M) and downstream genes and possibly an increase in transfer.

Unlike Tn*5397*, pMTL9301 transfer was not affected by α-amylase, but it was significantly decreased by DNase. It is likely that this artificial plasmid does not carry regulatory regions to detect and respond to the stress factors. The results of this study have shown that the response of horizontal gene transfer to the external stress factors varies depending on the mobile element itself and the nature of the stress-inducing molecule. More investigations are needed to understand these variations and the molecular basis behind the increased frequency of Tn*5397* transfer from *B. subtilis*.

A deep knowledge of stressed-induced horizontal gene transfer would be beneficial in the development of future control measures for CDI by lowering the likelihood that

horizontally transferred antibiotic resistance or virulence genes end up in the genome of *C. difficile*. Additionally, determining signal transduction pathways in *C. difficile* responsible for the detection and response to HGT-inducing stress factors would improve our knowledge of *C. difficile* biology, in particular with respect to the molecular basis of such responses.

The significant reduction in plasmid transfer frequency into *C. difficile* CD37 in the presence of DNase was further investigated by construction of pMTL9301Δ*oriT*. Deletion of the *oriT* from pMTL9301 did not abolish transfer from *E. coli* strain CA434 to *C. difficile* 630Δ*erm* but did abolish transfer to CD37 in the presence of DNase. In the absence of DNase, the transfer of pMTL9301 and pMTL9301Δ*oriT* still occurred from *E. coli* strain HB101 donor that lacks the helper plasmid.

The data presented in this thesis, for the first time, show that plasmids transfer into *C. difficile* both by a DNase resistant conjugation-like mechanism and a DNase sensitive transformation-like mechanism. However, the latter is not like most previously described transformation mechanisms in that naked DNA is not sufficient for transfer and live donor cells are required. More work is required to determine why transfer of some genetic elements is sensitive to DNase, whereas the transfer of other genetic elements is not, but this is likely to depend on both the elements themselves and the donor and recipient strains. A detailed molecular analysis is required to analyse the 'novel cell-to-cell transformation-like mechanism' observed in *C. difficile*. This could be done using efficient genetic tools such as CRISPR/ cas9 technology to completely remove the conjugative transfer (*tra*) genes from the helper plasmid RK2.

The bioinformatics study here of the sequenced genome of HB101 resulted in the discovery of a partial T4SS in this strain. It was expected that these genes would

encode an incomplete T4SS enough to mediate transfer although at a low frequency. However, none of these genes were amplified by standard PCR from the HB101 strain genomic DNA which we used for our experiments. Using the same reference strain subjected to whole genome sequencing as a donor and inactivating the *tra* genes, would help to determine whether an incomplete T4SS mediates the transfer of wild-type and mutant plasmids into *C. difficile*.

The data from this PhD also establish that non-conjugative, non-mobilisable plasmids can still be taken up by an organism that was previously thought not to be naturally competent, and it is important to determine how common this phenomenon is in nature. Furthermore, an intact *oriT* is not required for transfer. As MGEs are responsible for the spread of antibiotic resistance genes, the continued research on the molecular basis of their mechanism of transfer is essential, as it will provide an insight into the events, that lead to their dissemination. If we fully understand the mechanisms by which *C. difficile* acquires resistance, then we may be able to come up with strategies to halt the antibiotic resistance crisis.

To conclude, we have identified a novel transformation-like mechanism in *C. difficile* which unlike the conventional transformation mechanism, requires the presence of live donors. A limitation of this work is that we don't know the prevalence of this phenomenon in the mammalian gut. Further works should be undertaken to survey its prevalence in various niches, to determine how common this mechanism of gene transfer is and its possible clinical relevance for treating CDI.

Future work

To explain the DNase-sensitive *oriT*-independent plasmid transfer into *C. difficile*, two hypotheses have been developed. Hypothesis 1. To explain the observation that some ICE (Wasels *et al.,* 2015) and a shuttle plasmid (this study) sometimes transfer via a DNase sensitive transformation- like mechanism, we propose that only a partial *tra* operon is required in the donor and that the recipient requires a partial competence system. *C. difficile* encodes homologues of some of the proteins required for transformation, namely ComEA (79% at the amino acid level), CinA part of the type IV pilus assembly, FtsK and RecA (section 6.4.5). We shall delete the *comEA* homologue and each of the genes that is predicted to interact with it. In addition, the CinA encoding homologue, FtsK and RecA will be deleted. In-frame deletions will be made so that the expression of neighbouring genes is not affected. This has recently been shown to be possible using CRISPR editing technology (McAllister 2017). The ability of the mutants to act as recipients with donors containing ICEs (both ones whose transfer is DNase sensitive and resistant) or shuttle plasmids will be tested. Selected transconjugants will be subjected to whole genome sequencing (WGS) to determine if just the mobile element under investigation is transferred or if other parts of the chromosome are also transferred. Moreover, we shall delete the remaining *tra* genes from HB101 and then test the ability of this mutant strain to transfer the plasmid into *C. difficile*. If a partial *tra* system is required, this will abolish transfer from this strain into *C. difficile*.

Hypothesis 2. To explain the observation that an origin of transfer is not required for shuttle plasmid transfer we propose that the origin of replication can be used as a substitute transfer origin or that double stranded DNA is being transferred and an origin is not required. If the *oriV* is being used as an *oriT*, then a *tra* system would be required in the donor and there should be no requirement for *com* genes in the recipient. This will

be tested by deleting the *oriT* and determining if the plasmids can still transfer. If the alternative hypothesis is correct (i.e., double stranded DNA is transferred) we would expect that there would be no transfer in the *com* mutants (as presumably the *com* homologue would be required to take up the plasmid) but that transfer would still be possible from a donor lacking all the *tra* genes particularly the *mob* gene encoding the protein required for nicking, as there would be no requirement for nicking the plasmid prior to transfer. To test if double stranded DNA is transferred, we will make use of the differential sensitivity to the *Sal*I restriction-modification system of double and single stranded DNA. That is if single stranded DNA is transferred to a strain expressing *Sal*I, it will be resistant to the enzyme, whereas double stranded DNA will be digested.

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Appendices

Appendix 1. Composition of basal defined medium (BDM)

Component	(mg)
Vitamins	
Thiamine	1
Calcium-D-pantothenate	1
Nicotinamide	1
Riboflavin	1
Pyridoxine	1
Aminobenzoic acid	0.05
Folic acid	0.0125
Biotin	0.0125
B_{12}	0.05
Minerals	
KH_2PO_4	300
Na_2HPO_4	1500
NaCl	900
$CaCl_2$	26
$MgCl_2$	20
$MnCl_2$	10
$(NH_4)_2 SO_4$	40
$FeSO_4$	4
$CoCl_2$	1
$NaHCO_3$	5000
Glucose	2000
Amino acids	
MEM NEAA, Minimum Essential medium (100X) (Gibro, life technologies)	1X
Non-essential amino acids (50X) (Gibr, life technologies)	1X
Distilled water (ml)	to 1000 ml

Appendix 2. Purity of hog pancreatic α-amylase preparation

Preparations of hog pancreatic α-amylase (Sigma cat no. 10080) dissolved in dH_2O at concentration of 1 mg/ml were analysed by Dr Karen Homer (Kings College). Samples were run on SDS-PAGE gel (Figure A.2)., the gel profile shown is indicative of those seen in three independent experiments. The bands were cut and analysed using LC-MS/MS and the hits are listed according to the UniprotKB/SwissProt database from EBI. The data showed only pancreatic α-amylase as the sole protein present in the sample (Table A.2).

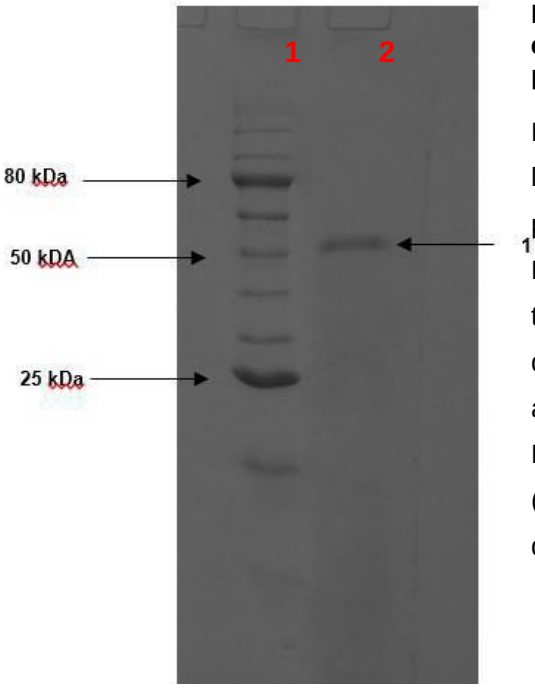

Figure A.2 SDS-PAGE analysis to confirm the purity of pancreatic α-amylase

Lane 1: 10-250 kDa protein ladder (NEB). Lane 2: hog pancreatic α- amylase. Bands were excised from the SDS-PAGE gel shown, digested in-gel with trypsin and analysed by LC-MS/MS. (UniprotKB/SwissProt database

Band No.[1]	Accession number[2]	Protein identity[3]	Peptides matched[4]	Coverage[5]	MW (kDa)[6]
1	P00690	Pancreatic α-amylase	6	40	57.1

Table A.2 Identity of protein present in hog pancreatic α-amylase preparation (Sigma-Aldrich) (Jowiya *et al.,* 2015).

[1] Band number as shown on the SDS PAGE gel in Figure A 1.1.

[2, 3] Accession number and protein identity as given in the the UniprotKB/SwissProt database from EBI.[4] Number of peptides observed in mass spectra matching to the protein in the database.

[5] Percentage amino acid coverage (peptides observed/theoretical from sequence data).

[6] Theoretical molecular weight (kDa)

Appendix 3. Publication resulting from this study

Khodadoost L, Hussain H and Mullany P (2017) Plasmids can transfer to *Clostridium difficile* CD37 and 630Δ*erm* both by a DNase resistant conjugation-like mechanism and a DNase sensitive mechanism. *FEMS* 364: fnx208