





Understanding Certain *Clostridium difficile* Virulence and Antibiotic Resistance Factors and How These Relate to Patient Clinical Outcome

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DECLARATION

Except where otherwise acknowledged, the work presented in this thesis is my own. No other part has been submitted for another degree at The University of Nottingham or any other institute of learning.

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ABSTRACT

Clostridium difficile is known for causing the nosocomial diarrhoeal disease, *C. difficile* infection (CDI). The aim of this study was to investigate the molecular and phenotypic basis of certain *C. difficile* virulence factors, such as *C. difficile* transferase (CDT) and sporulation, as well as rifaximin antibiotic resistance, and how these relate to patient clinical outcome.

The molecular basis of CDT was studied by creating in-frame deletion mutants (R20291Δ*cdtR*, R20291Δ*cdtA*Δ*cdtB*, R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*, R20291ΔPaLoc, R20291ΔPaLocΔ*cdtR*, R20291ΔPaLocΔ*cdtA*Δ*cdtB* and R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB*) via allelic exchange gene knock-out. Transcriptional linkage of the binary toxin gene locus by reverse transcriptase (RT) PCR, and *in silico* promoter analysis was completed. The investigation identified promoters upstream of the *cdtR* and *cdtA* genes, however, the locus was also discovered to be transcriptionally linked. This knowledge was used to create complementations of the CDT in-frame deletion mutant strains (R20291Δ*cdtR***cdtR* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB*) using a two-step method. All generated strains were subsequently characterised for differences in growth, cytotoxicity and biofilm formation. A key outcome from this characterisation was that the Vero cell cytotoxic effect in all CDT knock-out strains were significantly decreased when compared to the R20291 wildtype (WT) strain, despite the presence of the PaLoc.

Part of the research was completed in conjunction with the Nottingham Digestive Diseases Biomedical Research Unit (NDDBRU) on the Rifaximin for Preventing Relapse of *Clostridium* Associated Diarrhoea (RAPID) clinical trial. *C. difficile* was isolated from patient stool samples and ribotyped. A CDT PCR was established to amplify and sequence the CDT locus from patient isolates and then compared to known strains. This analysis revealed that the *cdtR* in ribotype 078 strains possess a premature stop codon, and in clinical strains containing the ghost CDT gene locus, the *cdtR* is well conserved. Further work is necessary to determine the significance of these findings to patient outcome. One of the most interesting outcomes of the RAPID clinical trial was the development of rifaximin resistance in five patients. Four single

nucleotide polymorphisms (SNPs) (Gln489Lys, Arg505Lys, Ser507Leu and Ile750Met) were determined in eight isolates. Lastly, sporulation was investigated in the clinical samples with the colony-forming units per ml (cfu/ml) shown to be increased at later time points. Concurrently, a ClosTron mutant was created to knock out the *C. difficile* 630 gene 26350, which encodes for a putative spore envelope protein. A sporulation assay demonstrated that the cfu/ml was reduced when compared to the control strain, indicating a potential important role in sporulation. However, the exact mechanism and identification of numerous genes which influence sporulation, remain unknown.

The precise role of certain *C. difficile* virulence and antibiotic resistance factors, and how these relate to patient outcome, is yet to be completely alluded to. However, the work presented in this study has demonstrated that the CDT gene locus, and indeed the ghost locus, are significant. It also indicates in certain individuals that exposure to rifaximin increases the risk of developing resistance. Nonetheless, many questions remain about the binary toxin and the synergistic effect with toxin A and B, the effect on colonisation and adherence, and the impact on the immune response. It is also necessary to research strain and host specific interactions before recommending rifaximin as appropriate treatment for the prevention of *C. difficile* relapse. Furthering knowledge on certain virulence factors will ultimately lead to improved diagnostics, therapeutic care and prevention/eradication measures.

PUBLICATIONS

Bilverstone, T. W., Kinsmore, N. L., Minton, N. P. and Kuehne, S. A. (2017) 'Development of *Clostridium difficile* R20291DeltaPaLoc model strains and in vitro methodologies reveals CdtR is required for the production of CDT to cytotoxic levels', *Anaerobe*, 44, pp. 51-54.

Rifaximin for Preventing Relapse of *Clostridium difficile* Associated Diarrhoea (RAPID) Clinical Trial (working draft).

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With regards to *Clostridium difficile* mutant generation, fellow Ph.D. student Terry Bilverstone contributed the following to this project:

1. The design and construction of the PaLoc knock-out cassette and associated screening primers.
2. The construction of the R20291Δ*pyrE*ΔPaLocΔ*cdtR* and R20291ΔPaLocΔ*cdtR* control strains.
3. The design and construction of the *cdtR* complementation cassette and associated screening primers.

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GLOSSARY/ABBREVIATIONS

A	Absorbance
AAD	Antibiotic-associated diarrhoea
ACE	Allele-coupled exchange
ADP	Adenosine diphosphate
Agr	Accessory gene regulator
BHIS	Brain heart infusion
BLAST	Basic Local Alignment Search Tool
BPROM	Prediction of Bacterial Promoters
CA-CDI	Community-associated *Clostridium difficile* infection
CaDPA	Calcium dipicolinic acid
CBP	Collagen-binding protein
CCEY	Cefoxitin-cycloserine egg yolk agar selective agar
CcpA	Catabolite control protein A
CDAD	*Clostridium difficile* associated disease
CDI	*Clostridium difficile* infection
c-di-GMP	Cyclic diguanosine monophosphate
CDMM	*Clostridium difficile* minimal media
cDNA	Complementary deoxyribonucleic acid polymerase
CDRN	*Clostridium difficile* Ribotyping Network
CDT	*Clostridium difficile* transferase (binary toxin)
CdtA	*Clostridium difficile* transferase enzymatic component
CdtB	*Clostridium difficile* transferase binding component

CdtLoc	*Clostridium difficile* transferase locus
CdtR	*Clostridium difficile* transferase regulator
cfu	Colony forming units
Chr	Chromosome
CLSI	Clinical and Laboratory Standards Institute
C_P	Crossing point
CRISPR	Clustered regularly interspaced short palindromic repeats
CSPG4	Chondroitin sulfate proteoglycan 4
C_T	Threshold cycle
CTaR	Chromosomal transfer and recombination
CTn	Conjugative transposon
CWP	Cell wall protein
Cyp	Cyclophilin
DCO	Double cross-over
dH_2O	Distilled water
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAP	Deoxyribonucleic acid polymerase
DNP	Double nucleotide polymorphism
DoH	Department of Health
ECM	Extracellular matrix
EIA	Enzyme immunoassay

ESCMID	European Society of Clinical Microbiology and Infectious Diseases
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAA	Fastidious Anaerobic Agar
FBP	Fibronectin-binding protein
FMT	Faecal microbiota transplant
5-FOA	5-fluoroorotic acid
FZD	Frizzled
GDH	Glutamate dehydrogenase
GTP	Trimeric G Protein
H2RA	Histomine-2 receptor antagonist
HCA-CDI	Healthcare-associated *Clostridium difficile* infection
HCl	Hydrogen chloride
HIV	Human immunodeficiency virus
HMW	High molecular weight
HSCT	Hematopoietic stem cell transplantation
Hsp	Heat shock protein
IBD	Inflammatory bowel disease
IDSA	Infectious Diseases Society of America
Ig	Immunoglobulin
IGS	Intergenic spacer
IJSEM	Internal Journal of Systematic and Evolutionary Microbiology
IL	Interleukin

InsP6	Inositol hexakisphosphate
IVIG	Intravenous immunoglobulin
LDF	Linear discriminant function
LHA	Left-hand homology arm
LMW	Low molecular weight
LSR	Lipolysis-stimulated lipoprotein receptor
MCS	Multiple cloning site
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
MOPS	4-Morpholinepropanesulfonic acid
MRSA	Methicillin resistant *Staphylococcus aureus*
NAAT	Nucleic acid amplification test
NaAc	Sodium acetate
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NDDCBRU	Nottingham Digestive Diseases Centre Biomedical Research Unit
NEB	New England BioLabs
NIHR	National Institute for Health Research
NPC	No protein control
NTP	Nucleoside triphosphate
OD	Optical density
ORF	Open reading frame

PaLoc	Pathogenicity locus
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulse-field gel electrophoresis
PFR	Pore-forming region
PHE	Public Health England
PLA	Proximity ligation assay
PMC	Pseudomembranous colitis
PPE	Personal protective equipment
PPI	Proton pump inhibitor
PPEP	Pro-Pro endopeptidase
PR	Promoter region
QMC	Queen's Medical Centre
qPCR	Quantitative polymerase chain reaction
RAM	Retrotransposition-activated marker
RAPID	Rifaximin for Preventing Relapse of *Clostridium difficile* Associated Diarrhoea
REA	Restriction endonuclease analysis
RHA	Right-hand homology arm
RNAP	Ribonucleic acid polymerase
RNP	Ribonuclear protein
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction

SBRC	Synthetic Biology Research Centre
SCLE	Spore cortex lytic enzyme
SCO	Single cross-over
SD	Standard deviation
SHEA	Society for Healthcare Epidemiology of America
S-layer	Cell surface layer
SLP	Cell surface layer protein
SNP	Single nucleotide polymorphism
SOC	Super optimal with catabolite repression
SOEing	Splicing by overlap extension
TB	Tuberculosis
TcdA	*Clostridium difficile* toxin A
TcdB	*Clostridium difficile* toxin B
TEM	Transmission electron microscope/microscopy
Th1	Type 1 T helper
TLR	Toll-like receptor
Tm	Melting temperature
TSSP	Transcription start site position
TY	Tryptose yeast
UDP	Uridine diphosphate
UK	United Kingdom
US	United States
UTI	Urinary tract infection

VRE	Vancomycin-resistant enterococci
WGS	Whole genome sequencing
WT	Wildtype

CHAPTER 1. INTRODUCTION

1.1. INTRODUCTION TO THE *CLOSTRIDIUM* GENUS

The genus *Clostridium* is highly diverse and one of the largest bacterial genera with 220 species and 5 subspecies, that use published official nomenclature, documented (EuzéBy, 1997; Parte, 2014; Public Health England, 2016). They are a group of Gram-positive, anaerobic, endospore-forming bacilli, which are ubiquitous in nature, having been isolated from water, soil and sewage (Liu, 2011; Greenwood *et al.*, 2012).

Of late there has been much interest in the *Clostridium* species that have distinct biotechnological potential and industrial value for their use in biofuel and chemical production, enzymology and cancer therapy (Minton and Clarke, 2013; Goldman and Green, 2015). For instance, *Clostridium acetobutylicum*, closely followed by *Clostridium beijerinckii* are the most extensively studied solventogenic clostridia strains for the production of butanol from sugars and gaseous substrates (Hidalgo and Puerta-Fernández, 2017). Furthermore, the *Clostridium novyi-NT* strain, attenuated by the deletion of the gene encoding the alpha toxin, has successfully demonstrated tumour regression through injecting the spores into the tumours of dogs. Significant oncolytic activity has also been observed with the spores of *Clostridium histolyticum* and *Clostridium sporogenes* (Sarotra and Medhi, 2016).

Previously, the absence of effective genetic manipulation tools has delayed the progress of exploiting the *Clostridium* species in the biotechnological field. Advances in synthetic biology with the development of efficient gene cloning and DNA transfer has allowed gene knock-in and knock-out systems to become fully established so that these clostridia species can be engineered and utilised commercially (Minton and Clarke, 2013; Goldman and Green, 2015; Green *et al.*, 2017).

Although the beneficial uses of selected species in this genus has been identified, the pathogenic clostridia continue to create significant problems. They can cause a variety of diseases, including diarrheal infections, neurological disorders and gas-gangrene (Table 1.1) (Bhunia, 2007).

Table 1.1 Classification of Pathogenic *Clostridium* Species and Respective Disease and Symptoms (Bhunia, 2007)

Organism	Disease
Clostridium botulinum	Botulism, infant botulism, wound botulism – affects peripheral nerves – flaccid paralysis
Clostridium barati	Infant botulism, hidden botulism
Clostridium butyricum	Infant botulism
Clostridium chauvoei	Black leg – cattle and sheep
Clostridium difficile	Antibiotic-associated membranous colitis (diarrhea) in human
Clostridium histolyticum	Gas gangrene (human)
Clostridium novyi A, B, C, D	Gas gangrene
Clostridium perfringens A, B, C, D, E	Gas gangrene, food poisoning, clostridial myonecrosis, enteritis in animals, enterotoxemias of sheep (struck) in sheep and pigbel in humans
Clostridium septicum	Gas gangrene
Clostridium sordelli	Gas gangrene in humans and animals; liver disease in sheep
Clostridium tetani	Tetanus in humans and animals; affects CNS causing spastic paralysis
Clostridium sporogenes	Nontoxigenic

However, less than 10% of the genus can make potent toxins that cause the unique clinical features of these diseases. The clostridial toxin proteins vary in size, structure and mode of action and can be categorised as either pore-forming, glycosyltransferases, ADP-ribosyltransferases, phospholipases, or metalloproteases that are zinc-dependent (Nakano and Zuber, 2004; Goldman and Green, 2015).

1.2. *CLOSTRIDIUM DIFFICILE*

1.2.1. HISTORY

Clostridium difficile is the known causative agent of the toxin-mediated diarrhoeal disease, *C. difficile* infection (CDI) (George *et al.*, 1978). The organism is distributed worldwide and found in various environmental sources such as soil, mud, hay and sand, as well as in the stool of numerous animals

including cats, dogs, horses, donkeys, cows and camels (Borriello *et al.*, 1983).

It was first described in 1935 when identified in the normal faecal flora of neonates and given the name *Bacillus difficilis*, due to the difficulty in laboratory isolation (Hall and O'Toole, 1935). Although pathogenicity had been demonstrated in other species through further characterisation (Snyder, 1937), it was thought to be non-pathogenic in humans until 1962 (Smith and King, 1962), when (the recently renamed) *C. difficile* organism was isolated from several patient abscesses, wounds, blood, peritoneal fluid and pleural fluid samples. However, the authors concluded that there was little evidence to suggest that *C. difficile* was responsible for the death of specific patients and that either it did not produce the characteristic toxin normally observed or the action of the toxin was not lethal in the human body. In the early 1970's, there was an increase in cases of pseudomembranous colitis (PMC). This coincided with the introduction of the broad-range antibiotic, clindamycin, and was termed clindamycin-associated colitis (Tedesco *et al.*, 1974). Strides were made in 1977, when a toxin-producing species of *Clostridium*, neutralisable by *C. sordellii* antitoxin, was demonstrated to cause clindamycin-associated colitis in hamsters (Bartlett *et al.*, 1977; Rifkin *et al.*, 1978). Finally, in 1978, *C. difficile* was again proposed as a human pathogen when the cytotoxin was detected in stool samples from patients with PMC. The researchers determined that due to the demonstration of cytopathic effect, *C. difficile* was the causative agent of PMC (Bartlett *et al.*, 1978; George *et al.*, 1978; Larson *et al.*, 1978).

1.2.2. TAXONOMY

Historically, the organisation of the *Clostridium* genus was arranged based on conventional microbiological characterisation. More recently, using molecular methods, classification was established through sequencing of the 16S rRNA gene. This analysis revealed how phylogenetically diverse the entire genus is and so a hierarchical system was developed with species grouped together into 19 clusters (Lawson and Rainey, 2016; Elliott *et al.*, 2017). Cluster I signifies the 'true' members of the *Clostridium* genus and so species that fall within this

are now represented as the *Clostridium sensu stricto* genus (Vos *et al.*, 2011). *C. difficile* was assigned to cluster XI, which includes additional similar Clostridia species, such as *Clostridium sordellii* and *Clostridium bifermentans*, together with other species such as *Peptostreptococcus anaerobius* and *Eubacterium tenue* (Collins *et al.*, 1994). *C. difficile* was later moved to the *Peptostreptococcaceae* genus and a suggestion to rename the organism to *Peptoclostridium difficile* was made (Yutin and Galperin, 2013). However, the name did not comply with the Internal Journal of Systematic and Evolutionary Microbiology (IJSEM) Bacterial Code and was rejected (Lawson *et al.*, 2016).

Clostridioides difficile has most recently been proposed as the new name for *C. difficile*. The IJSEM has yet to accept the latest name and it has been used very little by the research community in published papers (Carlucci, 2017; Dannheim *et al.*, 2017; Riedel *et al.*, 2017), therefore, this thesis will continue to use the former name.

1.2.3. EPIDEMIOLOGY

C. difficile has become a global problem and the principle cause of nosocomial infectious healthcare-associated diarrhoea in developed countries (Elliott *et al.*, 2017). Infection can result in patient isolation (Cartmill *et al.*, 1994; McNulty *et al.*, 1997), and in exceptional circumstances, ward closure (Wong *et al.*, 2015; van Beurden *et al.*, 2017). In the United States (US) alone, a total of 606,058 CDI episodes were observed in 2014, with 34.3% of cases occurring in the community and a predicted 44,500 associated deaths. The CDI economic cost reached $5.4 billion; $4.7 billion spent in healthcare settings and $725 million allocated to the community (Desai *et al.*, 2016). In some states, the CDI burden has surpassed that of the methicillin resistant *Staphylococcus aureus* (MRSA) healthcare acquired infection (Miller *et al.*, 2011a).

In the United Kingdom (UK), CDI is a notifiable disease and reportable to Public Health England (PHE). Between 1990 and 2001, voluntary surveillance identified a steady increase in CDI reports, which sharply escalated from 2002 onwards (Thelwall *et al.*, 2017). Reporting was made

mandatory in 2004 for patients 65 years old or greater, and in 2007 for patients aged 2 or more. Since 2007, reports of CDI incidence have decreased considerably, plateauing at an approximate rate of 23-26 CDI cases per 100,000 population for the last four financial years (Thelwall *et al.*, 2017). In the most recent annual epidemiological commentary, Public Health England stated that between April 2016 and March 2017, a total of 12,840 cases of CDI occurred in patients 2 years and older, and of this, 4,618 were trust apportioned (Figure 1.1) (Thelwall *et al.*, 2017).

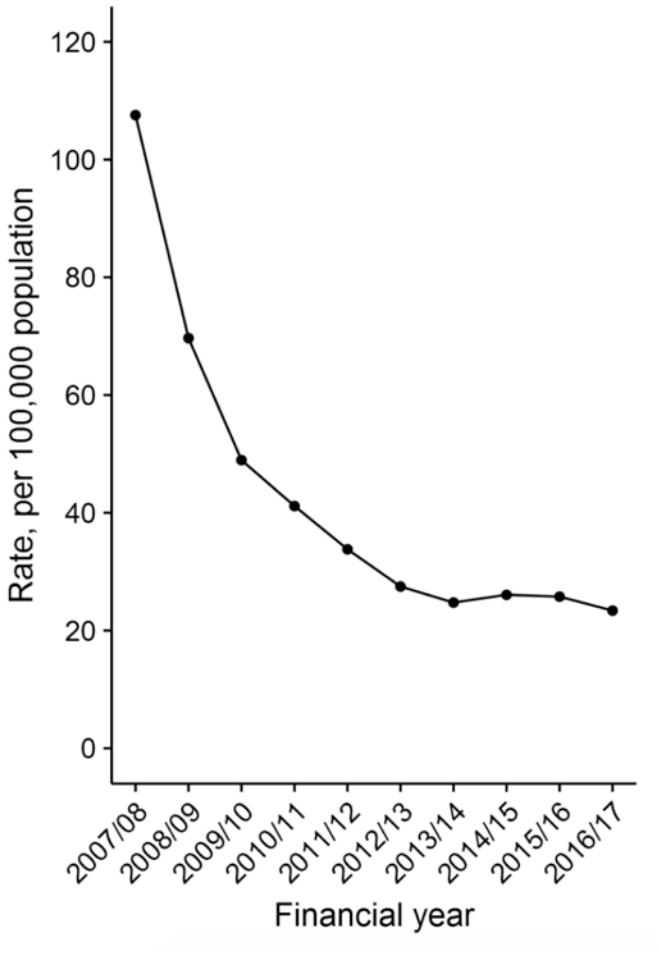


Figure 1.1 Trends in *Clostridium difficile* Infection (CDI) Rate of Incidence (Thelwall *et al.*, 2017)

The trends in rates of all CDI cases reported in England from 2007/08 to 2016/17. The rate of all CDI cases per 100,000 population, per year has fallen from 107.6 in 2007/08 to 23.4 in 2016/17.

The epidemiology of CDI has changed noticeably over the last 15 years. The initial reason for such an increased interest in this pathogen was due to the emergence and spread of the previously uncommon BI/NAP1/027 strains at the turn of this century (Elliott *et al.*, 2017). These strains were associated with higher rates of morbidity and mortality and resistant to fluoroquinolones (Pépin *et al.*, 2004; Pépin *et al.*, 2005; Labbé *et al.*, 2008). It was initially thought that the apparent hyper-virulence was due to increased toxin production as the strains contained a pathogenicity locus (PaLoc) *tcdC* negative regulator gene variant with a nonsense mutation in addition to an 18bp deletion (Loo *et al.*, 2005). A further study (Stabler *et al.*, 2008) determined that a variant toxin B (TcdB), with significant polymorphisms within the protein receptor-binding domain, increased toxicity due to an enhanced binding capacity compared to less virulent strains. Because of the ability of TcdB to undergo protein conformational changes in an acidic pH environment, the toxin could enter cells at a much faster rate and in an earlier stage of endocytosis (Lanis *et al.*, 2010). Although the prevalence of these strains has decreased in many countries, there were a number of severe outbreaks at the time, as witnessed in all Canadian provinces, at least 40 US states and numerous European countries including the UK, France, Germany, Finland and Belgium (Freeman *et al.*, 2010).

1.2.4. PATHOGENESIS OVERVIEW

CDI includes both asymptomatic colonisation (commonly seen in infants under 1 year of age, ~3% of healthy adults and 10-25% in hospitalised adults (Raveh *et al.*, 2006; Evans and Brachman, 2013; Alasmari *et al.*, 2014; Behar *et al.*, 2017)) and *C. difficile* associated disease (CDAD), which can range from mild to severe diarrhoea. In selected circumstances, this can progress to PMC, fulminant colitis and toxic megacolon, as well as rare extra-intestinal diseases, or even death (Cleary, 1998; Mylonakis *et al.*, 2001; Bartlett, 2010). The use of antibiotics disrupts the normal gut flora and allows the organism to colonise the gastrointestinal tract. Typical PMC lesions are usually found in the descending colon, although, a small number of cases have presented in the small intestine (Jacobs *et al.*, 2001).

C. difficile endospores are resistant to many chemical and physical agents and so remain in the environment on biotic and abiotic surfaces. Transmission occurs via the oral-faecal route but can also be from direct contact with contaminated surfaces. Once inside the gut, the spores can germinate (defined as 'the irreversible loss of spore-specific characteristics' (Burns *et al.*, 2010a; Burns *et al.*, 2010b)) and produce vegetative cells, which are essential for subsequent disease. These vegetative cells can penetrate the mucous layer and adhere to the enterocytes in the large intestine using flagella, composed of the flagellar proteins FliC and FliD (Tasteyre *et al.*, 2001a), proteases, such as the cysteine protease cell wall protein (CWP) 84 (Janoir *et al.*, 2007), and various adhesions, including the S-layer P36 and P47 proteins (Cerquetti *et al.*, 2000; Calabi *et al.*, 2002) and the fibronectin-binding protein Fbp68 (Hennequin *et al.*, 2003). *C. difficile* has been found to adhere to a number of cell lines *in vitro*, including human colon adenocarcinoma Caco-2 cells, Vero cells, host enteric HEp-2 cells and human gastrointestinal tract tissue sections, as well as the caecal mucus of mice *in vivo* (Karjalainen *et al.*, 1994; Gomez-Trevino *et al.*, 1996; Calabi *et al.*, 2002; Cerquetti *et al.*, 2002; Fagan *et al.*, 2009).

After colonisation has been established, the two main virulence factors, toxin A (TcdA); an enterotoxin with some cytotoxic activity, and toxin B (TcdB); a cytotoxin, are produced. These toxins can enter the enterocytes and glycosylate Rho GTPases, which results in cytoskeleton disorganisation and eventually cell apoptosis (Lyras *et al.*, 2009; Kuehne *et al.*, 2010). A number of strains (with an estimated prevalence of between 17% to 23% (Barbut *et al.*, 2007; Bauer *et al.*, 2011; Eckert *et al.*, 2013)) also produce *C. difficile* transferase (CDT); a binary toxin which leads to cytoskeletal rearrangements through ADP ribosyltransferase activity. The cumulative effect of these virulent properties is fluid secretion, inflammation and mucosal damage (Barbut and Petit, 2001; Deneve *et al.*, 2009; Pituch, 2009; Vohra and Poxton, 2011).

The intestinal damage seen in acute CDI is also generated by the host innate immune response. Neutrophils are the predominate white blood cells involved, and the dense infiltration beneath the intestinal mucosa, as well as the rise of these cells in the peripheral blood are specific characteristics of severe disease.

(Loo *et al.*, 2005; Lamontagne *et al.*, 2007; Cloud *et al.*, 2009). Additional elements of the immune system are initiated by the activity of the *C. difficile* toxin A and B glycosyltransferase; immune cells such as macrophages and monocytes are activated, together with the release of inflammatory cytokines such as interleukin 1 beta (IL-1β), interleukin 8 (IL-8) and tumour necrosis factor alpha (TNF-α) (Linevsky *et al.*, 1997; Jefferson *et al.*, 1999; Sun *et al.*, 2009).

1.2.5. RECURRENCE

A very important feature of CDI is the high recurrence rate through either reinfection, with the same or a different strain, or the relapse of an original infection, usually occurring in the first 2 weeks after treatment completion (Tang-Feldman *et al.*, 2003). It has been previously shown that exogenous *C. difficile* spores can remain viable in the environment for months at a time (Hensgens *et al.*, 2012b). Endogenous carriage of *C. difficile*, which has not been eradicated with the initial antibiotic treatment, is equally a likely explanation for recurrence, and seems to be the case for a large majority of recurrent CDI episodes (Kamboj *et al.*, 2011; Sim *et al.*, 2017).

CDI recurrence is directly proportional to the number of previous episodes. Studies have shown that CDI recurs in 15%–35% of patients who have had 1 prior episode and in 33%–65% of patients who have had >2 preceding episodes (Raveh *et al.*, 2006; Gerding *et al.*, 2008). Furthermore, previously published research found that patients who have their first recurrence of CDI in the initial three months following treatment are more likely to have multiple recurrent episodes (Archbald-Pannone and Guerrant 2013).

The chronic nature of recurrent CDI in the patient population has been demonstrated previously. For example, recurrent episodes of diarrhoea usually accompany fever and abdominal cramps in addition to colitis with or without pseudomembranes (McFarland *et al.*, 1999). More recent research suggested that patients with recurrent CDI have leucocytosis, fever and abdominal distention, in addition to diarrhoea, and these indicators should be included in a bedside scoring system for predicting recurrence (Viswesh *et al.*, 2017).

However, the severity of the diarrhoea and the duration of the episode are no more severe than those of the initial episode (McFarland *et al.*, 1999). A chronic disease that may recur over a period of months or even years can cause extreme incapacitation for the patient, as well as contribute to increased healthcare expenses (Marsh *et al.*, 2012). Several population-based studies (Kuntz *et al.*, 2017; Rodrigues *et al.*, 2017; Zilberberg *et al.*, 2017) have discovered that patients with recurrent CDI had consistently higher levels of healthcare facility use and rehospitalisation, additional hospital days, extra costs associated with hospitalisation, surgery and antibiotic treatment, as well as a greater one year mortality risk compared to patients with nonrecurring CDI and those who had never experienced CDI.

1.2.6. PATIENT RISK FACTORS

1.2.6.1 GUT MICROBIOME DYSBIOSIS

It has been evident for some time that there are consistent risk factors which increase the chance of acquiring CDI. Normal bowel flora is protective against *C. difficile* asymptomatic colonisation, CDAD, as well as recurrent episodes. There has been much recent research on the role of the gut microbiome in health and disease. The Human Microbiome Project, founded in 2008 by the US National Institute of Health, characterised the species in a healthy gastrointestinal tract. The two dominant phyla are the Firmicutes; which includes Gram-positive Bacilli and Clostridia, and the Bacteroidetes; which incorporates the Gram-negative anaerobes such as *Bacteroides*, *Parabacteroides*, and *Prevotella* (Huttenhower *et al.*, 2012; Khanna *et al.*, 2016b). In a CDI disease state, gut dysbiosis is represented as a loss of gut microbial diversity, as well as an increase in Gram-negative facultative anaerobic Enterbacteriaceae such as *Escherichia coli* (Khanna *et al.*, 2016b). This favourable environment allows colonisation and overgrowth of *C. difficile*, making it difficult to return to a healthy gut state and increasing the risk of recurrent infections (Fuentes *et al.*, 2014). It has also been hypothesised that, alongside the microbiome, bile acids play a key part in CDI. Secondary bile acids, which are produced by the healthy gut microbiota, are inhibitory to *C. difficile* growth and spore germination. After disruption of the healthy gut

state, primary bile salts, specifically cholic acid and chenodoxycholic acid, increase dramatically, allowing the germination of *C. difficile* and the development of CDI. Understanding these interactions may, in the future, allow clinicians to predict the patients who are most at risk of CDAD and recurrence (Theriot *et al.*, 2014; Theriot and Young, 2014; Allegretti *et al.*, 2016).

1.2.6.2 AGENTS THAT ALTER THE GASTROINTESTINAL FLORA

Perhaps the most significant risk factors are those agents that alter the normal gastrointestinal flora, allowing the growth of *C. difficile* and increase in toxin production. These include the use of antibiotics (Drekonja *et al.*, 2011; Loo *et al.*, 2011; Hensgens *et al.*, 2012a; McDonald *et al.*, 2018), gastrointestinal stimulants, enemas (McFarland *et al.*, 1990) and gastrointestinal acid suppression agents (Loo *et al.*, 2011; Janarthanan *et al.*, 2012). Although almost all antibiotic classes carry a risk associated with CDI, clindamycin and third-generation cephalosporins are conventionally known to carry the greatest risk (Menendez *et al.*, 2016; McDonald *et al.*, 2018). Additional studies (Yip *et al.*, 2001; McCusker *et al.*, 2003; Pépin *et al.*, 2005) have discovered fluoroquinolones to also be a high-risk antimicrobial. The use of antimicrobials not only increases CDI risk at the time, but for the next three months after therapy completion (Hensgens *et al.*, 2012a). Furthermore, antibiotic treatment or prophylaxis for another infection shortly after a CDI episode increases the risk of a recurrent episode, however, it is important to state that recurrence is not the consequence of *C. difficile* antibiotic resistance to initial treatment (either metronidazole or vancomycin) (Drekonja *et al.*, 2011). Gastrointestinal acid suppression agents, such as proton pump inhibitors (PPIs) and histamine-2 receptor antagonists (H2RAs) have also been associated with increased CDI risk (Loo *et al.*, 2011; Janarthanan *et al.*, 2012). Several hypotheses have been suggested to explain this escalated risk, including *C. difficile* colonisation due to decreased gastrointestinal acidity (Jump *et al.*, 2007), disruption of the gut microbiota resulting in heightened concentrations of unconjugated bile acids (Williams, 2001; Jump *et al.*, 2007) and a lowered neutrophil immune response (Yoshida *et al.*, 2000).

1.2.6.3 AGE

It is generally believed that older age is a significant host risk factor, specifically patients greater than 65 years of age (McFarland *et al.*, 1990; Garey *et al.*, 2008; Johnson, 2009; Loo *et al.*, 2011). After the age of 18, every additional year of age increases the risk of CDI by 2% (Loo *et al.*, 2011). Furthermore, a retrospective study (Raveh *et al.*, 2006) showed that the faecal contents of elderly patients are less inhibitory to *C. difficile* compared to younger people. Interestingly, in the latest PHE annual epidemiological report (Gerver *et al.*, 2014), they state that although CDI rates are still most significant in the elderly population, cases had decreased in the 75-84 and ≥85 year old age groups and instead had increased in the 65-74 year old age group. It is important to state that this change had only been observed in female patients. Furthermore, one paper (Carvajal *et al.*, 2017) reported CDI significantly effecting adults aged 50 to 70 and another study (Butt *et al.*, 2017) found that positive cases were identified in patients greater than 40 years old, suggesting that medical personnel should be expecting more cases from younger individuals. The emergence of CDI in the juvenile population has been described more recently in American hospitalised children, as well as those in the community, with the greatest increase in adolescents aged 15 years or older, and the strongest association among children diagnosed with inflammatory bowel disease (IBD) or hematopoietic stem cell transplantation (HSCT) (Zilberberg *et al.*, 2010; Nylund *et al.*, 2011; Khanna *et al.*, 2013; Pant *et al.*, 2016). The latest clinical practice guidelines for CDI from the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA) (McDonald *et al.*, 2018) now include guidance for children greater than 2 years of age.

1.2.6.4 GENDER

Gender difference has been a matter of debate in the past. A retrospective study (Raveh *et al.*, 2006) stated that the acquisition of hospital antibiotic-associated diarrhoea (AAD) was higher in women (54% vs. 46%). The reason for this was thought to be the increased rate of antibiotic treatment for urinary tract infections (UTIs) (Klingler *et al.*, 2000). However, other more recent

studies disagree and have found no significant difference in the acquisition of CDI between male and female patients (Eyre *et al.*, 2012; Thabit *et al.*, 2016; Kuntz *et al.*, 2017).

1.2.6.5 SEASONAL TREND ASSOCIATION

Researchers (Furuya-Kanamori *et al.*, 2015a; McLure *et al.*, 2019) have also observed a seasonal trend risk factor. CDI cases appeared to be at their highest in spring, with the months in summer and autumn demonstrating a lower number of cases. They commented that seasonal differences could be influenced by the use of antibiotics, for example, with increased prescriptions, the use of higher-risk antibiotics or repeated regimes at certain times of year. Furthermore, a co-seasonality trend of CDI and respiratory tract infections has been proposed (Polgreen *et al.*, 2010; Brown *et al.*, 2013). However, a seasonal association was not observed by (Eyre *et al.*, 2012) when investigating the predictors of CDI recurrence.

1.2.6.6 COMORBIDITY

Additionally, a recent meta-analysis (Furuya-Kanamori *et al.*, 2015b) suggested another significant host factor as the association between underlying conditions, such as diabetes, renal failure and malignancy, and CDI. These chronic diseases increase host susceptibility to CDI (as well as any other infection). Certain gastrointestinal comorbidities have been investigated with research from one study demonstrating a 3-fold increase in CDI rate from ulcerative colitis patients and a 2-fold increase observed from patients with Crohn's disease (Navaneethan *et al.*, 2010; Carpenter *et al.*, 2016). Gastrointestinal co-morbidities have also been connected to an increased risk of recurrence, with IBD patients 33% more likely to experience a recurrent episode when compared to the general population (Razik *et al.*, 2016). In further research (Khanafer *et al.*, 2017), links were made between CDI symptoms and malnutrition, liver disease and renal disease. The reasons for these associations are not always clear but it is thought that systemic chronic inflammation, impaired immune function and/or antibiotic therapy play an important role. An alternative theory is that comorbidities are related to an

increased hospital stay and so patients have a greater chance of exposure to *C. difficile* (McFarland *et al.*, 1990), however, this has been shown to be unlikely in more recent research (Kyne *et al.*, 2002; Ananthakrishnan *et al.*, 2008).

1.2.6.7 HOSPITAL AND COMMUNITY-ASSOCIATION

Hospitalisation and long-term care facilities, such as nursing homes, are historically the main source of CDI cases. A healthcare-associated CDI (HCA-CDI) episode is characterised by symptoms appearing more than 48 hours after hospital admission (McDonald *et al.*, 2007). In hospital, the patient risk of developing CDI is heightened with each additional day of hospitalisation (Stevens *et al.*, 2011). Researchers studying the acquisition of *C. difficile* by hospitalised patients found that colonisation occurred in 13% of patients that were hospitalised for 1-2 weeks. This increased dramatically to 50% when patients were hospitalised for greater than 4 weeks (Clabots *et al.*, 1992). Previous hospitalisation also appears to be significantly associated with recurrent CDI rates. One study proposed healthcare exposure (hospital or a healthcare facility) within 30 days as a considerable risk (Carpenter *et al.*, 2016).In recent times, CDI cases outside of the hospital setting are becoming more frequent, however, a large proportion of the CDI epidemiology literature is based on hospital acquisition, and so overall CDI incidence could be incredibly underestimated (Khanna *et al.*, 2016a). Community-associated CDI (CA-CDI) is causing severe and recurrent disease in individuals who were originally thought to be low-risk, such as younger patients and those who had no exposure to antibiotics (Kwon *et al.*, 2017). A CA-CDI case patient is defined as either having onset of symptoms when in the community or less than 48 hours after hospital admission (providing the onset is more than 12 weeks after the last hospital discharge) (McDonald *et al.*, 2007). A study (Jen *et al.*, 2012) completed in England over a period of 12 years illustrated the increase in the number of CA-CDI cases from 7.1% in 1997/98 to 13.5% in 2009/10. Another population-based analysis (Khanna *et al.*, 2012) found that 41% of 385 definite CDI cases were community acquired. Paediatric CA-CDI cases have also significantly risen, with a separate 12-year study (Adams *et al.*, 2017) conducted between 2001 and 2013 finding an average annual

increase of 47.9% (from approximately 35 patients in 2001 to almost 250 patients in 2013). It is believed that asymptomatic *C. difficile* carriers, such as children under the age of 2, may be a reservoir and contribute considerably to distribution of the bacteria in the community (Denno *et al.*, 2012; Gupta and Khanna, 2014; Kim and Zhu, 2017). There has also been much discussion on outpatient antibiotic prescriptions, food (particularly meat products), soil, waterways, animal production and domestic animals as all being potential sources of CA-CDI cases (Gupta and Khanna, 2014; Bloomfield and Riley, 2016; Kavanagh *et al.*, 2016; Warriner *et al.*, 2016).

1.2.6.8 STRAIN ASSOCIATION

With regards to *C. difficile* strains, the B1/NAP1/027 strains have been strongly associated with recurrence and increased disease severity in some studies (Marsh *et al.*, 2012; Kumar 2013; Neely *et al.*, 2017). An initial infection with a B1/NAP1/027 strain increases the risk of recurrent episodes significantly due to a reduced overall cure rate (Petrella *et al.*, 2012). Additional research has also identified an association of either recurrence or increased disease severity with non- B1/NAP1/027 strains such as ribotype 001 (Chen *et al.*, 2017), 023 (Sandell *et al.*, 2016), 078 (Sandell *et al.*, 2016), 106 (Neely *et al.*, 2017; Suárez-Bode *et al.*, 2019) and 220 (Herbert *et al.*, 2019). The fact that strain-specific characteristics may play a part in patient outcome could have important implications on future treatment research. However, it has been proposed recently that host factors, such as a patient being greater than 70 years of age, are more significant predictors of recurrent CDI (van Beurden *et al.*, 2018).

1.2.7. DIAGNOSIS

There are various tests that demonstrate the presence of *C. difficile*, that are now commercially available. These include streaking the faecal sample onto cefoxitin-cycloserine egg yolk (CCEY) selective agar (following the alcohol shock method to eliminate other non-sporulating faecal bacteria), latex agglutination test for the *C. difficile* somatic antigen, detecting a non-specific enzyme called glutamate dehydrogenase (GDH) (found in both toxin and non-

toxin producing strains), direct detection of toxins A and/or B using either a cell cytotoxic tissue culture assay (considered to be the 'gold standard') or a direct enzyme immunoassay (EIA) and identifying the toxin gene using a nucleic acid amplification test (NAAT) such as the polymerase chain reaction (PCR) (Barbut *et al.*, 2003a; Department of Health, 2012; Public Health England, 2014).

In the United Kingdom, the Department of Health (DoH) provides guidance (Department of Health, 2012) on the NHS laboratory diagnostic tests available for detecting *C. difficile* in patient samples. Firstly, faecal samples should only be processed if they adhere to score 5 (soft blobs with clear-cut edges) to 7 (liquid consistency with no solid pieces) on the Bristol stool form scale (Heaton *et al.*, 1992; Debast *et al.*, 2014). Secondly, the diarrhoeal samples should only be tested from hospital patients ≥2 years, community patients aged ≥65 and from community patients <65 if clinically indicated. The DoH, as well as the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), advocates the use of a two-test screening protocol. The first test should either be a GDH EIA or a NAAT, which demonstrates that *C. difficile* is present. However, this result alone is not sufficient to correlate to patient CDI symptoms. Therefore, the second test, such as a toxin EIA, is also performed to confirm diagnosis. A cytotoxin assay may be considered as an alternative to a toxin EIA. A third test, such as a NAAT or a cytotoxin assay, may be necessary if there are contradictory results in the first two tests or to identify those patients who are potential *C. difficile* carriers and spore shedders (Department of Health, 2012; Brecher *et al.*, 2013; Martinez-Melendez *et al.*, 2017).

From April 2007, ribotyping became the national system (*Clostridium difficile* Ribotyping Network (CDRN) Service (Department of Health, 2008)) used to characterise different *C. difficile* strains. Within England there are six specialist reference laboratories; Leeds, Birmingham, London, Manchester, Newcastle and Southampton (Public Health England, 2014). Profiles are generated with PCR primers designed to amplify the 16S-23S rRNA gene intergenic spacer region, and the result is compared to a known ribotype library (Stubbs *et al.*, 1999). For example, a B1/NAP1/027 strain essentially

refers to type B1 by restriction endonuclease analysis (REA), North American pulse-field type 1 by pulse-field gel electrophoresis (PFGE) and 027 by PCR ribotyping (Killgore *et al.*, 2008).

1.2.8. STANDARD ANTIBIOTIC TREATMENT

Patients with mild to moderate CDI were previously treated with a 10 to 14-day course of 500mg oral metronidazole, three times a day (Cohen *et al.*, 2010; Public Health England and Wilcox, 2013; Debast *et al.*, 2014; Gerding *et al.*, 2016). At the time, oral metronidazole was inexpensive and had been shown to be as effective as oral vancomycin in treating mild to moderate CDI cases (Department of Health, 2012). However, more recent research has demonstrated that metronidazole is inferior to vancomycin (Johnson *et al.*, 2014; Siegfried *et al.*, 2016) and prolonged use increases the risk of potentially irreversible neurotoxicity (Godfrey *et al.*, 2015). Therefore, for both an initial episode and patients with severe CDI, a 10 to 14-day course of 125mg oral vancomycin, four times a day, is currently recommended (Cohen *et al.*, 2010; Public Health England and Wilcox, 2013; Debast *et al.*, 2014; Gerding *et al.*, 2016; McDonald *et al.*, 2018). Vancomycin is poorly absorbed in the gastrointestinal tract and so is heavily concentrated in the colon, increasing the drug activity and reducing undesirable side effects, when compared to metronidazole (Taylor *et al.*, 2017; McDonald *et al.*, 2018). With regards to treatment failure and patient prognosis, in a prospective observational study (Musher *et al.*, 2005) of 207 patients that received metronidazole, 50% of patients were cured, 22% continued to have symptomatic disease despite therapy and 28% initially responded but had a recurrent episode within 90 days of successful treatment completion. The overall mortality rate within the cohort was 27%, with those patients who did not respond to treatment initially, at higher risk. Furthermore, a recent retrospective study (Stevens *et al.*, 2017) established that although CDI recurrence rates were similar between metronidazole and vancomycin, the 30-day mortality rate was significantly reduced in patients who received vancomycin. The author stated that these findings justified the use of vancomycin to treat initial episodes of CDI, as well as severe cases.

Cases of metronidazole and vancomycin antibiotic resistance have been observed previously in a number of different countries. An investigation (Pelaez *et al.*, 2002) conducted over an 8-year period in Madrid, Spain, found that the overall rate of resistance to metronidazole was 6.3%, and the total rate of intermediate resistance to vancomycin was 3.1% (as full resistance was not observed). Interestingly, a high number of the resistant strains were isolated from patients with the human immunodeficiency virus (HIV). Similarly, a 12-month study (Goudarzi *et al.*, 2013) in Iran observed metronidazole resistance in 5.3% of the 75 *C. difficile* isolates tested. Furthermore, in a three-year cross-sectional study (Jin *et al.*, 2017) in eastern China, although all 411 patient isolates were found to be vancomycin sensitive, 64 (15.6%) of these *C. difficile* isolates, including a non-toxigenic strain, were resistant to metronidazole. However, other studies have demonstrated much rarer incidences. For example, the largest pan-European epidemiological study (Freeman *et al.*, 2015b) to date, found scarce resistance to metronidazole (0.2% [*n*=6/2694]) and vancomycin (0.1% [*n*=2/2694]) over a 3-year period. Moreover, a second pan-European survey (Debast *et al.*, 2013) from 2008, as well as an Australian study (Knight *et al.*, 2015) determined that none of the 398 and 440 *C. difficile* patient isolates tested, respectively, demonstrated *in vitro* metronidazole and vancomycin resistance. It is important to note that these observed differences could be due to the fact that antibiotic use in many Asian countries is poorly regulated, either through misuse in the community, inappropriate or unnecessary prescription, or prescription of high-risk CDI antimicrobials, such as fluroquinolones (Holloway, 2011).

Additionally, there has also been evidence of strain specific antibiotic resistance including a Leeds study (Baines *et al.*, 2008) reporting 24.4% of all ribotype 001 isolates with reduced metronidazole susceptibility, and other research (Citron *et al.*, 2009; Adler *et al.*, 2015) determining that both metronidazole and vancomycin minimum inhibitory concentrations, in which 90% of the isolates are inhibited (MIC_{90}), for B1/NAP1/027 strains were up to 4-fold higher than for non-B1/NAP1/027 strains. The pan-European epidemiological study (Freeman *et al.*, 2015b) also identified multiple antibiotic resistances in the B1/NAP1/027 strains, as well as epidemic

ribotypes, such as 001 and 072, and non-epidemic ribotypes, including 018 and 356.

Furthermore, both oral metronidazole and vancomycin have been observed to promote the growth of vancomycin-resistant enterococci (VRE) (Al-Nassir *et al.*, 2008). In 56 patients with pre-existing VRE colonisation, metronidazole and vancomycin each encouraged persistent VRE overgrowth during treatment. In addition, out of the 34 patients who had no VRE detected in their stool before metronidazole or vancomycin therapy, 4 developed new VRE colonisation.

More recently, a 10-day course of 200mg oral fidaxomicin, twice daily, was approved for treatment of CDI in the US in 2011, and in 2012 for use in Europe (Department of Health, 2012; McDonald *et al.*, 2018). This relatively new narrow-spectrum antibiotic is a bactericidal non-absorbed macrolide that has a lethal effect on *C. difficile* without compromising other bacterial gut flora (Tannock *et al.*, 2010; Aljarallah, 2017). When compared to vancomycin, fidaxomicin was non-inferior while treating an initial CDI episode, however was superior in preventing recurrence and maintaining a clinical cure (Louie *et al.*, 2011; Cornely *et al.*, 2012). Further research (Nerandzic *et al.*, 2012) also demonstrated that treatment with fidaxomicin was less likely to encourage the overgrowth of VRE (although the selection of pre-existing VRE subpopulations with raised fidaxomicin MICs were common during treatment). However, the cost of fidaxomicin is considerably higher than that of vancomycin. Within the NHS, local decision making is necessary to justify the cost-effective use of fidaxomicin (Department of Health, 2012). There is also a suggestion that strain differences may alter the effectiveness. Several studies (Louie *et al.*, 2011; Marsh *et al.*, 2012; Petrella *et al.*, 2012) found that patients treated with fidaxomicin compared with vancomycin had significantly reduced recurrence rates for non-BI/NAP1/027 infections, but no difference in rates of recurrence was observed in BI/NAP1/027 infections.

Fidaxomicin was revealed to be as effective more than 1 year after licensure (Snydman *et al.*, 2015). However, there have been some isolated incidents of fidaxomicin resistance reported. In a phase 3 clinical trial (Goldstein *et al.*,

2011), an initially cured patient was found to have a *C. difficile* strain with an elevated fidaxomicin MIC (16µg/ml) when suffering from a recurrent episode Furthermore, another *C. difficile* isolate recovered from a Florida hospitalised patient was also found to have a resistant MIC (16µg/ml) (Peng *et al.*, 2017a). Although fidaxomicin resistance has been noted clinically, it is extremely rare and, therefore, still recommended as a viable treatment option for CDI.

1.2.9. NOVEL AND EXPERIMENTAL THERAPIES

Alternative therapeutic options have been proposed due to the increasing number of treatment failure and recurrent cases, particularly with those patients who have been treated with metronidazole and vancomycin (Vardakas *et al.*, 2012). There are agents that are either used as a last resort or are in encouraging clinical trials.

1.2.9.1 ANTIBIOTIC-BASED APPROACH

Rifaximin is a rifamycin derivative, which was originally utilised for treating traveller's diarrhoea. The antibiotic is broad spectrum, almost completely non-absorbed when administered orally and well tolerated by the patient (Kokkotou *et al.*, 2008; Rubin *et al.*, 2011; Mattila *et al.*, 2013). Much research has suggested the effective use of rifaximin in treating recurrent CDI, either as a first-line treatment option for high-risk patients (Rubin *et al.*, 2011), as an additional therapeutic management alternative for recurrent CDI (Mattila *et al.*, 2013), or as a 'chaser therapy' to prevent relapse; administering rifaximin immediately after vancomycin therapy (Johnson *et al.*, 2007; Johnson *et al.*, 2009; Garey *et al.*, 2011; Oldfield IV *et al.*, 2014) or after metronidazole if patients were unresponsive to this antibiotic (Basu *et al.*, 2010). Investigation into the rifaximin chaser regime has yielded particularly promising results with several of these studies reporting a decreased incidence of CDI and an interruption of recurrent episodes when 400mg rifaximin was taken three times daily for between 14 and 20 days immediately after standard treatment. One study (Johnson *et al.*, 2009) continued the rifaximin at a lower dose of 200mg, three times a day, for a further two weeks.

Unfortunately, the emergence of rifaximin resistance has been observed clinically. In a pan-European longitudinal surveillance study (Freeman *et al.*, 2015a), the rate of resistance was revealed as 13% in 953 *C. difficile* isolates from 22 different countries. The authors noted that this rifaximin resistance involved several different ribotypes. Another paper (Hecht *et al.*, 2007), which examined 110 toxigenic strains collected from 1983 to 2004, observed 3 samples with extremely high rifaximin MICs (>256μg/ml). A similar study (Marchese *et al.*, 2000) analysed 93 inpatient *C. difficile* isolates and found rifaximin inhibited 69 (74%) of the strains. Interestingly, *C. difficile*, when compared to other anaerobes, demonstrated a particularly low incidence of spontaneously resistant isolates ($<1x10^{9}$). A further study (O'Connor *et al.*, 2008) revealed 14 rifaximin resistant isolates, with an MIC of >32μg/ml, identified from 80 clinical samples. Unusually, researchers in the (Carman *et al.*, 2012) study found that one patient developed rifaximin resistance in a *C. difficile* ribotype 056 isolate within 32 hours, based on a rifaximin sensitive MIC of 0.002μg/ml on the first day and a resistant MIC of 32μg/ml after exposure to rifaximin. Rifaximin resistant *C. difficile* infections have been observed particularly in tuberculosis (TB) patients, where rifaximin is part of the intensive anti-TB therapy (Obuch-Woszczatyński *et al.*, 2013; Kullin *et al.*, 2017). It was speculated that the prior use of rifaximin before CDI may have led to these rifaximin resistant strains. In the (Johnson *et al.*, 2007) brief report, one of the eight patients experiencing recurrent *C. difficile* episodes responded to a second course of rifaximin treatment but a rifaximin resistant strain with a high MIC was isolated post therapy. Interestingly, the authors determined that previous rifaximin therapy in three of their patients did not predict rifaximin resistance in this case. However, in a follow-up study (Johnson *et al.*, 2009), a strain isolated from one of the patients that relapsed had an MIC of >256μg/ml after the first and during the second rifaximin course. It was later discovered that the patient had been treated with rifaximin 4 months prior to enrolment onto the study. However, another study (Curry *et al.*, 2009) demonstrated that of the 179 rifaximin resistant isolates detected, only 7 were directly attributed to prior exposure to rifaximin. The authors observed that instead of antibiotic-selective pressure driving resistance,

transmission of rifaximin resistant strains, at least at the University of Pittsburgh Medical Center–Presbyterian Hospital, was common.

Cadazolid is a novel fluoroquinolone-oxazolidinone antibiotic, which inhibits bacterial protein synthesis (Locher *et al.*, 2014) and has been shown to have potent *in vitro* activity against 133 *C. difficile* strains, including the B1/NAP1/027 strains (Rashid *et al.*, 2013). In a multicenter, double-blind, randomised phase 2 clinical trial with 84 patients, three different cadazolid doses (250, 500, or 1,000mg, twice a day) were compared against a standardised dose of vancomycin (125mg, four times a day) (Louie *et al.*, 2015). Both cadazolid and vancomycin resulted in a comparable patient clinical response rate, however, each dose of cadazolid produced a lower recurrence rate, which led to a sustained clinical outcome for these patients. Furthermore, during this study, cadazolid was found to be well tolerated and it demonstrated minimal effects on the gastrointestinal flora, in contrast to vancomycin (Gerding *et al.*, 2015a). However, in a more recent double-blind, placebo-controlled, randomised phase 3 trial (Gerding *et al.*, 2019), cadazolid was unable to demonstrate non-inferiority to vancomycin for patient clinical cure, and so is unlikely to be pursued any further as a treatment for CDI.

Surotomycin is a new lipopeptide antibiotic, which is structurally related to daptomycin. In a hamster model (Mascio *et al.*, 2012), surotomycin exhibited potent activity in resolving CDAD, even at low doses. In the same study, when surotomycin was compared to vancomycin, it showed an almost identical dose- and time-dependent trend in regard to *C. difficile* recurrence rates. Furthermore, it was noted in another study (Reigadas *et al.*, 2015) that surotomycin also demonstrated potent activity on clinical *C. difficile* isolates with intermediate sensitivity to metronidazole or vancomycin. Although surotomycin displayed lower CDI recurrence rates and a prolonged clinical response rate, when compared to vancomycin, in a phase 2, randomized, controlled, double-blind, non-inferiority, multicentre trial (Lee *et al.*, 2016), in a more recent phase 3 trial (Boix *et al.*, 2017), surotomycin was unable to meet the noninferiority criteria, versus vancomycin, for clinical response rate, at the end of treatment, and sustained clinical response, in a 40-day follow-up period.

Ridinilazole (formerly SMT19969) is a novel antibacterial, which acts by impairing cell division (Bassères *et al.*, 2016). Recent research (Bassères *et al.*, 2016) demonstrated the potent activity of ridinilazole against *C. difficile*, significantly reducing toxin production and limiting the inflammatory response. Furthermore, it has also been concluded that because ridinilazole has a narrow spectrum of action, the normal gastrointestinal flora are predominately spared (Goldstein *et al.*, 2014). A phase 2, randomised, double-blind, active-controlled, non-inferiority study (Vickers *et al.*, 2017), which assessed ridinilazole efficiency and safety, showed a sustained clinical response in 66.7% of patients on ridinilazole compared to 42.4% of vancomycin patients.

1.2.9.2 MICROBIOLOGICAL-BASED APPROACH

Live microbiota preparations are living non-pathogenic bacteria which can colonise the human gastrointestinal tract. The theory behind the use of these agents in the treatment of CDI is that they facilitate reconstitution of the healthy gut microbiota after becoming destabilised by the use of antibiotics (Ollech *et al.*, 2016).

The highly effective, yet unappealing, faecal microbiota transplant (FMT) has received much publicity as a promising salvage treatment for recurrent CDI (Han *et al.*, 2016; Hota *et al.*, 2017). The procedure involves administering an intestinal microorganism suspension of previously tested healthy donor stool by enema, slurries via nasogastric tube or colonoscopy into the upper or lower gastrointestinal tract (Gerding *et al.*, 2008). Current research (Reigadas *et al.*, 2018) has also determined that oral administration via capsules has proven to be safe, well-tolerated and very effective against *C. difficile* recurrence. When evaluating the success of FMT in recurrent CDI patients, a number of randomised clinical trials (Van Nood *et al.*, 2013; Cammarota *et al.*, 2015) have demonstrated the use of donor faeces to be significantly more effective than vancomycin. Furthermore, a systematic review (Cammarota *et al.*, 2014) determined an 87% diarrhoea resolution rate in 536 treated patients. However, a recent clinical trial (Hota *et al.*, 2017) found that there was no significant difference between patients that received a FMT after vancomycin treatment

and those that tapered their vancomycin therapy in reducing recurrent CDI. Even though FMT has been performed since 1958 to effectively treat patients with PMC, and proven successful in recurrent CDI cases, there still appears to be some reservations (Aljarallah, 2017). There are strict donor inclusion criteria and stool screening is a demanding and expensive process (Han *et al.*, 2016). There are also concerns about the spread of infectious diseases, particularly those which are unknown and cannot be screened for, and the possibility of changing the microbiome so that a patient is then susceptible to chronic autoimmune and obesity disorders (Smith *et al.*, 2014).

SER-109 is a healthy stool donor mixture composed of purified spores from around 50 Firmicutes species. In a phase 1b study by (Khanna *et al.*, 2016b), 29 of 30 patients, with a history of multiple recurrent episodes, achieved clinical resolution of *C. difficile* at 8 weeks. Concurrently, it was also demonstrated that gastrointestinal flora had diversified over this period. In a phase 2 clinical trial (Seres Therapeutics, 2018c), the findings of which are yet to be published, a decreased efficiency was observed when compared to the phase 1b trial. However, a press release from Seres Therapeutics (Seres Therapeutics, 2017) stated that this was due to either a misdiagnosis of recurrent CDI or substandard dosing in some patients. There are currently two phase 3 clinical trials underway; one is accessing SER-109 versus a placebo in the treatment of adults with recurrent CDI (Seres Therapeutics, 2018b), and the other is evaluating the efficacy of SER-109 in reducing recurrence of CDI in those patients who had a recurrence within 8 weeks after receipt of SER-109 or the placebo in the aforementioned trial (Seres Therapeutics, 2018a).

Furthermore, one study (Gerding *et al.*, 2015b) investigated the use of non-toxigenic *C. difficile* spores in patients who had been previously treated with metronidazole or vancomycin. The administration of a high dose of non-toxigenic strain M3 spores over a 7-day period were shown to be safe, well tolerated and effective at preventing recurrent episodes. It is evident that more research is necessary to conclude which agents are most effective, which types of patients they benefit and in combination with which specific antibiotics (Department of Health, 2012; Surawicz *et al.*, 2013).

Lastly, there has been some evidence to suggest that two probiotics (*Saccharomyces boulardii* and *Lactobacillus rhamnosus GG*) decrease the incidence of AAD, however, the evidence is insufficient to determine whether they prevent CDI (both initial and recurrent episodes) (Department of Health, 2012; Surawicz *et al.*, 2013).

Bacteriophage therapy is categorised as a microbiological-based approach but is quite different from those mentioned previously. There has been much interest in this viable option as an alternative to antibiotic treatment, due to its targeted specificity, lack of significant impact on the gastrointestinal flora and dose amplification in the patient (Nale *et al.*, 2015). A study by (Nale *et al.*, 2015) demonstrated that a combinations of three to four different types of strain-specific bacteriophages were able to completely lyse *C. difficile in vitro* within 2 to 5 hours. Furthermore, *in vivo* research in the same study showed bacteriophage combination treated mice with an increased survival rate and a four-log reduction of *C. difficile* vegetative and spore counts in the gut. Interestingly, bacteriophages may optimise other CDI therapies. A recent preliminary study (Zuo *et al.*, 2018) found that FMT treatment response increased when a larger number of *Caudovirales* bacteriophages were transferred and colonised in the recipients gut, simultaneously. Unfortunately, developing bacteriophage therapies has been challenging due to the need to create a bacteriophage with the inability to integrate mobile genetic elements (MGEs), as well as demonstrate safety and efficacy in human clinical trials (Mills *et al.*, 2018).

1.2.9.3 IMMUNOLOGY-BASED APPROACH

The role of immunity in CDI has encouraged research into this promising field, with several avenues being investigated. Human monoclonal antibodies, such as actoxumab (MK-3415) or bezlotoxumab (MK-6072), are known to bind and neutralise *C. difficile* toxin A and B, respectively (Gerding *et al.*, 2016). Two phase 3 trials (Maw *et al.*, 2017; Wilcox *et al.*, 2017) involving 2655 patients were completed at the start of 2017 and demonstrated that bezlotoxumab, when taken alongside the primary therapy (metronidazole, vancomycin, or fidaxomicin) was associated with a significantly lower rate at

almost 40% of recurrent CDI compared to a placebo. Further analysis determined that those patients with greater than 3 risk factors for recurrence benefited most (Gerding *et al.*, 2018). The authors (Wilcox *et al.*, 2017), however, reported no improvement in effectiveness with the addition of actoxumab and so concluded that bezlotoxumab alone was successful in reducing CDI recurrence.

Furthermore, the hypothesis that a low serum antibody response can predispose a person to CDI suggests the use of intravenous immunoglobulin (IVIG; prepared from pooled human serum containing antibodies against the *C. difficile* toxins (Shah *et al.*, 2014)) could also be effective in treating recurrent CDI (Taylor *et al.*, 2017). IVIG therapy has been shown to demonstrate an overall advantage, however, the limited amount of research comprised of small sample sizes and lacked control groups (O'Horo and Safdar, 2009).

Finally, an active immunisation approach via a vaccine containing inactivated toxins or toxoids A and B has been explored (Gribenko *et al.*, 2017). Previous research (Gardiner *et al.*, 2009; Jin *et al.*, 2013) has established that the C-terminal of the receptor binding region in both toxin A and B, as well as the N-terminal region of the toxin B protein each produce a protective antibody immune response in an *in vitro* cytotoxicity assay and during *in vivo* animal studies. A safety and immunogenicity investigation (Kotloff *et al.*, 2001), involving 30 healthy adults demonstrated that a *C. difficile* vaccine containing formalin-inactivated toxoids A and B was safe. It was also highly immunogenic with greater than 90% of individuals eliciting an immunoglobulin (Ig) G serum antibody response. In a further human clinical trial (Aboudola *et al.*, 2003), 30 healthy volunteers were given a *C. difficile* toxoid A vaccine, which induced high levels of serum anti-toxin A IgG. The researchers concluded that this vaccine was highly immunogenic, due to the level of IgG observed exceeding the quantity associated with protection. This vaccine was tested in 3 subjects with recurrent CDAD and found to be just as effective at provoking an immune response. The patients were able to cease oral vancomycin maintenance treatment without further recurrence (Sougioultzis *et al.*, 2005).

1.2.10. INFECTION CONTROL MEASURES

Hospital infection control measures can dramatically decrease the risk of acquiring CDI. A high level of hand hygiene must be maintained at all times. It is necessary for hospital personnel to wash their hands with no less than conventional soap and water, but preferably with 4% polyvidone soap or 4% chlorhexidine gluconate antiseptic, which have been shown to reduce the extent of *C. difficile* on the hands, significantly (McFarland *et al.*, 1989; Barbut *et al.*, 2003b; McDonald *et al.*, 2018). Any medical equipment should be disposable and single-use as far as is practical. Non-disposable equipment should be dedicated to a patient and then thoroughly cleaned using a *C. difficile*-sporicidal disinfectant (McDonald *et al.*, 2018), such as 2% alkaline glutaraldehyde solution or peracetic acid, which inactivate *C. difficile* spores after systematic cleaning (Rutala *et al.*, 1993; Wullt *et al.*, 2003). The same disinfectant must be used to decontaminate any environmental surfaces of potential contamination by *C. difficile* (Kundrapu *et al.*, 2012; Surawicz *et al.*, 2013).

Furthermore, contact precautions for a patient with CDI are advised. This should be continued for a minimum of 48 hours after diarrhoea ceases (McDonald *et al.*, 2018). For severe CDI cases and/or outbreaks, trusts must isolate the patient(s) in a ward side room for the duration of their hospital stay. All health workers and visitors entering the room must wear personal protective equipment (PPE), such as gloves and gowns, and discard these items before re-entering the main hospital ward (Surawicz *et al.*, 2013).

It is important that hospital trusts have comprehensive antimicrobial prescribing and stewardship guidelines. Cumulative antimicrobial exposures are connected to the incidence of CDI and so it is crucial that the objectives of an antibiotic stewardship program reduce the overall therapy dose, the number of exposure days and replace high-risk antimicrobials for lower-risk alternatives (Stevens *et al.*, 2011). It is further recommended that fluoroquinolones, clindamycin, and cephalosporins should be restricted, alongside any other target antimicrobial indicated from local epidemiological data (McDonald *et al.*, 2018). If an antibiotic stewardship is maintained, it can

enhance overall patient outcome, reduce the incidence of CDI, and prevent and control the development of antibiotic resistance (Barlam *et al.*, 2016). One study (Valiquette *et al.*, 2007) found that an antibiotic stewardship program promoted a 60% decrease in CDI incidence during an epidemic. The restrictive use of cefuroxime, a 2nd generation cephalosporin, in an elderly care unit has also been shown to reduce the frequency of CDI incidence from 2.1% to 0.9% (McNulty *et al.*, 1997).

Although asymptomatic carriers can contribute to horizontal spread of *C. difficile* within a hospital environment, it is not recommended that hospitalised inpatient *C. difficile* carriers are routinely treated (McDonald *et al.*, 2018). Studies have shown that the rate of re-colonisation can be high, it may increase the shedding of spores and asymptomatic carriers can go on to develop CDAD (Johnson *et al.*, 1992; Surawicz *et al.*, 2013).

More recently, research (Shaughnessy *et al.*, 2016) was conducted on environmental household contamination as a source for recurrent CDI. Although *C. difficile* was found in the household environment of all relapse patients, the authors could not conclude whether that was a cause or consequence. However, if household contamination does in fact lead to recurrent CDI episodes, effective decontamination may potentially reduce reinfection rates.

Lastly, there is a demand for improved surveillance methods to track *C. difficile* transmission and persistence within a hospital environment. Researchers collected whole genome sequencing (WGS) data analysis coupled with comprehensive epidemiological records in a Liverpool hospital over a 2-year period and found that monitoring CDI cases within healthcare facilities could lead to better informed infection control and patient management systems (Kumar *et al.*, 2016).

1.3. *CLOSTRIDIUM DIFFICILE* SPORULATION AND GERMINATION

1.3.1. SPORULATION

A minority of bacterial species, including *C. difficile*, can produce specialised cell types as a means of surviving and persisting in hostile and unfavourable environments. This was the primary purpose of endospore production; however, it is now also recognised as a crucial route in disease transmission. The fact that this mechanism can allow *C. difficile* to evade the consequences of heat, oxygen, alcohol and disinfectant exposure, makes this organism extremely challenging to eradicate (Paredes *et al.*, 2005; Setlow, 2007).

The most widely studied endospore-forming organism is *Bacillus subtilis*, having been used as a model system for several decades (Errington, 2003). The formation of a highly resistant dormant cell; the endospore, is a very complex and highly regulated process, however it can be divided into seven main stages (Nicholson *et al.*, 2000) (Figure 1.2).

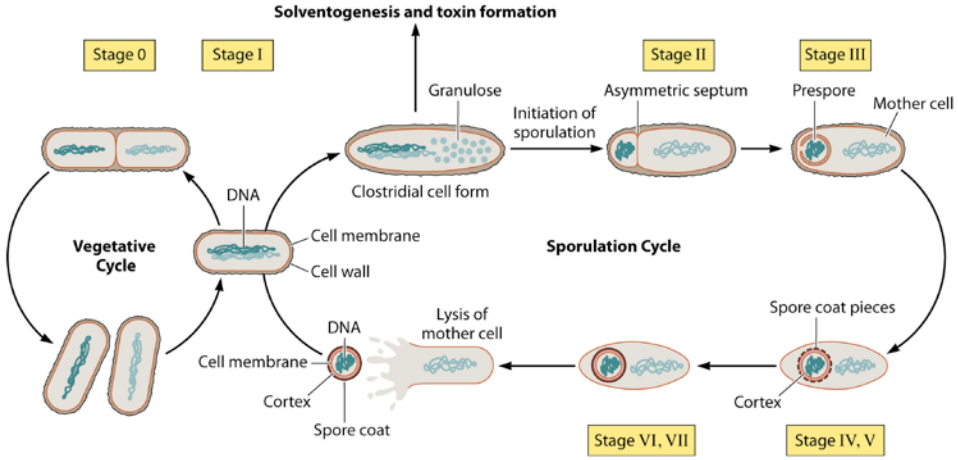


Figure 1.2 Endospore Formation (Al-Hinai *et al.*, 2015)

Endospore-forming bacteria such as *Bacillus subtilis* will form spores under hostile environmental conditions. Once granulose vesicles accumulate, the cell commences sporulation, undergoing asymmetric cell division and forming a forespore or prespore. This structure is then engulfed by the mother cell and the spore cortex and coat are formed. On completion of this process, the mother cell lyses and releases the mature endospore.

The vegetative cell, growing by binary fission, monitors a huge array of external and internal signals. No individual nutritional effect acts as a trigger in *C. difficile*, however, in *B. subtilis*, the main stimulus for sporulation is starvation (Errington, 2003; Pereira *et al.*, 2013). This response to limited nutrients is controlled by CodY, a global transcriptional regulator (Nawrocki *et al.*, 2016). Catabolite control protein A (CcpA) is an additional global transcriptional regulator that reacts to carbon and carbohydrate activity, specifically in Gram-positive bacteria (Antunes *et al.*, 2012). Firstly, growth switches to asymmetric (polar) division and the DNA coils along the central axis of the vegetative cell as an 'axial filament', to initiate spore development. The DNA then separates, and one chromosome becomes enclosed in a plasma membrane to form a protoplast. The protoplast is then engulfed by the mother cell membrane and forms an intermediate structure called a forespore or prespore. The core wall, cortex, spore coat layers and exosporium are synthesised between the two membranes (Todar, 2012). In *C. difficile* spores, the exosporium layer alone contains a total of 184 proteins (Diaz-Gonzalez *et al.*, 2015), including the collagen-like protein BclA1, which was originally isolated in *Bacillus anthracis* (Pizarro-Guajardo *et al.*, 2014). Recent research has demonstrated there are two exosporium morphotypes observed in a *C. difficile* sporulating culture and biofilm development; those that possess a thick outermost exosporium layer and those that contain a thin outermost exosporium layer (Pizarro-Guajardo *et al.*, 2016a; Pizarro-Guajardo *et al.*, 2016b). As the spore matures and water is removed, it becomes increasingly heat resistant and more refractile. The mature spore is eventually released through lysis of the mother cell. In *B. subtilis*, the entire process takes place over a 6-7 hour time period and requires the regulation of a large number of unique genes (Todar, 2012).

B. subtilis uses a phosphorelay system to initiate sporulation. It involves five sensory histidine kinases (KinA-KinE), which phosphorylate the sporulation initiation phosphotransferase (Spo0F) and the single-domain response regulator (Spo0B). The phosphoryl group is then forwarded to the master positive regulator (Spo0A). Although clostridia seem to generally share key genes of the *B. subtilis* sporulation pathway downstream of *spo0A*, the initial

phosphorelay genes are not conserved. Clostridia appeared as a separate class ~2.7 billion years ago, before the 'great oxidation' event, whereas, bacilli (which includes *B. subtilis*) diverged as a class ~ 2.3 billion years ago, at a similar time as the 'great oxidation' event. The different environmental conditions for divergence may explain the dissimilar early sporulation pathway (Errington, 2003; Battistuzzi *et al.*, 2004; Pereira *et al.*, 2013).

It has been hypothesised that the initiation of clostridial sporulation uses different signalling than that of *B. subtilis*. Three possibilities include Spo0A being phosphorylated directly by a histidine kinase, or by an unknown small-molecule phosphodonor, or it may be possible that clostridia use a novel phosphorelay system (Paredes *et al.*, 2005). The *C. difficile* 630 strain is known to encode five orphan histidine kinases (CD1352, CD1492, CD1579, CD1949, and CD2492). There is some evidence to suggest that Spo0A is directly phosphorylated and currently the orphan histidine kinase CD1579 has been shown to transfer a phosphate directly to Spo0A (Paredes-Sabja *et al.*, 2014). Furthermore, a CD2492 orphan histidine kinase deletion mutant demonstrated a 3.5-fold reduction in sporulation capability when compared to the wildtype (WT) (Underwood *et al.*, 2009). Another orphan histidine kinase, CD1492, which was similarly believed to phosphorylate Spo0A directly, has since been confirmed as a negative regulator of sporulation (Childress *et al.*, 2016). The role of the remaining orphan histidine kinases continues to be researched.

After Spo0A phosphorylation, a sequential cascade begins. Spo0A-Spo0P act as activators of stationary growth phase genes or as repressors of early expressed genes and govern the switch to asymmetric division in all spore formers (including Clostridia) (Onyenwoke *et al.*, 2004). Several key sporulation genes are transcribed, such as the *spoIIA* operon (which encodes three genes, including *spoIIAA*, *spoIIAB* and *sigF*; an early forespore-specific sigma factor σF), *spoIIE* (a phosphatase that activates SpoIIAA) and *spoIIG* (an operon that includes *sigE*; the early mother cell-specific sigma factor σE) (Errington, 2003; Paredes *et al.*, 2005). The σG (encoded by *sigG*) and σK (encoded by *sigK*) sigma factors replace σF and σE when engulfment of the forespore is completed (Pereira *et al.*, 2013; Paredes-Sabja *et al.*, 2014).

Collectively, the four sporulation sigma factors; σF, σE, σG and σK activate expression of no less than 224 genes (Fimlaid *et al.*, 2013). Two recent studies (Fimlaid *et al.*, 2015b; Serrano *et al.*, 2016a), researching σG spore morphogenesis activity discovered that *spoIIQ*, *spoIIIA*, and *spoIIIAH* mutants were unable to perform engulfment or achieved irregular engulfment of the forespore or pre-spore. Those spores which completed engulfment were then impaired in later stages of endospore production, such as in forming the spore coat or creating heat-resistant spores. The authors of both papers determined that this SpoIIQ-SpoIIIAH gene complex is necessary for spore formation. The other key positive regulator is σH (encoded by *sigH*), which interacts with the core RNA polymerase to initiate transcription from at least 49 promoters controlling 87 or more genes (Errington, 2003; Paredes *et al.*, 2005).

C. difficile lacks, or is certainly less dependent on, the intercompartmental communication between sporulation-specific sigma factors, when compared to *B. subtilis*, in proceeding with the morphological changes observed in sporulation (Pereira *et al.*, 2013; Paredes-Sabja *et al.*, 2014; Fimlaid *et al.*, 2015b). The sigma factor σK also does not require proteolytic removal of an inhibitory pro-sequence as seen in *B. subtilis* (Serrano *et al.*, 2016b).

1.3.2. GERMINATION

The irreversible process of germination and outgrowth are necessary prerequisites for pathogenesis as *C. difficile* toxins can only be produced by metabolically active vegetative cells (Borriello *et al.*, 1990; Voth and Ballard, 2005).

The current knowledge on the mechanisms of *C. difficile* germination has, again, been gathered by studying *B. subtilis*. Germination is initiated when receptors within the spore inner membrane detect specific effectors called germinants. These germinants vary by bacterial species but include ions, amino acids, sugars, nucleotides, surfactants, enzymes and/or hydrostatic pressure. The spore is then committed to germination and subsequent events include the release of monovalent cations (H^+, Na^+ and K^+), divalent cations (Ca^{2+}, Mg^{2+} and Mn^{2+}) and a large deposit (~25% of spore dry weight) of

calcium dipicolinic acid (CaDPA). The next step involves the hydrolysis of the spore cortex by one or more spore cortex lytic enzymes (SCLEs), where the low water content is returned to that of a vegetative cell. The spore core is now able to expand and allow enzyme activity, metabolism and outgrowth (Figure 1.3) (Paredes-Sabja *et al.*, 2008; Wheeldon *et al.*, 2008; Burns *et al.*, 2010a; Sorg and Sonenshein, 2010).

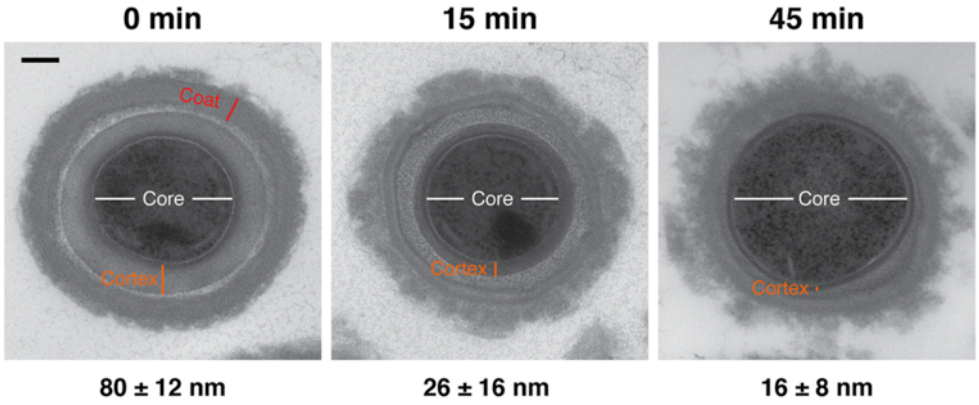


Figure 1.3 Transmission Electron Microscope (TEM) Image of a *Clostridium difficile* Germinating Spore (Fimlaid *et al.*, 2015a)

The TEM image shows *C. difficile* strain JIR8094 (an erythromycin-sensitive derivate of strain 630) spores at time point 0, 15 and 45 minutes' post-exposure to taurocholate. The scale bar defines 100 nm. Spore cortex hydrolysis and expansion of the spore core can be observed. The average cortex thickness is noted underneath each TEM image.

The specific germinants of *B. subtilis* include L-alanine, asparagine, glucose, fructose and potassium ions which bind to specific germinant receptors, such as GerA, GerB and GerK (Burns *et al.*, 2010a). Spore germination in Clostridia is less well studied. Limited research has shown that Clostridia germinate in response to certain amino acids and salts. Specifically, *C. difficile* germinates in nutrient media with bile salts (Paredes-Sabja *et al.*, 2008), such as taurocholate, cholate and deoxycholate, with equal efficiency, as well as the enzyme lysozyme (Wheeldon *et al.*, 2008). Furthermore, it has been reported that amino acids, specifically glycine, alanine and/or serine, are necessary co-germinants to initiate germination (Francis *et al.*, 2013a; Shrestha *et al.*,

2017). It is hypothesised that *C. difficile* spores interact with bile salts along the gastrointestinal tract and that there is a host-derived signal that initiates germination. However, other bile salts, such as chenodeoxycholate, as well as mouse muricholic acids (Francis *et al.*, 2013b), competitively inhibit taurocholate-mediated germination and vegetative cell outgrowth (Paredes-Sabja *et al.*, 2008; Burns *et al.*, 2010a; Sorg and Sonenshein, 2010; Francis *et al.*, 2013a).

The germinant receptors that *C. difficile* uses to detect environmental signals were unclear until recently. Although *C. difficile* lacks the *B. subtilis* GerA family of germinant receptors based on homology searches, the pseudoprotease CspC (one of the three Csp serine proteases encoded by *C. difficile*) acts as a bile acid germinant receptor (Paredes-Sabja *et al.*, 2008; Francis *et al.*, 2013a). The levels of CspC in mature endospores are controlled by the serine protease, CspA, which is necessary for efficient spore germination (Kevorkian *et al.*, 2016). It has been demonstrated that CspC directly activates CspB, which in turn processes pro-SleC, into active SleC (a SCLE, specifically, an exo-acting lytic transglycosylase that produces small disaccharides (Gutelius *et al.*, 2014)) leading to the complete degradation of the spore cortex and CaDPA release (Paredes-Sabja *et al.*, 2008; Francis *et al.*, 2013a). The fact that the spore cortex is degraded before the release of CaDPA is novel in *C. difficile* (Francis *et al.*, 2015). This release of CaDPA is also dependent on the spore cortex layer degradation and occurs via a mechanosensing mechanism (Francis and Sorg, 2016). Until very recently, the mechanism of how Csps were incorporated into spores was unknown. However, it is now recognised that the previously uncharacterised GerG protein, which is only found in *C. difficile*, facilitates this process (Donnelly *et al.*, 2017). Another novel germination regulator is GerS, a lipoprotein, which is anchored to the outer spore membrane. It was discovered to also activate SleC when secreted. The current hypothesis is that CspC still activates CspB, however, CspC inhibits the action of SleC when not in the presence of GerS (Fimlaid *et al.*, 2015a; Bhattacharjee *et al.*, 2016).

1.4. *CLOSTRIDIUM DIFFICILE* COLONISATION RESISTANCE AND ADHERENCE FACTORS

1.4.1. SURFACE LAYER AND CELL WALL PROTEINS

The *C. difficile* cell surface layer (S-layer) is composed of two S-layer protein (SLP) subunits termed P47, the high molecular weight (HMW) protein, and P36, the low molecular weight (LMW) protein (Cerquetti *et al.*, 2000). These components result from the proteolytic cleavage of the polypeptide precursor, SlpA, by the cysteine protease cell wall protein (Cwp) 84 (Dang *et al.*, 2010). The formation of this tightly-associated SLP non-covalent complex is believed to be crucial in bacterial cell adherence to host enteric cells as observed in HEp-2 cell culture *in vitro* and human gastrointestinal tract tissue sections (Calabi *et al.*, 2002; Fagan *et al.*, 2009). Current research (Lynch *et al.*, 2017) has identified a link between sequence differences in these SLPs to CDI susceptibility and CDAD severity and suggested that SLPs may have a key role in the emergence of hyper-virulent strains. Furthermore, much interest has been generated in targeting SLPs with SLP-specific single-domain antibodies as a viable therapeutic approach against CDAD (Kandalaft *et al.*, 2015).

In particular, SlpA has been deemed a major contributor to host-cell adherence. Several studies (Merrigan *et al.*, 2013; Spigaglia *et al.*, 2013) have revealed that distinct *C. difficile* strains, containing different SlpA sequences, mediate host-cell attachment with varying levels of success. In addition to this, one study (Merrigan *et al.*, 2013) further identified that crude or purified preparations containing SlpA from a non-toxigenic *C. difficile* strain prevented host cell adherence of an epidemic-associated strain.

The involvement of the cysteine protease, Cwp84, in processing the S-layer is well recognised (Kirby *et al.*, 2009; de la Riva *et al.*, 2011). Additionally, it has been reported that Cwp84 can degrade extracellular matrix (ECM) host proteins, indicating that the proteolytic activity could also promote host tissue degradation and spread of infection (Janoir *et al.*, 2007). Furthermore, a small number of studies (Dapa *et al.*, 2013; Pantaleon *et al.*, 2015) have identified an important requirement of Cwp84 in biofilm formation. Due to the continually

evolving function of this particular CWP, it is not surprising then that a patent application (Davis *et al.*, 2014) has been submitted to use Cwp84 as a sensitive and specific diagnostic marker for *C. difficile*.

1.4.2. EXTRACELLULAR MATRIX BINDING PROTEINS

C. difficile binds to various ECM proteins, such as fibronectin, fibrinogen, collagen and vitronectin, to aid in adhesion to the host (Cerquetti *et al.*, 2002). Fibronectin-binding protein (Fbp) 68 has demonstrated *in vitro* binding to host ECM proteins, including fibronectin, fibrinogen and, to a lesser extent, vitronectin (Hennequin *et al.*, 2003). The same study also revealed that antibodies raised against Fbp68 were able to partially inhibit attachment to fibronectin and prevent Vero cell binding.

There has been more recent research (Tulli *et al.*, 2013) identifying a new FBP, termed collagen-binding protein (Cbp) A. CbpA can bind with high affinity to collagens I and V, which are the most common collagen components found in gastrointestinal tissue (Awad *et al.*, 2014). Incidentally, a novel secreted zinc metalloprotease, Pro-Pro endopeptidase (PPEP-1), has been found to control adhesion through cleavage of CBPs (CD2831 in this instance) (Hensbergen *et al.*, 2015). PPEP-1 has also previously been shown to successfully cleave fibronectin and fibrinogen (Cafardi *et al.*, 2013), indicating that this degradative activity facilitates *C. difficile* pathogenesis and spread of the bacterium throughout the host (Awad *et al.*, 2014).

1.4.3. CELL-SURFACE APPARTUS

The genome of *C. difficile* encodes proteins for the formation of a type IV pilus, which is involved in adhesion, cell aggregation and biofilm development (Janoir, 2016). The major pilin of the type IV pili is PilA1 (Piepenbrink *et al.*, 2015), with a deletion mutant having been reported as forming a biofilm with diminished mass and thickness compared to the WT strain (Maldarelli *et al.*, 2016). Another study (Bordeleau *et al.*, 2015) observed that inactivation of *pilA1* and *pilB1* genes eliminated pilus formation and prevented cell aggregation, even under high levels of cyclic diguanosine

monophosphate (c-di-GMP). C-di-GMP is a bacterial messenger that initiates the conversion from free, motile individuals to a multi-cellular process.

The last virulence feature to consider is the flagella. Some *C. difficile* strains are highly motile, making the flagella a prominent part of host invasion and colonisation. The two major flagella components, FliC; the flagellin structural subunit (Tasteyre *et al.*, 2000), and FliD; the cap protein (Tasteyre *et al.*, 2001b), are the best characterised. It has been previously reported (Baban *et al.*, 2013) that the importance of flagella in colonisation is strain dependent. In the epidemic strain R20291, flagella do appear to affect colonisation and adherence. A R20291 flagellar mutant adhered less than the WT strain *in vivo*, whereas the non-epidemic strain 630Δ*erm* flagellar mutant produced the opposite effect. Several other studies have eluded to additional roles of the flagella, other than motility, including inducing a pro-inflammatory cytokine response by FliC activation of the Toll-like receptor 5 (TLR5) found on gastrointestinal epithelial cells (Batah *et al.*, 2016; Batah *et al.*, 2017). Moreover, 630 *fliC* and *fliD* mutants have been shown to produce toxin A and B to high cytotoxic levels in Vero cell lines (Dingle *et al.*, 2011). Further research (Aubry *et al.*, 2012) into the PaLoc genes, *tcdA*, *tcdB*, *tcdR* and *tcdE*, revealed a significant increase in transcription in the *fliC* mutant. *In vivo*, the *fliC* mutant strain was more virulent in the hamster model (Dingle *et al.*, 2011). A purposed hypothesis for this increased mutant virulence is the significant deregulation of expression seen in genes controlling mobility, metabolism and virulence (Barketi-Klai *et al.*, 2014).

1.5. *CLOSTRIDIUM DIFFICILE* TOXINS

1.5.1. TOXIN A AND B

The major virulence factors associated with *C. difficile* are two toxins; toxin A (308kDa) and toxin B (270kDa) which exhibit a high degree of overall similarity (66%). They are joined by *Clostridium sordellii* lethal toxin and haemorrhagic toxin and *Clostridium novyi* alpha toxin to make up the group of

large clostridial toxins (Bette *et al.*, 1991). Their respective genes (*tcdA* and *tcdB*) form part of the 19.6kb five-gene pathogenicity locus (PaLoc) (Geric *et al.*, 2006; Carter *et al.*, 2007; Carman *et al.*, 2011) along with the accessory genes; *tcdC* (negative toxin regulator), *tcdR* (encodes an RNA polymerase sigma factor, positive toxin regulator) and *tcdE* (encodes for a holin-like protein) (Figure 1.4) (Kuehne *et al.*, 2010; Vohra and Poxton, 2011). In non-toxigenic strains, the region is replaced by a highly conserved short 75 or 115bp non-coding sequence (Rupnik *et al.*, 2003; Monot *et al.*, 2015).

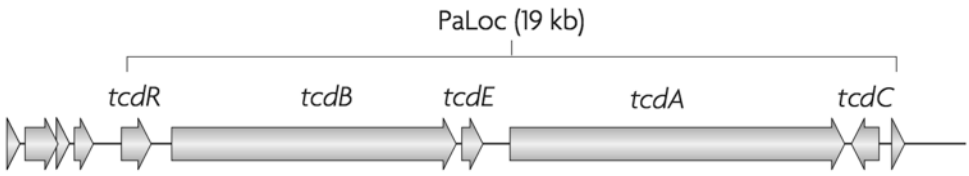


Figure 1.4 *Clostridium difficile* Pathogenicity Locus (PaLoc) (Rupnik *et al.*, 2009)

The 19.6kb PaLoc containing *tcdA* and *tcdB* which encode for *Clostridium difficile* toxin A and toxin B, respectively. Alongside these genes are the negative regulator; *tcdC*, positive regulator; *tcdR*, and *tcdE*, which encodes for a holin-like protein.

In anaerobic growth conditions and with response to various environmental stimuli, when the organism is leaving exponential phase and entering stationary phase, *tcdA*, *tcdB*, *tcdR* and *tcdE* are up-regulated, whilst *tcdC* is down-regulated (Hundsberger *et al.*, 1997; Viswanathan *et al.*, 2010). However, recent work has suggested there may be no association between the *tcdC* genotype and toxin production, and that an abnormal gene, such as the frequent 18bp deletion, is not a predictor of increased hypertoxigenicity (Matamouros *et al.*, 2007; Cartman *et al.*, 2012). Furthermore, there is a theory that toxin synthesis is additionally controlled by the accessory gene regulator (Agr) quorum-signalling system, specifically the Agr-1 locus, which is crucial for *C. difficile* virulence and pathogenesis. It is believed that this system is mediated by a small thiolactone molecule. An investigation into this

compound, identified that it was excreted in the stool of CDI patients (Darkoh *et al.*, 2015; Darkoh *et al.*, 2016).

The exact mechanism of toxin A and B in modifying host regulatory GTPases has been studied in detail, and a summary of the steps is given in Figure 1.5 **c**. The structure of toxin A and B can be defined as the ABCD model, consisting of four major functional domains. Firstly, the C-terminus of the toxin (Domain B, Figure 1.5 **a** - green) binds to the host colonic epithelial cell surface receptor. In toxin A, PACSIN2 (also termed syndapin-II) (Chandrasekaran *et al.*, 2016) and dynamin (Papatheodorou *et al.*, 2010) are involved, and in toxin B, both chondroitin sulfate proteoglycan 4 (CSPG4) (Yuan *et al.*, 2014) and Frizzled (FZD) (Tao *et al.*, 2016) are host cell receptors. Attachment to the receptor results in endocytosis of the toxin–receptor complex and endosome formation. The low pH of endosomes results in a structural change in the toxin, allowing membrane insertion. The pore-forming region (PFR) of the delivery domain (Domain D, Figure 1.5 **a** - yellow) then allows the glucosyltransferase (Domain A, Figure 1.5 **a** - red) and cysteine protease (Domain C, Figure 1.5 **a** - blue) domains to be translocated into the cytosol. The cysteine protease domain is activated by binding to inositol hexakisphosphate ($InsP_6$) and autocatalytically cleaves the toxin to release the glucosyltransferase domain into the cytosol of target cells. The Rho-family proteins are then glycosylated, causing pathogenic effects, including the disruption of epithelial barrier function and structure (Just *et al.*, 1994; Just *et al.*, 1995; Reineke *et al.*, 2007; Leslie *et al.*, 2015). The Rho proteins are 21-25kDa GTP-binding proteins which belong to the Ras superfamily (K. Aktories *et al.*, 2017). They play a key role in numerous cellular processes, including actin cytoskeleton organisation, the control of epithelial barrier function, and host immune cell signalling and migration pathways (Jank and Aktories, 2008).

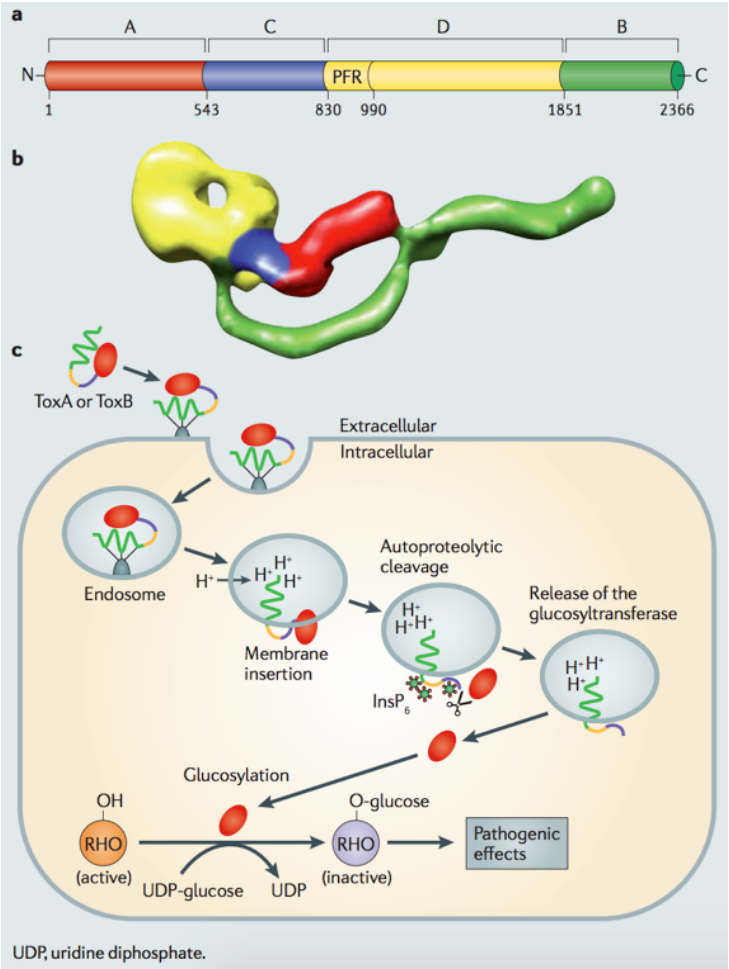


Figure 1.5 *Clostridium difficile* Toxin A and Toxin B Mechanism of Action (K Aktories, 2011)

a/b The ABCD model: amino-terminal region harbours the active glucosyltransferase domain A (red); carboxy-terminal domain B (green) is involved in binding to a cell surface receptor; cysteine protease domain C (blue); delivery domain (yellow) is involved in translocation of the toxin into the cytosol. **c** Toxin A and B translocate to the target cell cytosol after release from the bacterium. Once inside, toxin A and B act as glucosyltransferases by modifying and inactivating trimeric G protein (GTP) binding proteins such as Rho. This is achieved by using uridine diphosphate (UDP) glucose as a substrate. The result is actin condensation, cell rounding and ultimately apoptosis (Voth and Ballard, 2005).

A paper (Chumbler *et al.*, 2016) reporting the crystal structure of Toxin A discovered that the presence of zinc was necessary for toxin autoprocessing and a surface loop of the delivery domain acted as a support for the hydrophobic α-helices concerned with pore formation. A recent study (Li *et al.*, 2015) determined that for toxin B-mediated cytopathic and cytotoxic

effects in CDAD, the glycosyltransferase activity, but not the cysteine protease activity, was essential.

The evidence as to whether one or both toxins are necessary for virulence, and subsequently disease, is still being disputed. Early research (Riegler *et al.*, 1995) demonstrated that the human colonic epithelium is ten times more susceptible to the detrimental consequences of toxin B than toxin A. In a hamster infection model, one study (Lyras *et al.*, 2009) found that toxin B and not toxin A is essential for virulence, with toxin A-negative, toxin B-positive *C. difficile* strains reported as the cause for a number of hospital-associated CDAD outbreaks (Al-Barrak *et al.*, 1999; Alfa *et al.*, 2000; Kuijper *et al.*, 2001; Sato *et al.*, 2004) and the source for a severe PMC case in an immunocompromised patient (Limaye *et al.*, 2000). However, more recent research (Kuehne *et al.*, 2010; Kuehne *et al.*, 2014) demonstrated that virulent strains can produce either toxin on its own or mutually together to cause disease. Although this suggests that both toxins need to be targeted in preventative and therapeutic approaches (Savidge *et al.*, 2003), it is important to note that there has been no evidence to date of a toxin A-positive, toxin B-negative *C. difficile* strain causing a clinical outbreak.

1.5.2. BINARY TOXIN

Some *C. difficile* strains, including the BI/NAP1/027 strains, also produce a third toxin; *C. difficile* transferase (CDT) binary toxin (Carter *et al.*, 2007). CDT is a member of the Clostridial binary toxins that also include the C2 toxin from *C. botulinum* types C and D, the iota toxin from *C. perfringens* type E and the *Clostridium spiroforme* iota-like toxin (Perelle *et al.*, 1997; Rupnik *et al.*, 2003). In particular, the *C. perfringens* and *C. spiroforme* binary toxins have many conserved protein components when compared to *C. difficile* CDT, which suggests a shared evolutionary route (Barth *et al.*, 2004). CDT was originally discovered in 1988 from a single *C. difficile* strain (CD196) (Popoff *et al.*, 1988; Perelle *et al.*, 1997), however, the possible clinical significance of binary toxin producing strains was only recognised more recently (Rupnik *et al.*, 2003). CDT genes are found in between 17% to 23% of *C. difficile* isolates, and it has been speculated that their presence,

alongside toxins A and B, is associated with increased disease severity (Geric *et al.*, 2006; Eckert *et al.*, 2015).

CDT^+ isolates are those that express CDT and contain the *cdtR*, *cdtA* and *cdtB* genes, which together form the 6.2kb CDT locus (CdtLoc) (Figure 1.6 **a**) (Carter *et al.*, 2007). Isolates that are known to carry the CdtLoc but CDT expression has only been assumed and not proven, are termed $CDT^{(+)}$. With regards to CDT^- isolates, there are two separate genotypes. Some CDT^- isolates carry the *cdtR* gene, but truncated fragments of *cdtA* and *cdtB* called pseudogenes. This non-functional CDT locus is termed the 'ghost' CDT locus (Stare *et al.*, 2007; Carman *et al.*, 2011). For example, in strain 630, parts of the 5'-end of the *cdtA* gene and 3'-end of the *cdtB* gene are conserved, however, around 2kb of the CDT sequence is missing (Figure 1.6 **b**) (Carter *et al.*, 2007). There appears to be the same 2kb deletion in strain VPI 10463 (Chang and Song, 2001). The other CDT^- isolates have the entire CdtLoc replaced by a conserved 68bp sequence (Figure 1.6 **c**) (Carter *et al.*, 2007; Carman *et al.*, 2011).

Expression of the *cdtA* and *cdtB* genes by *C. difficile* is positively regulated by CdtR (30kDa, encoded by *cdtR*). CdtR has been identified as a functional LytTR family response regulator (Carter *et al.*, 2007). It shares 28% amino acid sequence identity with *Staphylococcus aureus* AgrA (accessory gene regulator A), also from the LytTR family of response regulators. A recent study (Bilverstone *et al.*, 2017) has identified the importance of the transcriptional regulator by demonstrating that *cdtR* directly affects expression of *cdtA* and *cdtB*, and was required for CDT-mediated cytotoxicity. This research confirmed previous findings in which expression of *cdtA* and *cdtB* was increased 15-fold in the presence of *cdtR* (Carman *et al.*, 2011).

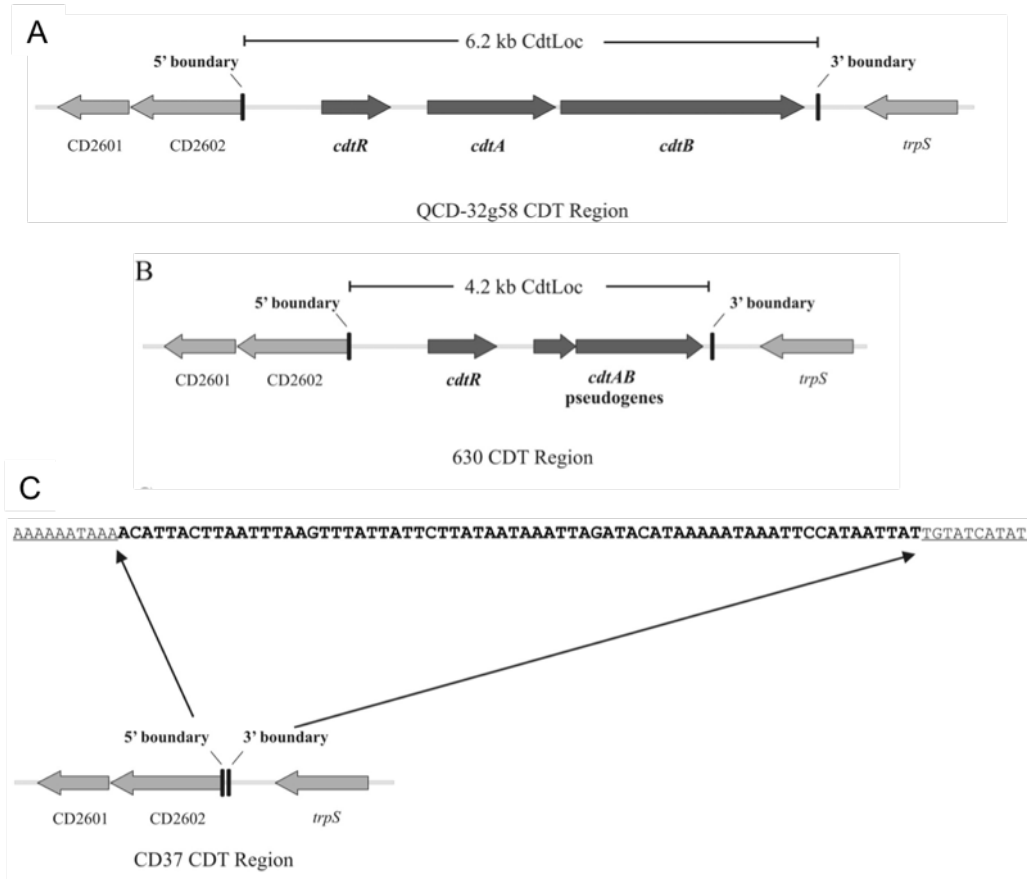


Figure 1.6 *Clostridium difficile* Transferase (CDT) Binary Toxin Locus (CdtLoc) (Carter *et al.*, 2007)

The complete 6.2kb CdtLoc containing the *cdtR*, *cdtA* and *cdtB* genes for the CDT toxin positive strain QCD-32g58 (**a**), the incomplete 4.2kb 'ghost' CdtLoc comprising of *cdtR* and pseudogenes of *cdtA* and *cdtB* for the 630 strain (**b**) and the non-toxigenic 68bp nucleotide sequence for strain CD37 (**c**) are shown. The 5' flanking genes CD2601 and CD2602 and the 3' flanking gene *trpS* are also illustrated.

CDT acts as an actin-specific ADP ribosyltransferase and consists of two unlinked components, both of which are necessary for toxic activity (Figure 1.7) (Carter *et al.*, 2007; Carman *et al.*, 2011).

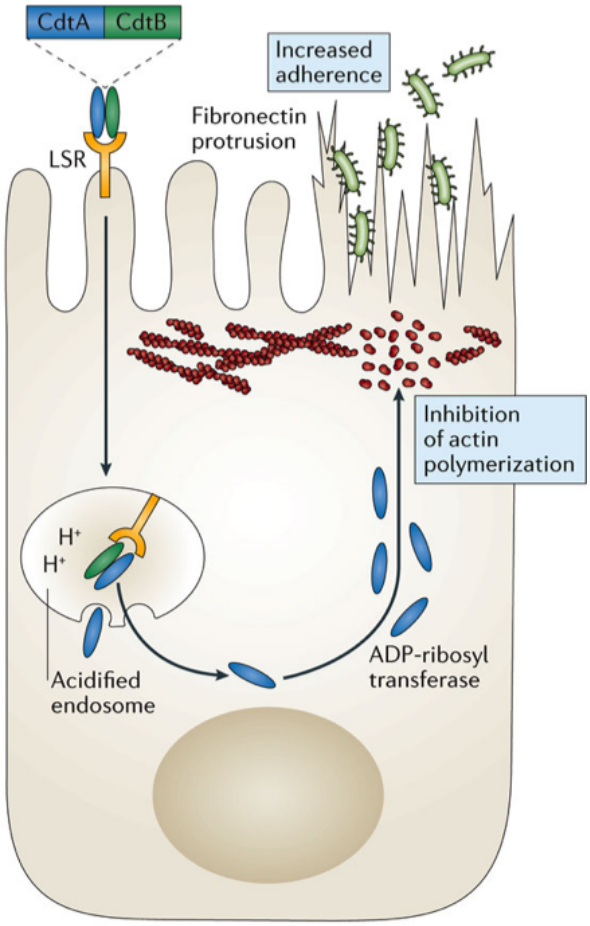


Figure 1.7 *Clostridium difficile* Transferase (CDT) Binary Toxin Mechanism of Action (Abt *et al.*, 2016)

CDT binds to the lipolysis-stimulated lipoprotein receptor (LSR) and is internalised into the cell. The CdtB component can form translocation pores in the endosome membrane, allowing the CdtA subunit to be released into the cytosol. The outcome is inhibition of actin polymerisation through the activity of the ADP-ribosyltransferase, as well as the production of microtubule protrusions containing fibronectin, which increase bacterial adherence.

CdtB (the binding component, encoded by *cdtB*) binds to the extracellular Ig-like domain of the lipolysis-stimulated lipoprotein receptor (LSR), which is thought to be the main host receptor for cell entry (Hemmasi *et al.*, 2015). The presence of CDT induces clustering of the LSR into lipid rafts (Papatheodorou *et al.*, 2011; Papatheodorou *et al.*, 2013). This process leads to the internalisation of CdtA (the enzymatic component, encoded by *cdtA*) into the cytosol (Carter *et al.*, 2007; Carman *et al.*, 2011), which occurs due to the formation of a transformation pore in the endosomal membrane (Roeder *et al.*,

2014). The membrane translocation of CdtA is facilitated by cyclophilin (Cyp) A and chaperone heat shock protein (Hsp) 90 (Kaiser *et al.*, 2011). In the cytosol, CdtA catalyses the ADP-ribosylation of monomeric actin (Carter *et al.*, 2007; Carman *et al.*, 2011), specifically modifying arginine-177 and blocking actin polymerisation (Schwan and Aktories, 2017). The result is disruption of the actin cytoskeleton and metabolic processes, which leads to cell death (Carter *et al.*, 2007; Carman *et al.*, 2011). ADP-ribosylating activity can be detected in supernatant culture after 2 hours of growth and production is independent of sporulation (Rupnik *et al.*, 2003). CDT can also cause microtubule-based protrusions from enterocytes. It is thought that septins guide the formation of the microtubule protrusions by accumulating at specific protrusion sites, creating 'collar-like' base structures (Nolke *et al.*, 2016). Stim-1-dependent calcium signalling is also increased, with rerouting of Rab-5 and Rab-11 positive fibronectin-containing vesicles. Upon release of the fibronectin, the proteins go on to promote colonisation and increase adherence of *C. difficile* bacteria (Schwan *et al.*, 2009; Schwan *et al.*, 2014).

1.6. PROJECT AIMS

C. difficile remains the most common cause of nosocomial diarrhoea in the developed world. One of the least studied features of *C. difficile* is the binary toxin that some strains carry. This feature is thought to be linked to disease severity, enhanced bacterial virulence and patient recurrence risk (Bacci *et al.*, 2011; Cowardin *et al.*, 2016; Pilate *et al.*, 2016; Reigadas *et al.*, 2016). The binary toxin was the central focus of the study, which was divided into two major components. Firstly, it was necessary to analyse the molecular basis of regulation and mechanism of binary toxin in *C. difficile*. This was achieved through the subsequent aims:

- Create the following in-frame deletion mutants via Allelic Exchange Gene Knockout: R20291Δ*cdtR*, R20291Δ*cdtA*Δ*cdtB* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*.

- Create the following in-frame deletion control mutants via Allelic Exchange Gene Knockout: R20291ΔPaLoc, R20291ΔPaLocΔ*cdtR*, R20291ΔPaLocΔ*cdtA*Δ*cdtB* and R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB*.

- Assess transcriptional linkage of the binary toxin locus by reverse transcription PCR (RT-PCR) to identify promoters and use this knowledge to create complementations of the CDT in-frame deletion mutants using a 2-step method.

- Characterise the mutant, complemented and control mutant strains by assessing their effect on virulence and colonisation factors, focusing on CDT toxin production, growth rates and biofilm formation.

Secondly, through the Rifaximin for Preventing Relapse of *Clostridium difficile* Associated Diarrhoea (RAPID) clinical study with the Nottingham Digestive Diseases Centre Biomedical Research Unit (NDDCBRU), the molecular and phenotypic basis of *C. difficile* binary toxin, as well as other virulence factors such as sporulation and antibiotic resistance, were analysed. The aims for the characterisation of the RAPID clinical study isolates were:

- Compare the CDT or ghost CDT locus of all RAPID study clinical isolates.

- Characterise the RAPID study clinical isolates in terms of sporulation using a created ClosTron mutant CD630_2635 as an intermediate control.

- Investigate rifaximin and fidaxomicin antibiotic resistance in the RAPID study clinical isolates.

The importance of this research and studying the impact of binary toxin, as well as other virulence factors, on patient clinical outcome are crucial to predicting and preventing severe CDAD and recurrent *C. difficile* episodes.

CHAPTER 2. MATERIALS AND METHODS

2.1. MATERIALS

Unless stated otherwise, bacterial growth media was sourced from Oxoid and antibiotics and chemicals were purchased from Sigma-Aldrich or Thermo Fisher Scientific. All reagents used in molecular techniques, such as enzymes and buffers were obtained from New England BioLabs (NEB) or Promega.

2.2. BACTERIAL STRAINS

All bacterial strains that were used in this research are listed in Table 2.1.

Table 2.1 Bacterial Strains

Strain	Properties	Source/Reference
***Escherichia coli* TOP10**	Cloning host strain F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G λ-	Invitrogen
***E. coli* CA434**	Conjugation donor strain *E. coli* HB101 [F$^-$ *mcrB mrr hsdS20*(r_B^- m_B^-) *recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20*(Sm^R) *glnV44* λ$^-$] with plasmid R702 (Cartman *et al.*, 2012)	(Williams *et al.*, 1990)
***Clostridium difficile* 630**	Ribotype 012 Wild type Outbreak strain from hospital patient with severe pseudomembranous colitis (PMC); Zurich, Switzerland	(Wüst *et al.*, 1982)
C. difficile* 630Δ*erm	Ribotype 012 Erythromycin sensitive derivative of *C. difficile* 630 strain	(Hussain *et al.*, 2005)
C. difficile* 630Δ*erm*Δ*spo0A	Ribotype 012 Erythromycin sensitive derivative of *C. difficile* 630 strain with *spo0A* allelic exchange in-frame deletion	(Ng *et al.*, 2013)

***C. difficile* 630Δ*erm*::CT26350**	*C. difficile* 630Δ*erm* gene 26350 ClosTron insertional mutant Gene 26350: Putative spore envelope protein (insertion site 522/523s)	This Study
***C. difficile* R20291**	Ribotype 027 Wild type Outbreak strain; Stoke Mandeville hospital, U.K.	Jon Brazier, Anaerobe Reference Laboratory, Cardiff, U.K.
C. difficile* R20291Δ*pyrE	Ribotype 027 *pyrE* deficient *C. difficile* R20291 strain for allelic exchange manipulation	(Ng *et al.*, 2013)
C. difficile* R20291Δ*pyrE*Δ*cdtR	*pyrE* deficient *C. difficile* R20291 strain with *cdtR* allelic exchange in-frame deletion CdtR: LytTR response regulator (size 747bp, genome position 2918789-2919535)	This Study
C. difficile* R20291Δ*pyrE*Δ*cdtA*Δ*cdtB	*pyrE* deficient *C. difficile* R20291 strain with *cdtA* and *cdtB* allelic exchange in-frame deletion CdtA: ADP-ribosyltransferase subunit (size 1,392bp, genome position 2919925-2921316) CdtB: ADP-ribosyltransferase binding protein (size 2,631bp, genome position 2921369-2923999)	This Study
C. difficile* R20291Δ*pyrE*Δ*cdtR*Δ*cdtA*Δ*cdtB	*pyrE* deficient *C. difficile* R20291 strain with *cdtR*, *cdtA* and *cdtB* allelic exchange in-frame deletion	This Study
C. difficile* R20291Δ*cdtR	*pyrE* restored *C. difficile* R20291 strain with *cdtR* allelic exchange in-frame deletion	This Study
C. difficile* R20291Δ*cdtA*Δ*cdtB	*pyrE* restored *C. difficile* R20291 strain with *cdtA* and *cdtB* allelic exchange in-frame deletion	This Study
C. difficile* R20291Δ*cdtR*Δ*cdtA*Δ*cdtB	*pyrE* restored *C. difficile* R20291 strain with *cdtR*, *cdtA* and *cdtB* allelic exchange in-frame deletion	This Study
***C. difficile* R20291Δ*pyrE*ΔPaLoc**	*pyrE* deficient *C. difficile* R20291 strain with PaLoc allelic exchange in-frame deletion PaLoc: Toxin A and Toxin B Pathogenicity Locus (size 19.6kb, genome position 706660-724127)	This Study

C. difficile* R20291Δ*pyrE*ΔPaLocΔ*cdtR	*pyrE* deficient *C. difficile* R20291 strain with PaLoc and *cdtR* allelic exchange in-frame deletions	(Bilverstone *et al.*, 2017)
C. difficile* R20291Δ*pyrE*ΔPaLocΔ*cdtA*Δ*cdtB	*pyrE* deficient *C. difficile* R20291 strain with PaLoc and *cdtA* and *cdtB* allelic exchange in-frame deletions	This Study
C. difficile* R20291Δ*pyrE*ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB	*pyrE* deficient *C. difficile* R20291 strain with PaLoc and *cdtR*, *cdtA* and *cdtB* allelic exchange in-frame deletions	This Study
***C. difficile* R20291ΔPaLoc**	*pyrE* restored *C. difficile* R20291 strain with PaLoc allelic exchange in-frame deletion	(Bilverstone *et al.*, 2017)
C. difficile* R20291ΔPaLocΔ*cdtR	*pyrE* restored *C. difficile* R20291 strain with PaLoc and *cdtR* allelic exchange in-frame deletions	(Bilverstone *et al.*, 2017)
C. difficile* R20291ΔPaLocΔ*cdtA*Δ*cdtB	*pyrE* restored *C. difficile* R20291 strain with PaLoc and *cdtA* and *cdtB* allelic exchange in-frame deletions	This Study
C. difficile* R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB	*pyrE* restored *C. difficile* R20291 strain with PaLoc and *cdtR*, *cdtA* and *cdtB* allelic exchange in-frame deletions	This Study
C. difficile* R20291Δ*cdtR*cdtR	*pyrE* restored *C. difficile* R20291 strain with *cdtR* complemented at the *pyrE* locus	This Study
C. difficile* R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*cdtR*cdtA*cdtB	*pyrE* restored *C. difficile* R20291 strain with *cdtR*, *cdtA* and *cdtB* complemented at the *pyrE* locus	This Study
C. difficile* R20291::CT*cdtB	*C. difficile* R20291 *cdtB* ClosTron insertional mutant	(Kuehne *et al.*, 2014)
C. difficile* R20291_*ermB	Macrolide, lincosamide and streptogramin (MLS) resistant *C. difficile* R20291 strain	(Kelly *et al.*, 2016)
***C. difficile* R20291_*ermB*_GT**	Fidaxomicin intermediate *C. difficile* R20291 strain (MIC 2µg/ml)	Kuehne *et al*., under review
***C. difficile* R20291_*ermB*_TG**	Fidaxomicin intermediate *C. difficile* R20291 strain (MIC 8µg/ml)	Kuehne *et al*., under review
***C. difficile* R20291_*ermB*_TA**	Fidaxomicin resistant *C. difficile* R20291 strain (MIC >32µg/ml)	Kuehne *et al*., under review
***C. difficile* RT002_A7**	Rifaximin sensitive *C. difficile* RT002 strain (MIC >0.5µg/ml)	(Stevenson *et al.*, 2016)
***C. difficile* RT002_F1**	Rifaximin intermediate *C. difficile* RT002 strain (MIC	(Stevenson *et al.*, 2016)

	≥16µg/ml)	
***C. difficile* CD37**	Ribotype 009	(Smith *et al.*, 1981)
***C. difficile* M120**	Ribotype 078	Brendan Wren, London School of Hygiene and Medicine (LSHTM), U.K.
***C. difficile* 2007855**	Ribotype 027	Lisa Dawson, LSHTM, U.K.
***C. difficile* BI-1**	Ribotype 027	Lisa Dawson, LSHTM, U.K.
***C. difficile* BI-9**	Ribotype 001	Trevor Lawley, Welcome Trust Sanger Institute, Cambridgeshire, U.K.
***C. difficile* CD196**	Ribotype 027	Trevor Lawley, Welcome Trust Sanger Institute, Cambridgeshire, U.K.

2.3. PLASMIDS

The plasmid vectors that were used in this research are listed in Table 2.2.

Table 2.2 Plasmids

Plasmid	Properties	Source/Reference
pMTL-YN4	Allelic exchange knock-out vector for *C. difficile* R20291 strain Main components: *catP*, *pyrE*, ColE1, TraJ, *lacZ* containing a multiple cloning site (MCS), T1, T2, RepA and Orf2 Size: 5852bp	(Ng *et al.*, 2013)
pMTL-YN4_*cdtR* KOC	pMTL-YN4 vector containing *cdtR* deletion insert	This Study
pMTL-YN4_*cdtA*_*cdtB* KOC	pMTL-YN4 vector containing *cdtA* and *cdtB* deletion insert	This Study
pMTL-YN4_*cdtR*_*cdtA*_*cdtB* KOC	pMTL-YN4 vector containing *cdtR*, *cdtA* and *cdtB* deletion insert	This Study
pMTL-YN4_PaLoc KOC	pMTL-YN4 vector containing PaLoc deletion insert	(Bilverstone *et al.*, 2017)
pMTL-YN2	Allele-coupled exchange (ACE) vector for *pyrE* restoration	(Ng *et al.*, 2013)

	Main components: *catP*, ColE1, TraJ, left-hand homology arm (LHA) with R20291 *pyrE* gene missing 50 nucleotides at the 5' end and 235bp at the 3' end, right-hand homology arm (RHA) with 1200bp region immediately downstream of the *pyrE* gene, RepA and Orf2 Size: 6597bp	
pMTL-YN18	ACE vector to create a *pyrE* deletion in the *C. difficile* R20291 strain Main components: pMTL-YN2 vector with a *lacZ* containing a MCS Size: 6640bp	(Ng *et al.*, 2013)
pMTL-YN18_*cdtA*_*cdtB*	pMTL-YN18 vector containing *cdtA* and *cdtB* complemented insert *cdtA* gene missing 700 nucleotides at the 5' end	This Study
pMTL-YN2C	ACE vector for *pyrE* restoration and insertion of a complemented sequence	(Ng *et al.*, 2013)
pMTL-YN2C_PR_*cdtR*	pMTL-YN2C vector containing the promoter region (PR) and *cdtR* complemented insert (PR contained within 273bp downstream of *cdtR* gene)	(Bilverstone *et al.*, 2017)
pMTL-YN2C_IGS_*cdtA*	pMTL-YN2C vector containing the intergenic spacer (IGS) region and *cdtA* complemented insert (IGS region between *cdtR* and *cdtA*) size: 389bp	This Study
pMTL-YN2C_PR_*cdtR*_*cdtA*	pMTL-YN2C vector containing the PR, *cdtR* and *cdtA* complemented insert (PR contained within 700bp downstream of *cdtR* gene)	This Study
pMTL007C-E2	Second generation modular ClosTron plasmid Main components: *fdx* promoter from *Clostridium sporogenes* to drive expression of the Group II intron, *Hind*III and *BsrG*I restriction sites and a *lacZ* stuffer sequence that is replaced during intron retargeting (Cartman *et al.*, 2010) Size: 8926bp	(Heap *et al.*, 2010)
pMTL007C-E2::26350	pMTL007C-E2 ClosTron vector retargeted to *C. difficile* 630 gene 26350	This Study

2.4. MICROBIOLOGICAL METHODS

2.4.1. BACTERIAL STORAGE CONDITIONS

E. coli strains were kept at -80°C in sterile cryogenic tubes with beads in a hypertonic preservative solution consisting of phosphate buffered saline (PBS) and supplemented with sucrose, glycerol and peptone (Microbank Bacterial Preservation System, Pro-Lab Diagnostics). *C. difficile* strains were kept at -80°C as a combination of vegetative cells and spores, resuspended in brain heart infusion (BHIS) broth (14.8g brain heart infusion/2.0g yeast extract/0.4g L-cysteine/400ml deionised water) and supplemented with 10% (v/v) filter-sterilised glycerol.

2.4.2. AEROBIC BACTERIAL CULTURE CONDITIONS

E. coli strains were grown at 37°C aerobically on Luria-Bertani (LB) agar or in broth (10.0g tryptone extract/5.0g yeast extract/5.0g NaCl/1L deionised water), with shaking at 200rpm (New Brunswick Scientific Innova 40, Eppendorf), supplemented with chloramphenicol (25mg/ml in 100% ethanol, agar working concentration 25µg/ml, broth 12.5µg/ml) when necessary. The *E. coli* TOP10 strain was used as a cloning host throughout, with *E. coli* CA434 acting as a donor strain when transferring DNA to *C. difficile* by conjugation.

2.4.3. ANAEROBIC BACTERIAL CULTURE CONDITIONS

C. difficile strains were grown on either BHIS agar (18.8g brain heart infusion agar/2.0g yeast extract (5mg/ml)/0.4g L-cysteine (0.1%)/400ml deionised water) or in BHIS broth (14.8g brain heart infusion/2.0g yeast extract/0.4g L-cysteine/400ml deionised water) (Burns *et al.*, 2011). *C. difficile* selective supplement (cycloserine 250µg/ml and cefoxitine 8µg/ml) was added to the media when required. All strains were incubated at 37°C in a MG1000 anaerobic workstation (Don Whitley Scientific) with an atmosphere containing 80% (v/v) nitrogen, 10% (v/v) hydrogen and 10% (v/v) carbon

dioxide. Prior to inoculation, all liquid media was pre-reduced overnight, and agar was incubated anaerobically for a minimum of 4 hours before use.

2.4.4. PREPARATION OF ELECTROCOMPETENT *ESCHERICHIA COLI* CELLS

To prepare electrocompetent cells for use in *E. coli* transformation, 200mls of 37ºC pre-warmed LB broth in a 500ml conical flask was inoculated with a 1ml overnight *E. coli* culture. The 200ml culture was incubated at 37ºC, with shaking at 200rpm (New Brunswick Scientific Innova 40, Eppendorf), until an optical density reading at 600nm (OD_{600}) of between 0.5 and 0.7 was reached, indicating the cells were in exponential growth phase. The cells were equally distributed between 50ml falcon tubes, to a maximum of 40ml, and cooled on ice for 30 minutes. After this time, the falcon tubes were centrifuged at 2,500g (Allegra X-30R, Beckman Coulter) for 10 minutes at 4ºC. The supernatant was gently poured off, so as not to disturb the cell pellet, and two pellets were resuspended in 40ml of ice-cold, sterile distilled water (dH_2O). The cells were then pelleted with the same centrifugation conditions as before. Resuspension of the cell pellets was performed twice more in 20ml and 10ml of ice-cold sterile dH_2O, respectively, using the centrifugation conditions mentioned above. The supernatant was discarded between each resuspension. Finally, the cell pellets were resuspended in 1ml of 1mM 4-Morpholinepropanesulfonic acid (MOPS) with 10% (v/v) filter-sterilised glycerol and stored at -80ºC in 50µl aliquots.

2.4.5. TRANSFORMATION VIA ELECTROPORATION

Plasmids were transformed into electrocompetent *E. coli* TOP10 and CA434 via electroporation at 2.5kV voltage, using the MicroPulser electroporator (Bio-Rad). Briefly, 4µl of plasmid combined with a 50µl aliquot of electrocompetent cells (previously thawed on ice) was transformed in a pre-frozen 2mm gap electroporation cuvette (Sigma). The transformation mixture was then immediately incubated in 400µl of super optimal with catabolite repression (SOC) medium (Invitrogen) at 37°C, with 200rpm shaking (New Brunswick Scientific Innova 40, Eppendorf) for a 2-hour recovery period.

After this time, transformed cells were selected for by inoculating the mixture onto LB agar supplemented with chloramphenicol at neat, 1/10 and 1/100 serial dilutions in SOC.

2.4.6. TRANSFER OF PLASMID DNA VIA CONJUGATION

The plasmids were transferred from *E. coli* CA434 donor cells to the *C. difficile* recipient cells through conjugation as previously described (Heap *et al.*, 2009). Firstly, the CA434 donor containing the required plasmid and the *C. difficile* recipient were grown overnight in liquid broth. The following day, 2ml of the donor overnight culture was centrifuged at 1,100g (Benchtop Centrifuge 541R, Eppendorf) for 1 minute, and the pellet was resuspended in 400µl of phosphate buffered saline (PBS), twice, to wash the cells of antibiotic. The pelleted cells were then transferred to the anaerobic cabinet and combined with 200µl of recipient cells. This mixture was applied in 20µl distinct spots onto BHIS agar, containing no antibiotic, and incubated for 24 hours to allow plasmid DNA transfer to take place. After this time, all bacterial conjugal growth was resuspended in 500µl of PBS and plated onto five BHIS agar plates supplemented with *C. difficile* selective supplement and thiamphenicol (15mg/ml in 50% ethanol, working concentration 15µg/ml). The plates were incubated anaerobically for between 24 and 72 hours, and periodically examined for transconjugant growth. Potential transconjugant colonies were then sub-cultured onto the same selective media for purification and further analysis.

Alterations were made to the protocol following work published more recently (Kirk and Fagan, 2016), which increased R20291 conjugation efficiency. The *C. difficile* recipient strains were initially grown in TY broth (12g bacto-typtose/8g yeast extract/0.4g thioglycolate/400ml deionised water) and the overnight culture was heat-shocked at 52°C for 5 minutes prior to conjugation. Furthermore, the donor/recipient mixture was inoculated onto BHI agar (18.8g brain heart infusion agar/2.0g yeast extract/400ml deionised water) to recover the highest number of transconjugants achievable.

2.4.7. RIFAXIMIN FOR PREVENTING RELAPSE OF *CLOSTRIDIUM DIFFICILE* ASSOCIATED DIARRHOEA CLINICAL STUDY

2.4.7.1. STOOL SAMPLE COLLECTION

The following Rifaximin for Preventing Relapse of *Clostridium difficile* Associated Diarrhoea (RAPID) clinical study stool samples were received for each patient: the initial sample taken, used to diagnose the patient with CDAD (termed week -1); the sample after successful treatment with either metronidazole or vancomycin and a 4-week treatment of 'follow on' rifaximin (2x 200mg tablets three times a day for the 1st two weeks followed by 1x 200mg tablet three times a day for the 2nd two weeks) or a placebo (termed week 0); and two check-up samples at week 4 and 12. If the patient relapsed before the check-up samples were taken, this relapse sample was received instead. Appendix 5 details the RAPID clinical trial experimental protocol and ethical approval (Spiller *et al.*, 2012).

2.4.7.2. ISOLATION OF *CLOSTRIDIUM DIFFICILE* FROM STOOL SAMPLES

All initial culture of the stool samples for isolation of *C. difficile* was completed in the Queen's Medical Centre (QMC) Nottingham Digestive Diseases Centre Biomedical Research Unit (NDDCBRU) laboratory using a purpose-installed DG250 anaerobic workstation (Don Whitley Scientific) (Figure 2.1). The isolation of *C. difficile* from the 58 RAPID stool samples was conducted by myself, Dr Emma Stevenson, Dr Carolyn Meaney, Dr Patrick Budd, and fellow Ph.D. student Lorna Finch. *C. difficile* was isolated on Braziers agar (19.2g Braziers agar/16ml egg yolk emulsion (4%)/400ml deionised water) supplemented with *C. difficile* selective supplement. Briefly, the stool was mixed with PBS 1:1 and heat-shocked at 80°C for 15 minutes, before being centrifuged at 1,500g (Benchtop Centrifuge 5414R, Eppendorf) for 5 minutes. This protocol was adapted from (Hink *et al.*, 2013a), who determined that the heat-shock method yielded the best recovery of *C. difficile* when compared to ethanol-shock and direct plating out. After successful heat-shock, 50µl of the supernatant was plated in triplicate and incubated for 24 to

48 hours. After this time, ≤20 *C. difficile* colonies from each stool were identified by a lack of opacity around the colonies on the egg-yolk-based Braziers agar. This is due to an absence of lecithinase, which differentiates *C. difficile* from *Clostridium bifermentans*, *Clostridium perfringens* or *Clostridium sordellii* (Public Health England, 2018). Confirmed colonies were cultured into individual wells (200µl BHIS broth) of a Nunc MicroWell 96-well plate (Thermo Fisher Scientific) and incubated for a further 24 hours. Finally, the BHIS broth cultures were prepared for in-house ribotyping by diluting 10-fold (20µl into 180µl sterile polymerase chain reaction (PCR) grade water) in a fresh 96-well plate and 10% (v/v) glycerol was added to the remaining cultures for storage at -80°C. Once 10% (v/v) glycerol frozen stocks were made, all further characterisation investigations were conducted in the Synthetic Biology Research Centre (SBRC) laboratory.

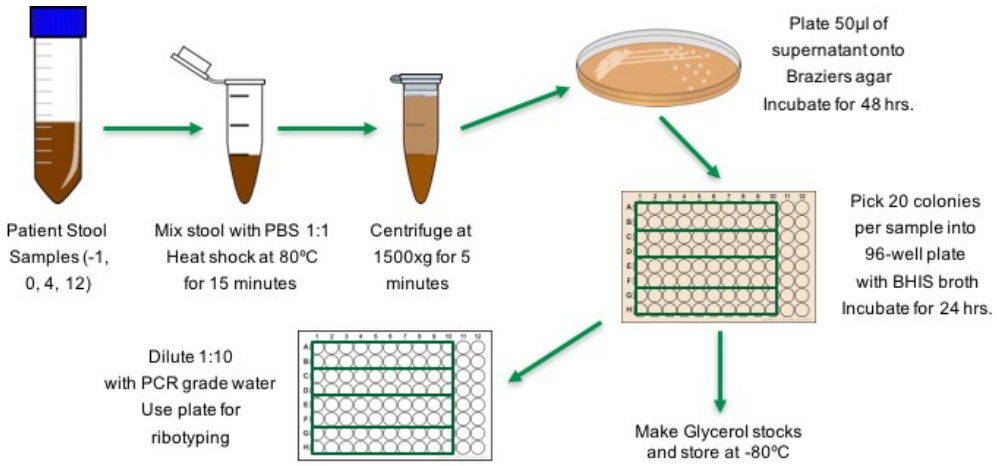


Figure 2.1 *Clostridium difficile* Isolation from Stool Samples

C. difficile was isolated on Braziers agar (19.2g Braziers agar/16ml egg yolk emulsion (4%)/400ml deionised water) supplemented with *C. difficile* selective supplement. Briefly, the stool was mixed with PBS 1:1, heat-shocked at 80°C for 15 minutes and centrifuged at 1,500g (Benchtop Centrifuge 5414R, Eppendorf) for 5 minutes. Then, 50µl of the supernatant was plated in triplicate and incubated for 24 to 48 hours. After this time, ≤20 colonies from each stool were cultured into individual wells (200µl BHIS broth) of a Nunc MicroWell 96-well plate (Thermo Fisher Scientific) and incubated for a further 24 hours. Finally, the BHIS broth cultures were prepared for ribotyping by diluting 10-fold (20µl into 180µl sterile polymerase chain reaction (PCR) grade water) in a fresh 96-well plate and 10% (v/v) glycerol was added to the remaining cultures for storage at -80°C.

2.4.7.3. RIBOTYPING

All in-house ribotyping was completed by Dr Emma Stevenson (Stevenson *et al.*, 2016). The 16S rRNA intergenic spacer region was amplified via PCR and carried out according to a modified protocol obtained from the former *C. difficile* ribotyping laboratory in Cardiff. Ribotyping profiles were analysed with a QIAxcel capillary electrophoresis machine (Qiagen) using the OL400 program. Both a QX 15bp to 1kb alignment marker and a QX 50bp to 800bp size marker were used in each ribotyping run. The individual profiles were assessed to determine if coinfection had occurred. One isolate from each distinct ribotyping profile was re-cultured onto BHIS agar containing *C. difficile* supplement and then stored at -80°C as a 10% (v/v) glycerol stock.

All official *C. difficile* ribotyping was performed at the *Clostridium difficile* Ribotyping Network (CDRN) Service situated in Leeds. The isolate was grown on a Columbia Blood Agar Base (Oxoid) and incubated anaerobically for 72 hours. After this time, all growth was placed on a Amies Transport Medium swab (Oxoid) containing charcoal, and transported to Leeds as a UN 3373 Biological Substance Category B classification package.

2.5. PRIMERS

Primers utilised in this research were designed manually in Microsoft Word. The reverse complement program (www.bioinformatics.org) was used to produce 5' to 3' sequences for the reverse primers. The melting temperature (Tm) and GC content for each primer was verified by the OligoAnalyzer 3.1 program (Integrated DNA Technologies). All primers were purchased from Sigma-Aldrich and are listed in Table 2.3, Table 2.4, Table 2.5, Table 2.6, Table 2.7, Table 2.8, Table 2.9, Table 2.10 and Table 2.11.

Table 2.3 Allelic Exchange Plasmid Construction Primers

Oligonucleotide	Sequence 5' to 3'
CDR20291_CdtR_LHA_F	TTTTTTCCTGCAGGTAATAACTTGTAGTTATCATTAACTCTACAATTC
CDR20291_CdtR_LHA_R	TTTCTATTTATAAATACCCTCCTATAAAAAATTC
CDR20291_CdtR_RHA_F	GGAGGGTATTTATAAATAGAAAAAAGAGATGTCTC
CDR20291_CdtR_RHA_R	AAAAAAGGCGCGCCTGAGAATATTTACTTATTTCTACAGAATCTTTTT
CDR20291_CdtA_CdtB_LHA_F	TTTTTTCCTGCAGGTTTTTACTATCTACTCAGATTCCTCACTATGGAA
CDR20291_CdtA_CdtB_LHA_R	TCATTTGATATTATTCTCCCTCCCAATATTAGTT
CDR20291_CdtA_CdtB_RHA_F	GAGGGAGAATAATATCAAATGATTTAAATTTGTCC
CDR20291_CdtA_CdtB_RHA_R	AAAAAAGGCGCGCCTCTTAGAAAAGTTTATAAAAAAGTTGGATTTATA
CDR20291_CdtR_CdtA_CdtB_LHA_F	TTTTTTCCTGCAGGTAATAACTTGTAGTTATCATTAACTCTACAATTC
CDR20291_CdtR_CdtA_CdtB_LHA_R	TCATTTGATATAAATACCCTCCTATAAAAAATTC
CDR20291_CdtR_CdtA_CdtB_RHA_F	GGAGGGTATTTATATCAAATGATTTAAATTTGTCC
CDR20291_CdtR_CdtA_CdtB_RHA_R	AAAAAAGGCGCGCCTCTTAGAAAAGTTTATAAAAAAGTTGG

Table 2.4 Allelic Exchange Strain Confirmation Primers

Oligonucleotide	Sequence 5' to 3'
pMTL-YN4_F	ATGACTTTTTTAGCCGCTAAAAC
pMTL-YN4_R	GTCTTCATTAAATGCCTTAGAATCC
CDR20291_CdtR_F	ACTTCTATTAACAACTAATACTAGCTGACTAACAC
CDR20291_CdtR_R	TTTCTCGAGAATTTGCTTCTATTTGATAATCATAA
CDR20291_CdtA_CdtB_F	AAAAGATACGTTAAAGAAAAAAATTATCGC
CDR20291_CdtA_CdtB_R	AAAAAATATATAGTATGGGAGCAGAAAAAG
CDR20291_PaLoc_F	GGCAAGTGTATGTATTATAC
CDR20291_PaLoc_R	CCTTTGTTAGGTAGTATGTTTAG
CDR20291_PyrE_F	TTATAACATTAACATGGAGG
CDR20291_PyrE_R	AGTAAATATTCCGATTGACG

Table 2.5 Allele Exchange Complementation Primers

Oligonucleotide	Sequence 5' to 3'
CDR20291_PR_CdtR_F	TTTTTGCGGCCGCCACGTATAAGAATAAAAATTCCAG
CDR20291_PR_CdtR_R	TTTTTGGATCCTTATGTTTTAATAATGTTCTTTAAAATATTTC
CDR20291_CdtA_CdtB_YN18_F_GB	TTGCTGAAGACGTTGTGCGGCCGCGTATAAAAATAGATAATTGTTCGTATAGTTATA
CDR20291_CdtA_CdtB_YN18_R_GB	CACTATAAATAATTAAGTTTGCTAGCCTAATCAACACTAAGAACTAATAACTCT
pMTL-YN18_SC_F	GTTTCTTATGAGTTAGGAAGACAATTAGGA
pMTL-YN18_SC_R	CTAACCTTCATTTATAAGGCTAACTGC
CDR20291_CdtA_CdtB_DC_F	GACGAAATAAGAGGAATTATATAAGGTAGA
CDR20291_CdtA_CdtB_DC_R	TAAACAATGAGCTGTCTGTCATAGA
CDR20291_CdtA_CdtB_YN18_F1	CCCATACAACAGATAATTCAACTGC
CDR20291_CdtA_CdtB_YN18_R1	GCCAATTTGATTTTCTTGTGCTTTG
CDR20291_PR_CdtR_CdtA_YN2C_F_GB	GGAATTAGGGATGTAAGCGGCCGCTAATAACTTGTAGTTATCATTAACTCTACAATTC
CDR20291_PR_CdtR_CdtA_YN2C_R_GB	GCTAAGGATTCAGAACGGCGCGCCTTAAGGTATCAATGTTGCATCAAC
CDR20291_PR_CdtR_CdtA_YN2C_F1	AAATATCATATTGTTTCTCTCGACC
CDR20291_PR_CdtR_CdtA_YN2C_R1	TATATAGAGGATTCTTAGGAACTAC
CDR20291_IGS_CdtA_YN2C_F	GCGGCCGCTAAATAGAAAAAAGAGATGTCTCAAGATAAG
CDR20291_IGS_CdtA_YN2C_R	GGCGCGCCTTAAGGTATCAATGTTGCATCAAC
CDR20291_PyrE_F1	ATGAGTAAAGTAGATGTAGTGG
CDR20291_PyrE_R1	TTACATCCCTAATTCCTTGAAC

Table 2.6 Transcriptional Linkage of the Binary Toxin Primers

Oligonucleotide	Sequence 5' to 3'
CdtR_F	GTAGTTAATAAATATCATATTGTTTCTCTCGACC
CdtA_R	TAAGTCTTGTGCATAAATGTTATTAGGTAACAAA
CdtA_F	GTACTACGTATAACTATACCTAAAGGTTCT
CdtB_R	CAAATTTTAAATTACCATCTTTTATGGGTG

Table 2.7 Horizontal Binary Toxin Gene Transfer Primers

Oligonucleotide	Sequence 5' to 3'
OJH39	CCGAATTCAAGCTTCCCACTCTTTATGTCATATTCC
OJH42	CCCGAGCTCCTAATGATGTACGATATATCC
OJH43	GATACCAAGTATCTGTATGC
OJH44	CCATGATTCAGATTCCCTTGG
TcdA_F	CATCTATAAGTTCTCATATTCCTTC
TcdA_R	CCAGAAACTTATATTGTCC

Table 2.8 RAPID Clinical Study Binary Toxin Primers

Oligonucleotide	Sequence 5' to 3'
CDT_F	TTTTTTACTTTTGAATTTTTTATAGGAGGG
CDT_F2	ATACAAAAAAAAGTAGTTCCTAAGAATCCTC
CDT_F3	GAAAAACCTACACCATTACTTATGCACTTA
CDT_F4	TGTATATGCTCAAACTAGTACAAGTAATCA
CDT_F5	TGGTGTGTCTGTTAATGTAGGATATCAAAA
CDT_F6	TTTTTATCACGTATAAGAATAAAAATTCCA
CDT_F7	GCACCAACACAAACTAATAAAGAAGCTCTC
CDT_R	ATTTCTTGTATGTCATAATATTTTCTCCAC
CDT_R2	CAAACCATCTTTTTTAGTAGCGCCAAAAGC
CDT_R4	GATATACGACATATATGGCCATTACTC
CDT_026_F	GCCACCTGATACTATAGATATTATTC
CDT_026_R	AAGTTGGATTTATAGAGAGAAG
01013_12_19_F	TGGAAATTTTGGTACAAAAAATGGTTCTGG
01013_12_19_R	TGAGCCTTGTAAACCATCTTGATTTGTA

Table 2.9 RAPID Clinical Study Antibiotic Resistance Primers

Oligonucleotide	Sequence 5' to 3'
rpoB_F	TCTCACATTGATTTTGACATAGATGA
rpoB_F1	ATGCCACATCCTGTCACGAT
rpoB_F2	CTTTCAAGAGAAAGAGCTGGATTCG
rpoB_F3	AGACATTAGGATGGTATGTAGCAAC
rpoB_F4	AGAAGACATAATAAATCCAGAA
rpoB_F5	AAAGAGAAGATGGAAACAGAGATAG
rpoB_F6	GGAGGTTTGGGCTCTAGAAGCA
rpoB_R	GAACCATTTCAACAGCACCA
rpoB_R1	TTACATATCAAAGTCATCACTTTCAAAATC
rpoB_R2	GTAGCAGATAATCTCTCATCTTCTCCTA

Table 2.10 RAPID Clinical Study 16S rRNA Gene Primers

Oligonucleotide	Sequence 5' to 3'
27_F	AGAGTTTGATCMTGGCTCAG
1492_R	TACGGYTACCTTGTTACGACTT

Table 2.11 ClosTron Mutagenesis System Strain Confirmation Primers

Oligonucleotide	Sequence 5' to 3'
CD630_26350_F	GTGAAGGGATGAGTTTGGTAATAAG
CD630_26350_R	TAATATCCCTGCATTAGCATAATCC
EBS Universal	TTTACTGAACGCAAGTTTCTAATTTCG

2.6. MOLECULAR MICROBIOLOGY METHODS

2.6.1. PLASMID DNA EXTRACTION

All plasmids were extracted from *E. coli* using the GenElute Plasmid Miniprep kit (Sigma), in accordance with the manufacturer's instructions.

2.6.2. GENOMIC DNA EXTRACTION

Genomic DNA was extracted from *C. difficile* using lysozyme and the GenElute Bacterial Genomic DNA kit (Sigma), following the manufacturer's instructions for a Gram-positive bacterial preparation.

2.6.3. DNA AMPLIFICATION VIA POLYMERASE CHAIN REACTION

All polymerase chain reactions (PCR) were carried out using the Epicentre Failsafe PCR system with Failsafe Premix E (Cambio). A Taq DNA polymerase (GoTaq) or high-fidelity Taq DNA polymerase (Phusion or Q5) were used for amplification of DNA less than 2kb, in accordance with the manufacturer's instructions. DNA fragments greater than 2kb were amplified using LongAmp Taq DNA polymerase, following the manufacturer's instructions. The T3000 Thermo Cycler (Biometra) and amplification

conditions, as shown in Table 2.12, were used for all PCR reactions, unless stated otherwise.

Table 2.12 PCR Amplification Conditions

PCR Step	Temperature			Time			Number of Cycles
	GoTaq	Phusion /Q5	LongAmp	GoTaq	Phusion /Q5	LongAmp	
Initial Denaturation	95ºC	98ºC	94ºC	2 mins	30 secs	30 secs	1
Denaturation	95ºC	98ºC	94ºC	0.5-1 min	5-10 secs	15-30 secs	29
Annealing	42-65ºC	45-72ºC (50-72ºC Q5)	45-65ºC	0.5-1 min	10-30 secs	15-60 secs	
Extension	72ºC	72ºC	65ºC	1 min/kb	15-30 secs/kb (20-30 secs/kb Q5)	50 secs/kb	
Final Extension	72ºC	72ºC	65ºC	5 mins	5-10 mins (2 mins Q5)	10 mins	1
Hold	4ºC	4ºC	4-10ºC	Indefinite	Indefinite	Indefinite	1

2.6.4. DNA AMPLIFICATION VIA SPLICING BY OVERLAP EXTENSION PCR

Splicing by overlap extension (SOEing) PCR was carried out to amplify and splice together two DNA fragments using appropriate left-hand and right-hand homology arm primers (Table 2.3) (Horton, 1995).

2.6.5. DNA AMPLIFICATION OPTIMISATION VIA GRADIENT PCR

Gradient PCR was performed when optimisation of PCR annealing temperatures was required. The Sensoquest Gradient Labcycler (Geneflow Ltd.) was capable of evaluating twelve potential annealing temperatures in a single reaction.

2.6.6. RESTRICTION ENDONUCLEASE DNA DIGEST

Digests were performed using NEB restriction enzymes and the CutSmart Buffer or their respective NEBuffers, under the conditions recommended by the manufacturer.

2.6.7. DNA DE-PHOSPHORYLATION

To prevent re-ligation of digested DNA ends and remove 5'phosphate groups, NEB Antarctic Phosphatase and Antarctic Phosphatase Buffer were used in a de-phosphorylation reaction, following the manufacturer's instructions.

2.6.8. DNA LIGATION

Previously digested DNA fragments were ligated using NEB T4 DNA Ligase and carried out in 20µl reactions, in accordance with the instructions of the manufacturer. All ligations were incubated overnight in a water/ice mixture at room temperature.

2.6.9. DNA MEMBRANE DIALYSIS

The ligation reaction products were dialysed using a 0.025µm nitrocellulose membrane (Millipore) floated on dH_2O for 30 minutes. This removed salt from the mixture and prevented arcing when transforming in the electroporator.

2.6.10. AGAROSE GEL ELECTROPHORESIS

DNA separation was achieved by using a 1% agarose gel (4g agarose/400ml 1x tris base, acetic acid and EDTA [TAE] buffer), stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, 1:10,000 dilution in TAE) and placed in a CompactM gel tank (Biometra) containing 1x TAE. Gel loading dye was added to the DNA samples at a concentration of 1:5. A total of 20µl was pipetted into an individual well for visualisation and 50µl for agarose gel DNA extraction. Either a NEB 1kb or 2-log (0.1-10.0kb) DNA ladder was used to function as a size marker. Gels were electrophoresed with a PowerPac 300 (Bio-Rad) at 100V for 45 to 60 minutes, depending on expected fragment

size. The DNA bands were visualised with a gel documentation system (Molecular Imager Gel Doc XR+ Imaging System, BioRad).

2.6.11. AGAROSE GEL DNA EXTRACTION

DNA bands of the correct fragment size were excised from the agarose gel and purified using either the GenElute Gel Extraction kit (Sigma) or the Monarch DNA Gel Extraction Kit (NEB), following the manufacturer's instructions.

2.6.12. DNA QUANTIFICATION

The quantification of a DNA concentration was accomplished by evaluating 1µl of sample on a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). The OD_{260} is measured and the concentration calculated using a known standard (with a path length of 1cm, 50mg/ml of double-stranded DNA has an OD_{260} of 1).

2.6.13. NUCLEOTIDE SEQUENCING

All Sanger DNA sequencing reactions were carried out by Source Bioscience, ensuring the service sample requirements were adhered to.

2.6.14. WHOLE GENOME SEQUENCING

2.6.14.1. PHENOL-CHLOROFORM DNA EXTRACTION

Firstly, the *C. difficile* strains were streaked onto BHIS agar supplemented with *C. difficile* selective supplement. The following day, the strains were grown in BHIS broth and incubated anaerobically, overnight. After this incubation period, 1 to 10ml of culture was centrifuged at 11,700g (Benchtop Centrifuge 5414R, Eppendorf) for 1 minute to obtain a sizeable pellet. The cell pellet was resuspended in 180µl lysis buffer (PBS with 20mg/ml lysozyme) and incubated for 30 minutes at 37°C, agitating occasionally. Then 4µl of RNase A was added and incubated for 15 to 30 minutes at room temperature. After this time, 25µl Proteinase K Solution (Qiagen), 85µl dH_2O and 110µl 10% (w/v) Sodium dodecyl sulfate (SDS) solution is added, the mixture is inverted several times and incubated at 65°C for 30 minutes, with

occasional agitation. In the Class I fume hood, 400µl of phenol-chloroform was added to the sample and mixed thoroughly by inversion. Upon transfer to a Phase Lock Gel Heavy Tube (Eppendorf, 2.0ml), the sample was centrifuged at 11,700g (Benchtop Centrifuge 5414R, Eppendorf) for 3 minutes. The top layer was carefully transferred to a new phase lock tube and the phenol-chloroform extraction was repeated a further two times. Once completed, 40µl of 3M sodium acetate (NaAc) and 800µl of ice-cold 100% ethanol were added to the sample and mixed thoroughly by inversion. The sample was incubated at -80°C for 30 minutes and then centrifuged at 11,700g (Benchtop Centrifuge 5414R, Eppendorf) for 15 minutes. After pouring off the supernatant in one swift motion (to stop resuspension of the pellet), the sample was washed with 1ml of 70% ethanol and centrifuged at 11,700g (Benchtop Centrifuge 5414R, Eppendorf) for 3 minutes. The supernatant was again poured off quickly and centrifuged for a further 3 minutes. The remaining supernatant was removed using a 200µl pipette and the pellet was air dried for 45 minutes at room temperature. After this period, the pellet was resuspended in 50µl dH_2O.

2.6.14.2. PURE DNA VERIFICATION

To ensure that no RNA contamination was present and that there was minimal DNA degradation in the samples, 1 to 2µl was evaluated on an agarose gel. Any residual RNA contamination was removed by repeating the RNase treatment, followed by the ethanol precipitation. Once the samples were pure, they were measured using the NanoDrop spectrophotometer, and quantity and quality of the DNA was assessed.

2.6.14.3. WHOLE GENOME SEQUENCING

Deep Seq, the Next Generation Sequencing Facility (Centre for Genetics and Genomics, The University of Nottingham), required a minimum of 5ug RNA-free genomic DNA to perform Illumina sequencing using the MisSeq system.

2.7. PLASMID CONSTRUCTION

2.7.1. CDT ALLELIC EXCHANGE KNOCK-OUT PLASMIDS

2.7.1.1. HOMOLOGY ARM AND RECOMBINANT ALLELE CREATION

As previously described (Cartman *et al.*, 2012; Ng *et al.*, 2013), allele exchange cassettes were assembled by SOEing PCR (Horton, 1995) specific to the CDT binary toxin locus in *C. difficile* strain R20291. The 1400bp recombinant alleles consisted of a 700bp left-hand homology arm (LHA) and a 700bp right-hand homology arm (RHA). A high-fidelity DNA polymerase (Phusion) was used to create the homology arms (Table 2.3, LHA primer pairs; CDR20291_CdtR_LHA_F/CDR20291_CdtR_LHA_R, CDR20291_CdtA_CdtB_LHA_F/CDR20291_CdtA_CdtB_LHA_R and CDR20291_CdtR_CdtA_CdtB_LHA_F/CDR20291_CdtR_CdtA_CdtB_LHA_R. RHA primer pairs; CDR20291_CdtR_RHA_F/CDR20291_CdtR_RHA_R, CDR20291_CdtA_CdtB_RHA_F/CDR20291_CdtA_CdtB_RHA_R and CDR20291_CdtR_CdtA_CdtB_RHA_F/CDR20291_CdtR_CdtA_CdtB_RHA_R), as well as the recombinant alleles (Table 2.3, primer pairs; CDR20291_CdtR_LHA_F/CDR20291_CdtR_RHA_R, CDR20291_CdtA_CdtB_LHA_F/CDR20291_CdtA_CdtB_RHA_R and CDR20291_CdtR_CdtA_CdtB_LHA_F/ CDR20291_CdtR_CdtA_CdtB_RHA_R). To create the recombinant alleles, the SOEing PCR annealing temperature conditions were optimised by gradient PCR (CDR20291_CdtR; 55°C annealing temperature, CDR20291_CdtA_CdtB; 58°C annealing temperature and CDR20291_CdtR_CdtA_CdtB; 50°C annealing temperature with a 45 seconds' extension time).

2.7.1.2. ALLELE EXCHANGE CASSETTE PLASMID ASSEMBLY

The recombinant allele PCR products were run on a 1% agarose gel, extracted and then digested, using *Sbf*I and *Asc*I restriction endonucleases, along with the pMTL-YN4 vector. The digested vector backbone was dephosphorylated,

and the vector and insert were ligated and dialysed, before being transformed into *E. coli* TOP10 and CA434 to create the following three knock-out plasmids: pMTL-YN4_*cdtR*, pMTL-YN4_*cdtA_cdtB* and pMTL-YN4_*cdtR_cdtA_cdtB*. The plasmids were extracted before being sent for verification by Sanger DNA sequencing (Table 2.4, primer pair; pMTL-YN4_F/pMTL-YN4_R).

2.7.2. CDT COMPLEMENTED PLASMIDS

2.7.2.1. CREATION OF COMPLEMENTATION VECTOR VIA DIGEST METHOD

The R20291_IGS_*cdtA* complement fragment was amplified with *Not*I and *Asc*I restriction sites using LongAmp DNA Polymerase (Table 2.5, primer pair; CDR20291_IGS_CdtA_YN2C_F/CDR20291_IGS_CdtA_YN2C_R, 52ºC annealing temperature, 2 minutes' extension time). The complement fragment PCR product was run on a 1% agarose gel, extracted and then digested, using *Not*I and *Asc*I restriction endonucleases, along with the pMTL-YN2C vector. The digested vector backbone was dephosphorylated, and the vector and insert were ligated and dialysed, before being transformed into *E. coli* TOP10 and CA434 to create pMTL-YN2C_IGS_*cdtA* complementation plasmid. The plasmid was extracted and confirmed by a control PCR, using the primers and conditions mentioned above. Confirmed plasmids were sent for verification by Sanger DNA sequencing, using the previously stated PCR primers.

2.7.2.2. CREATION OF COMPLEMENTATION VECTORS VIA GIBSON ASSEMBLY

The pMTL-YN18_*cdtA_cdtB* and pMTL-YN2C_PR_*cdtR_cdtA* plasmids for complementation were generated using the Gibson Assembly Master Mix (NEB), following the manufacturer's procedure. The primers were designed with appropriate fragment overlaps and the complement fragments PCR amplified using LongAmp DNA Polymerase (Table 2.5, primer pairs; CDR20291_CdtA_CdtB_YN18_F_GB/
CDR20291_CdtA_CdtB_YN18_R_GB at 50ºC annealing temperature, 4

minutes' extension time and CDR20291_PR_CdtR_CdtA_YN2C_F_GB/ CDR20291_PR_CdtR_CdtA_YN2C_R_GB at 55ºC annealing temperature, 4 minutes' extension time). The relevant vectors (pMTL-YN18 or pMTL-YN2C) were digested and linearised, and the concentration of all fragments was determined using the NanoDrop spectrophotometer. The DNA fragments were added to the Gibson Assembly Master Mix and incubated, before being transformed into *E. coli* TOP10 and CA434. The plasmids were extracted and confirmed by a control digest (pMTL-YN18_*cdtA_cdtB* with *Not*I and *Nhe*I restriction endonucleases and pMTL-YN2C_PR_*cdtR_cdtA* with *Not*I and *Asc*I. Confirmed plasmids were sent for verification by Sanger DNA sequencing, using the previously stated PCR primers and relevant walking primers (Table 2.5, primer pairs; CDR20291_CdtA_CdtB_YN18_F1/ CDR20291_CdtA_CdtB_YN18_R1 and CDR20291_PR_CdtR_CdtA_YN2C_F1/ CDR20291_PR_CdtR_CdtA_YN2C_R1).

2.7.3. CLOSTRON GROUP II INTRON PLASMIDS

2.7.3.1. INTRON DESIGN

ClosTron target sites were identified using the Intron Design Tool (Heap *et al.*, 2009) based on the Perutka algorithm (Perutka *et al.*, 2004) implemented at www.ClosTron.com.

2.7.3.2. SYNTHESIS OF RE-TARGETED SEQUENCE AND PLASMID ASSEMBLY

The ClosTron re-targeted sequence of around 300bp, and inclusive of the *Hind*III and *BsrG*I sites, was synthesised by DNA2.0. The synthesised fragment was further cloned into plasmid pMTL007C-E2, sequence verified, and subsequently delivered on filter paper.

2.7.3.3. PLASMID ELUTION FROM FILTER PAPER

Heated forceps were used to pierce a hole in the bottom of a 0.5ml eppendorf (Eppendorf), which was placed into a 1.5ml eppendorf. The filter paper containing the dried plasmid was then folded around the forceps and placed

into the 0.5ml eppendorf. 50µl of 37°C pre-warmed sterile dH_2O was added and the sample was centrifuged at 11,700g (Benchtop Centrifuge 5414R, Eppendorf) for 1 minute to elute the plasmid into the 1.5ml eppendorf. The plasmid was then transformed into *E. coli* TOP10 and CA434, ready for conjugation into *C. difficile*.

2.8. STRAIN CONSTRUCTION

2.8.1. CDT ALLELIC EXCHANGE KNOCK-OUT STRAINS

2.8.1.1. ISOLATION OF SINGLE CROSS-OVER CLONES

After *C. difficile* DNA transfer via conjugation, single cross-over clones (SCOs) were identified as faster growing transconjugant colonies on BHIS agar supplemented with *C. difficile* selective supplement and thiamphenicol, as previously described (Cartman *et al.*, 2012; Heap and Minton, 2014).

2.8.1.2. ISOLATION OF DOUBLE CROSS-OVER CLONES

Confirmed SCOs were streaked onto non-selective BHIS agar to allow for a second recombination event to occur. True double cross-over clones (DCOs) that have lost the chloramphenicol/thiamphenicol resistance gene (*catP*) along with the plasmid were confirmed by plating onto *C. difficile* minimal media (CDMM, 2% bacteriological agar [2g in 100ml deionised water, autoclaved]/61ml sterile dH_2O/20ml amino acid solution 5x/10ml salt solution 10x/5ml 20% glucose solution/2ml trace salt solution 50x/1ml FeSO4•7H2O solution 100x/1ml vitamin solution 100x) containing 5-fluoroorotic acid (5-FOA) (100mg/ml in DMSO, working concentration 2mg/ml) and uracil (1mg/ml in dH_2O, working concentration 10µg/ml), (Cartman *et al.*, 2012) and BHIS agar plates supplemented with thiamphenicol (Heap and Minton, 2014).

2.8.1.3. KNOCK-OUT CLONE VERIFICATION

Transconjugants were screened at the SCO and DCO clone stage. Firstly, the genomic DNA was extracted from *C. difficile* R20291Δ*pyrE*. For confirmation

of the SCOs, a Taq Polymerase screening PCR (55°C annealing temperature, 2 minutes' extension time) was completed. Primers specific to the pMTL-YN4 vector (Table 2.4, primers; pMTL-YN4_F and pMTL-YN4_R) and primers which anneal to the chromosomal sequence flanking the allele (Table 2.4, primers; CDR20291_CdtR_F, CDR20291_CdtR_R, CDR20291_CdtA_CdtB_F, CDR20291_CdtA_CdtB_R, CDR20291_PaLoc_F and CDR20291_PaLoc_R) were used to demonstrate both LHA and RHA SCOs. To verify the DCOs, a Phusion Polymerase screening PCR (55°C annealing temperature, 45 seconds' extension time) using only the primers directed against the allele was performed. The PCR products were run on a 1% agarose gel and extracted. Once sent for Sanger DNA sequencing, the following four allelic exchange knock-out strains were confirmed: R20291Δ*pyrE*Δ*cdtR*, R20291Δ*pyrE*Δ*cdtA*Δ*cdtB*, R20291Δ*pyrE*Δ*cdtR*Δ*cdtA*Δ*cdtB* and R20291Δ*pyrE*ΔPaLoc.

2.8.1.4. CONVERSION OF *pyrE* MINUS KNOCK-OUT STRAIN TO PROTOTROPHY

C. difficile DNA transfer via conjugation was performed with the pMTL-YN2 *pyrE* restoration plasmid. The method was modified by the addition of uracil (2mg/ml in dH_2O, working concentration 20µg/ml) in BHIS broth (for overnight liquid culture) and BHIS agar (for the non-selective conjugal transfer plate and *C. difficile* selective supplement and thiamphenicol transconjugant selection plates). Potential transconjugant colonies were passaged several times on BHIS agar supplemented with *C. difficile* selective supplement, thiamphenicol and uracil and then the fast-growing cells from each transconjugant clone were harvested and restreaked onto CDMM containing no 5-FOA or uracil supplements. Potential *pyrE* restoration clones were plated onto BHIS and BHIS supplemented with thiamphenicol to identify those clones which had lost the plasmid-encoded thiamphenicol resistance.

2.8.1.5. *pyrE* RESTORED STRAIN VERIFICATION

All thiamphenicol sensitive clones were screened for correction of the *pyrE* gene. Genomic DNA was extracted from *C. difficile* R20291 and a Phusion

Polymerase PCR (52°C annealing temperature, 1 minute 30 seconds' extension time) was completed. Primers specific to the restored *pyrE* flanking regions (Table 2.4, primer pair; CDR20291_PyrE_F/CDR20291_PyrE_R) were used for the PCR reaction and subsequent Sanger DNA sequencing.

2.8.2. CDT COMPLEMENTED STRAINS

2.8.2.1. ALLELE-COUPLED EXCHANGE

The *C. difficile* R20291Δ*pyrE*Δ*cdtR*Δ*cdtA*Δ*cdtB* and *C. difficile* R20291Δ*pyrE*Δ*cdtA*Δ*cdtB* strains were initially converted to *pyrE* prototrophy. Then allelic exchange, isolating single and double cross-over clones, was carried out using the pMTL-YN18_*cdtA_cdtB*. Transconjugants were screened at the SCO and DCO clone stage. For confirmation of the SCOs, a LongAmp Polymerase PCR (LHA; 55°C annealing temperature, 6 minutes' extension time, RHA; 53°C annealing temperature, 5 minutes' extension time) was completed. Primers specific to the pMTL-YN18 vector (Table 2.5, primers; pMTL-YN18_SC_F and pMTL-YN18_SC_R) and primers which anneal to the chromosomal sequence flanking the allele (Table 2.5, primers; CDR20291_CdtA_CdtB_DC_F and CDR20291_CdtA_CdtB_DC_R) were used to demonstrate both LHA and RHA SCOs. To verify the DCOs, a LongAmp Polymerase PCR (53°C annealing temperature, 6 minutes' extension time) using only the primers directed against the allele was performed. The PCR products were run on a 1% agarose gel and extracted. Once sent for Sanger DNA sequencing, the R20291Δ*pyrE*Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtA***cdtB* strain (with the *cdtA* missing 700 nucleotides at the 5' end) was confirmed.

2.8.2.2. COMPLEMENTATION AND *pyrE* RESTORATION

The R20291Δ*pyrE*Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtA***cdtB* strain was once more converted to *pyrE* prototrophy, with the concomitant insertion of the PR_*cdtR_cdtA* fragment on the pMTL-YN2C plasmid. Furthermore, the R20291R20291Δ*pyrE*Δ*cdtR* strain was also *pyrE* restored and simultaneously complemented with the pMTL-YN2C_PR_*cdtR* complementation plasmid. Thiamphenicol sensitive clones were screened for correction of *pyrE* using

primers designed inside of the gene (Table 2.5, primer pair; CDR20291_PyrE_F1/CDR20291_PyrE_R1). The complemented fragments (PR_*cdtR_cdtA_cdtB* and PR_*cdtR*) was similarly amplified and sent for Sanger DNA Sequencing for confirmation (Table 2.5, primer pair for PR_CdtR; CDR20291_PR_CdtR_F/CDR20291_PR_CdtR_R. Walking primers for PR_CdtR_CdtA_CdtB; Table 2.4, CDR20291_PyrE_F, CDR20291_PyrE_R; Table 2.5, CDR20291_CdtA_CdtB_YN18_F1, CDR20291_CdtA_CdtB_YN18_R1 and Table 2.8, CDT_F, CDT_F2, CDT_F3, CDT_F4).

2.8.3. CLOSTRON GROUP II INTRON INTEGRANTS

2.8.3.1. INTEGRATION AND CONFIRMATION

C. difficile DNA transfer via conjugation was performed, as previously described (Chapter 2.4.6.), however, transconjugal growth was suspended in 500µl of PBS and inoculated onto five BHIS agar plates supplemented with erythromycin (50mg/ml in 100% ethanol, working concentration 10µg/ml), instead of thiamphenicol.

2.8.3.2. TARGET GENE INSERTION VERIFICATION

Firstly, the genomic DNA was extracted from *C. difficile* 630Δ*erm* and a flanking PCR (Table 2.11, primer pair; CD630_26350_F/CD630_26350_R) and junction PCR (Table 2.11, primer pair; CD630_26350_F/EBS Universal) using Taq Polymerase were performed (58°C annealing temperature, 30 seconds' extension time). The PCR products were run on a 1% agarose gel and the bands visualised. A flanking PCR and junction PCR using the primer pairs detailed above and a high-fidelity DNA polymerase (Phusion Polymerase) were carried out on all colonies positive for ClosTron mutant screening. The PCR products were run on a 1% agarose gel and gel extracted samples were prepared before being sent for confirmation by Sanger DNA sequencing, with previously stated PCR primers.

2.9. EXPERIMENTAL CHARACTERISATION MICROBIOLOGICAL METHODS

All experiments were carried out in triplicate, unless otherwise stated.

2.9.1. MEASUREMENT OF GROWTH RATE

The *C. difficile* strains were cultured overnight in the anaerobic cabinet before being diluted (20µl of 1ml BHIS broth overnight into 180µl BHIS broth with a blank control of 200µl BHIS broth) using a Nunc MicroWell 96-well plate (Thermo Fisher Scientific). The plate was sealed with a plate sealer (R&D Systems), transferred to the GloMax-Multi+ Detection System (Promega) and assayed for 24 hours, in accordance with the manufacturer's instructions (protocol: high orbital shaking for 6 seconds, absorbance at 600nm and incubation for 3600 seconds at 37°C).

2.9.2. GROWTH DENSITY STANDARDISATION

All growth required for phenotypic assays was standardised beforehand. Overnight anaerobic cultures were diluted 1:10 to $1x10^{-10}$ in a 96-well plate, sealed and incubated for 12 to 16 hours anaerobically. The most dilute culture that exhibited growth was used to inoculate the assay media. This technique ensured standardisation in growth densities across all strains.

2.9.3. MEASUREMENT OF SPORULATION

2.9.3.1. RATE OF SPORULATION

The RAPID patient samples along with control samples (sporulation-positive 630Δ*erm* wildtype [WT] strain, sporulation-negative 630Δ*erm*Δ*spo0A* strain and sporulation-intermediate *C. difficile* 630Δ*erm*::CT26350 strain) were assayed to compare sporulation rates. The growth density was standardised, as previously stated (Chapter 2.9.2.), which also ensured that no spores were present when the initial medium was inoculated.

A 500µl sample was taken at 0, 24, 48, 72, 96 and 120-hour time points, removed from the anaerobic cabinet, briefly centrifuged, and heat-treated (60°C for 30 minutes to kill the vegetative cells (Burns *et al.*, 2011)). The samples were then returned to the anaerobic cabinet and serially diluted to $1x10^{-8}$ (20µl into 180µl PBS) using a 96-well plate. Three discrete 20µl spots of each dilution were plated onto BHIS agar supplemented with 0.1% taurocholate. The total colony forming units (cfu) count (spores and vegetative cells in the non-heat-treated samples) was also measured at 0 and 120 hours.

After anaerobic incubation for 24 hours, the colonies were counted and, using the appropriate dilution factor, the number of cfu/ml was determined (Burns *et al.*, 2010a).

2.9.3.2. TOTAL SPORULATION

10 to 15µl of the heat-treated samples taken at the 120-hour time point were loaded into a haemocytometer chamber and viewed using the Eclipse Ci Microscope (Nikon). The number of spores were counted in ten 200µm squares and averaged. To calculate the number of spores per ml, the following calculation was performed: average per 200µm square x 250 x 1000.

2.9.4. MEASUREMENT OF *CLOSTRIDIUM DIFFICILE* BINARY TOXIN

2.9.4.1. MAINTENANCE OF VERO CELL LINE

The African green monkey kidney epithelial Vero cell line was used in this research (Ammerman *et al.*, 2008). The Vero cells were grown as previously described (Kuehne *et al.*, 2010). The complete growth media used to maintain the Vero cell line was Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Thermo Fisher Scientific), 500ml supplemented with 50ml heat-inactivated foetal bovine serum and 5ml penicillin-streptomycin. Vero cells were revived from storage by re-suspending in 37°C pre-warmed complete growth media to a density of $1.5\text{-}2.5x10^5$ cells/ml. 20ml of this cell suspension was used to seed a $75cm^2$ tissue culture flask (Nunc), which was incubated at 37°C in 5% CO_2 and passaged two to three times before

cytotoxicity assay use. Cell passage involved discarding the complete growth media and washing the cells twice, with 10ml of PBS. The cells were then incubated with 5ml 1x trypsin-EDTA at 37°C for 5 minutes. After incubation, 5ml of fresh complete growth media was added to the cells, ensuring that all cells were dislodged from the tissue culture flask. The subsequent cell suspension was resuspended in additional fresh complete growth medium to a final density of 1.5-2.5x10^5 cells/ml. 20ml of this cell suspension was used to seed each new 75cm^2 tissue culture flask.

2.9.4.2. PREPARATION OF VERO CELL MONOLAYER

Once the Vero cells had reached a confluent monolayer, a tissue culture flask was used to seed a 96-well plate. The cells were dislodged from the flask using 1x trypsin-EDTA as described above. The cell suspension was then transferred into a 10ml universal tube and centrifuged at 120g (Centrifuge 5430R, Eppendorf) for 10 minutes at room temperature. After centrifugation, the cell pellet was resuspended in 10ml of the complete growth medium. The subsequent cell suspension was resuspended in additional fresh complete growth medium to a final density of 1.5-2.5x10^5 cells/ml. 100µl aliquots of the diluted cell suspension was pipetted into each well of a 96-well plate. The seeded plates were incubated for 24 hours at 37ºC in 5% CO_2, to form a confluent monolayer.

2.9.4.3. CYTOTOXICITY ASSAY

C. difficile strains were grown in tryptose yeast extract (TY) media (12.0g bacto-tryptose/8.0g yeast extract/0.4g sodium thioglycolate/400ml deionised water) in anaerobic conditions. Cell densities were initially standardised, as previously stated (Chapter 2.9.2.), before inoculating a 50ml falcon tube containing 20ml pre-reduced TY media. 2ml samples were taken at 96 hours, centrifuged and the supernatants passed through a 0.22µm filter and frozen at -20°C until required. Samples were thawed and CdtB was proteolytically activated (Perelle *et al.*, 1997) with bovine pancreas trypsin (1mg/ml in 1mM hydrogen chloride [HCl], working concentration 400µg/ml) for 1 hour at room temperature. Following trypsinisation, the trypsin was blocked with trypsin

inhibitor from *Glycine max* (soybean) (10mg/ml in dH_2O, working concentration 200µg/ml) for 15 minutes at room temperature (Bilverstone *et al.*, 2017). The treated samples were then diluted fourfold in sterile PBS, with 20µl applied to each well of the pre-incubated 96-well plate. Cytotoxic effects and cell rounding were observed under an inverted microscope at 20x magnification, after a 24-hour incubation period. To obtain objective data, the end-point (1/toxin titre) of each replicate sample was determined. The end point is the greatest serial dilution at which at least 50% of cell morphology is indistinguishable from the trypsinated PBS control (Rothman, 1986).

2.9.5. BIOFILM FORMATION

C. difficile strains were grown in BHIS broth supplemented with 0.1M glucose, incubated anaerobically. The assay was conducted at 24 and 96-hour time points. Firstly, cell densities were standardised, as previously stated (Chapter 2.9.2.). A 1:100 dilution was made of all desired *C. difficile* strains into fresh BHIS broth containing 0.1M glucose in a tissue culture treated 24-well plate (Corning/Costar), which had been pre-reduced in the anaerobic cabinet for 48 hours prior to use. The plate was sealed with a plate sealer and incubated anaerobically. After the required time, the wells were gently washed with PBS, to remove the culture medium, and allowed to dry for a minimum of 10 minutes. The biofilm in each well was then stained with 1ml of filter-sterilised 0.2% (v/v) crystal violet solution, and was incubated for 30 minutes at 37°C in anaerobic conditions. The crystal violet staining solution was removed and the wells were washed with PBS, until the solution ran clear. For dye extraction from the biofilm, the plate was removed from the anaerobic cabinet and 1ml of methanol was added to each well. After incubation for 30 minutes at room temperature, the absorbance at 570nm (A_{570}) of the methanol-extracted dye was measured with the GloMax-Multi+ Detection System (Dapa *et al.*, 2013).

2.9.6. MEASUREMENT OF ANTIBIOTIC RESISTANCE

2.9.6.1. ANTIBIOTIC PLATE ASSAY OPTIMISATION

To ensure that intermediate fidaxomicin and rifaximin antibiotic resistance isolates were not overlooked, strains with different levels of sensitivity were assessed. The fidaxomicin resistance strains that were verified included a sensitive (R20291_*ermB*; 0.25µg/ml) (Kelly *et al.*, 2016), resistant (R20291_*ermB*_TA; >32µg/ml) and two intermediate isolates (R20291_*ermB*_GT; 2µg/ml and R20291_*ermB*_TG; 8µg/ml) (Kuehne *et al*., under review). With regards to the rifaximin resistance, a sensitive strain (RT002_A7; >0.5µg/ml) and an intermediate strain (RT002_F1; ≥16µg/ml) were tested (Stevenson *et al.*, 2016). All control strains were originally grown on BHIS agar supplemented with *C. difficile* selective supplement. The following day, four to five well-formed colonies were restreaked onto BHIS agar containing fidaxomicin (1mg/0.95ml in 100% dimethyl sulfoxide [DMSO], various working concentrations of 1, 4, 8, 16 and 32µg/ml) and BHIS agar containing rifaximin (1.024mg/ml in 100% ethanol, various working concentrations of 4, 8, 16 and 32µg/ml) and incubated anaerobically for 24 hours. After this time, growth on the selective media was verified.

2.9.6.2. ANTIBIOTIC PLATE ASSAY

All RAPID *C. difficile* isolates were streaked onto BHIS agar containing fidaxomicin (working concentration 1µg/ml) and BHIS agar containing rifaximin (working concentration 8µg/ml) and incubated anaerobically for 24 hours. After this time, growth on the selective media was verified.

2.9.6.3. ANTIBIOTIC BROTH DILUTION ASSAY

The antibiotic broth dilution assay was conducted as previously stated (Stevenson *et al.*, 2016). The *C. difficile* strains were grown overnight in BHIS broth. The following day, a starter culture was prepared using a 10-fold dilution (1ml) of the overnight culture in BHIS broth (9ml) and incubated for 4 hours to reach an OD_{600} of between 0.3 and 0.4. For the broth dilution assay, a rifaximin starting concentration of 512µg/ml was selected, and a stock

solution was prepared starting at a 2-fold higher concentration compared to the first concentration in the assay. 100µl of BHIS broth was transferred into all wells (except column 1) of a 96-well plate. 200µl of rifaximin stock solution (2-fold more concentrated than highest intended concentration) was transferred into the wells of column 1. Then a two-fold dilution series was performed across the plate, using 100µl from the column 1 wells. 100µl of the 10-fold diluted starter culture for each strain was transferred into the respective wells of the prepared 96-well plate. Additionally, control wells, which contained the strains with no antibiotic, a negative control well to highlight potential contamination, and a positive control well containing the rifaximin sensitive R20291 WT strain, were included. The plate was sealed and incubated anaerobically at 37°C for 24 hours, after which the OD_{600} of each well was measured using the GloMax-Multi+ Detection System. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of antibiotic that inhibits visible bacterial growth, expressed in µg/ml (European Society of Clinical Microbiology and Infectious Diseases, 2019). An isolate with an MIC of >32µg/ml was considered to be resistant (O'Connor *et al.*, 2008).

2.9.7. HORIZONTAL GENE TRANSFER

2.9.7.1. STRAIN ANTIBIOTIC SENSITIVITY EVALUATION

C. difficile strain CD37 and R20291::CT*cdtB* were initially assessed for their antibiotic sensitivity against tetracycline (5mg/ml in 70% ethanol, working concentration 10µl/ml), erythromycin (50mg/ml in 100% ethanol, working concentration 10µg/ml), lincomycin (20mg/ml in dH_2O, working concentration 20µl/ml) and rifaximin (1.024mg/ml in 100% ethanol, working concentration 16µg/ml) by employing the antibiotic plate assay as mentioned previously (Chapter 2.9.6.2.).

2.9.7.2. TRANSFER OF DNA VIA FILTER MATING

For the horizontal *C. difficile* transferase (CDT) gene transfer experiment, the following protocol was employed, as described previously (Brouwer *et al.*, 2013). The donor (R20291::CT*cdtB*) and recipient (CD37) strains were streaked

onto agar plates containing relevant antibiotics (*C. difficile* selective supplement with erythromycin or rifaximin, respectively). Overnight cultures were made in 1ml of BHI broth (14.8g brain heart infusion/2.0g yeast extract/400ml deionised water). The following day, the strains were grown to an OD_{600} of 0.5-0.6, washed twice with PBS, centrifuged at 70g (Allegra X-30R, Beckman Coulter) for 5 minutes, and the pellet resuspended in 100µl BHI broth. The donor and recipient mixtures were then combined for a total volume of 200µl and spread onto a 0.45µm pore size cellulose nitrate membrane filter (Sartorius) placed on a BHI agar plate. After a 24-hour incubation, the filters were placed in a 25ml falcon tube containing 1ml of BHI broth and vortexed vigorously for 30 seconds. The filter mixture was then plated onto selective media to identify any potential transconjugants (BHI agar supplemented with erythromycin and rifaximin). A control assay was also carried out simultaneously to identify any spontaneous erythromycin or rifaximin resistant colonies.

2.10. EXPERIMENTAL CHARACTERISATION MOLECULAR MICROBIOLOGICAL METHODS

All experiments were carried out in triplicate, unless otherwise stated.

2.10.1. TAQMAN PROTEIN ASSAY

Strict laboratory practice was adhered to for all parts of the procedure. A Class II Biosafety Cabinet was used to prevent contamination. Separate dedicated personal protective equipment (PPE), laboratory supplies and equipment was used at all times. The preparation area was cleaned with DNA*Zap* solution (Thermo Fisher Scientific) to degrade contaminating DNA and RNA from surfaces.

2.10.1.1. PROBE DEVELOPMENT ASSAY

The following kits were used to develop TaqMan Protein Assay Probes from a commercially bought biotinylated antibody (Biotin Conjugated Affinity

Purified Chicken anti-*Clostridium difficile* Binary Toxin Subunit A, Gallus Immunotech Inc.); TaqMan Protein Assays Oligo Probe Kit, TaqMan Protein Assays Buffer Kit, TaqMan Protein Assays Core Reagents Base Kit and TaqMan Protein Assays Fast Master Mix (Applied Biosystems, Life Technologies). The probe development assay was performed in accordance with the TaqMan Protein Assays Probe Development Protocol.

2.10.1.2. SAMPLE LYSATE PREPARATION

The Protein Expression Sample Preparation Kit (Applied Biosystems, Life Technologies), as well as the Calbiochem Protease Inhibitor Cocktail Set I and the Calbiochem Phosphatase Inhibitor Cocktail Set II were used to prepare cell lysates. Sample lysate preparation was carried out by following the TaqMan Protein Assays Sample Prep and Assay Protocol.

The samples were collected by applying several different methods for optimisation. Growth density of each sample was standardised as previously stated (Chapter 2.9.2.). Samples were grown anaerobically in BHIS and TY broth and 1ml was taken at 0, 4, 8, 12, 24, 48, 72 and 96 hour-time points. The samples were then either frozen as pure culture at -20°C or pelleted at 2,500g (Benchtop Centrifuge 5414R, Eppendorf) for 4 minutes and frozen at -20°C until required. The 96-hour samples were also centrifuged at 11,700g (Benchtop Centrifuge 5414R, Eppendorf) for 1 minute, the supernatant filter-sterilised and frozen at -80°C.

Minor differences were made to the protocol, including not counting the cells using a haemocytometer and resuspending the cell pellet in 100µl of ice-cold Cell Resuspension Buffer so that all the cell material was lysed. Furthermore, one of the optimisation methods was to lyse the cells using lysozyme and the GenElute Bacterial Genomic DNA kit (Sigma), following the manufacturer's instructions for Gram-positive bacterial lysis.

2.10.1.3. CELL LYSATE DILUTION

The Protein Expression Sample Preparation Kit and the previously prepared sample lysates was used to prepare lysate two-fold dilutions of one replicate.

Cell lysate dilutions were performed by following the TaqMan Protein Assays Sample Prep and Assay Protocol.

2.10.1.4. ASSAY PROBE BINDING REACTION

The TaqMan Protein Assays Buffer Kit, as well as the previously prepared Assay Probe A and Assay Probe B were used for the binding reaction. The Assay Probe solution and binding reaction 96-well plate were prepared in accordance with the TaqMan Protein Assays Sample Prep and Assay Protocol.

2.10.1.5. LIGATION REACTION

The TaqMan Protein Assays Core Reagents Base Kit was used for the ligation reaction, following the TaqMan Protein Assays Sample Prep and Assay Protocol.

The protease reaction was omitted as the real-time quantitative PCR (qPCR) was performed immediately after the ligation reaction.

2.10.1.6. REAL-TIME QUANTITATIVE PCR

The TaqMan Protein Assays Core Reagents Base Kit and the TaqMan Protein Assays Fast Master Mix were used to prepare the ligation samples for real-time qPCR. The TaqMan Protein Assays Sample Prep and Assay Protocol was followed to prepare the PCR mix and PCR LightCycler 480 Multiwell 96 Plate (white with sealing foils, Roche).

The real-time qPCR was performed on the Roche LightCycler 480 system using the reaction thermal-cycling conditions stated in Table 2.13. The analysis mode was set at Quantification with a FAM dye reporter and a non-fluorescent quencher. The results were analysed by calculating crossing point (C_P)/threshold cycle (C_T) values using Absolute Quantification/2nd Derivative Max (Software Version LCS480 1.5.0.39) with the mode set at high confidence.

Table 2.13 Roche LightCycler 480 Thermo-Cycling Conditions

Stage	Temperature	Time (Enzyme Activation, Denaturation, Annealing/Extension)	Ramp Rate (°C/s)
Hold	95°C	10 minutes	4.40
Cycle (40 Cycles)	95°C	10 seconds	4.40
	60°C	45 seconds	2.20

2.10.2. TRANSCRIPTIONAL LINKAGE

Strict laboratory practice was adhered to for all parts of the procedure. A Class II Biosafety Cabinet was used to prevent contamination. Dedicated personal protective equipment (PPE), laboratory supplies and equipment was used at all times. The preparation area was cleaned with RNase*Zap* solution (Thermo Fisher Scientific) to destroy contaminating RNase from surfaces.

2.10.2.1. PREPARATION OF *CLOSTRIDIUM DIFFICILE* TOTAL RNA

Two volumes of RNA protect (Qiagen) were added to the *C. difficile* broth cultures, which were then vortexed and incubated in anaerobic conditions at 37°C for 5 minutes. After centrifugation at 1,700g (Allegra X-30R, Beckman Coulter) for 10 minutes at 4°C, the pellets were resuspended in 1ml of RNA pro solution (MP Biochemicals) in a Class I fume hood. The cell suspension was transferred to a matrix tube and processed in a homogeniser at 6,400rpm for 40 seconds. The subsequent cell lysate was then centrifuged at 11,700g (Benchtop Centrifuge 5414R, Eppendorf) for 15 minutes at 4°C. The supernatant was transferred to a fresh eppendorf tube and incubated for 5 minutes at room temperature to increase the RNA yield. After this time, 300µl of ice-cold chloroform was added and the supernatant mixture was vortexed for 10 seconds. There was a further incubation time of 5 minutes at room temperature to permit nucleoprotein dissociation and increase RNA purity. The incubated supernatant mixture was then centrifuged at 11,700g (Benchtop Centrifuge 5414R, Eppendorf) for 15 minutes at 4°C. The upper phase was transferred to a fresh eppendorf tube, mixed with 500µl of ice-cold 100% ethanol and incubated at -20ºC for 2 hours to precipitate the RNA. There was

another centrifugation step of 11,700g (Benchtop Centrifuge 5414R, Eppendorf) for 15 minutes at 4°C to pellet the RNA. After discarding the supernatant, 500µl of ice-cold 75% ethanol (diluted with UltraPure DEPC-treated water) was added to the sample and centrifuged at 11,700g (Benchtop Centrifuge 5414R, Eppendorf) for 15 minutes at 4°C. The final RNA pellet was air dried at room temperature, resuspended in 50µl UltraPure DEPC-treated water and subsequently stored at -80°C.

2.10.2.2. DNA CONTAMINATION REMOVAL FROM TOTAL RNA

Contaminating genomic DNA was removed from the RNA samples using the TURBO DNAse kit (Ambion), in accordance with the manufacturer's procedure.

2.10.2.3. RNA PURIFICATION

The DNase-treated RNA samples were further purified using reagents and buffers from the RNeasy MinElute Cleanup Kit (Qiagen). Firstly, in the Class I fume hood, β-merceptothanol was added to an aliquot of RLT kit buffer (10µl/ml). Then 350µl of this buffer, along with 200µl 100% ethanol was added to the DNAse-treated sample. The solution was mixed and applied to the kit spin column and centrifuged for 15 seconds at 7,000g (Benchtop Centrifuge 5414R, Eppendorf). The spin column was then transferred to a new collection tube and 500µl of RPE kit wash buffer was added and centrifuged as stated above. After discarding the supernatant, the wash step was repeated and the sample centrifuged at 7,000g (Benchtop Centrifuge 5414R, Eppendorf) for 2 minutes to dry the spin column. The RNA was eluted from the spin column with 17µl UltraPure DEPC-treated water by allowing to stand for 3 minutes and then centrifuging for 1 minute at 7,000g (Benchtop Centrifuge 5414R, Eppendorf). The elution process was repeated twice more to obtain a total volume of 50µl. At this point, the RNA samples were stored at -80°C prior to use.

2.10.2.4. PCR TO VERIFY RNA PURIFICATION

A Platinum Taq DNA Polymerase (Thermo Fisher Scientific) PCR was performed (Table 2.6, primer pair; CdtR_F/CdtA_R, 55°C annealing temperature, 1-minute extension time) on every RNA sample to ensure that all contaminating DNA had been removed.

2.10.2.5. ONE-STEP REVERSE TRANSCRIPTASE PCR

The RNA concentration of each sample was quantified on the NanoDrop spectrophotometer with the volume added to the PCR reaction standardised so that 100ng/50µl reaction was achieved. A One-Step Reverse Transcriptase (RT) PCR (SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase, Invitrogen) was performed, following the manufacturer's instructions (Table 2.6, primer pairs; CdtR_F/CdtA_R and CdtA_F/CdtB_R, cDNA synthesis; 55ºC annealing temperature, 30 minutes' extension time, PCR amplification; 55ºC annealing temperature). A *C. difficile* R20291 WT strain was included as a positive control for each primer pair and all samples were additionally tested with Platinum Taq DNA Polymerase to detect any potential DNA contamination in reaction preparation. The PCR products were detected on an agarose gel as previously described (Chapter 2.6.10.).

2.10.3. HORIZONTAL GENE TRANSFER PCR ANALYSIS

To determine if isolated colonies were true transconjugants, three different screening PCR reactions were carried out. Firstly, a LongAmp DNA Polymerase PCR (Table 2.8, primer pair; CDT_F/CDT_R, 59ºC annealing temperature, 5 minutes' extension time) to amplify the binary toxin gene locus was conducted. In parallel, a Phusion DNA Polymerase PCR (Table 2.7, primer pair; TcdA_F/TcdA_R, 52ºC annealing temperature, 1 minute 30 seconds' extension time) to amplify the *tcdA* gene of the PaLoc locus was completed (Brouwer *et al.*, 2013). Lastly, a previously developed Phusion DNA Polymerase PCR (Table 2.7, primer pairs; OJH39/OJH43, 52ºC annealing temperature, 1 minute 30 seconds' extension time and OJH44/OJH42, 54ºC annealing temperature, 1 minute extension time) to

assess *sigK* organisation (whether it was disrupted or undisrupted due to the presence of $skin^{Cd}$) was also performed (Haraldsen and Sonenshein, 2003).

2.10.4. BINARY TOXIN GENE LOCUS PCR ANALYSIS

2.10.4.1. CDT CONTROL STRAIN PCR

A LongAmp DNA Polymerase PCR (Table 2.8, primer pair; CDT_F/CDT_R, 59ºC annealing temperature, 5 minutes' extension time) to amplify the binary toxin gene locus was optimised for different *C. difficile* strains with various ribotypes from the SBRC laboratory collection.

2.10.4.2. CDT RAPID STRAIN PCR

Each RAPID patient sample glycerol stock was cultured overnight in 1ml BHIS broth and the genomic DNA extracted for use in the optimised CDT PCR. Any positive full CDT or ghost CDT gene locus electrophoresis bands were gel extracted and sent for Sanger DNA sequencing using the PCR primers and specially designed walking primers (For the full CDT gene locus; Table 2.8, primers; CDT_F, CDT_F2, CDT_F3, CDT_F4, CDT_F5 and CDT_R, and for the ghost CDT gene locus; Table 2.8, primers; CDT_F, CDT_F2, CDT_R and CDT_R2.) This CDT PCR product omitted the first ~50 nucleotides from the 5' end and ~100 nucleotides from the 3' end. To attain the missing DNA sequence, two PCR reactions (Table 2.8, primer pair; CDT_F7 and CDT_026_R, 55ºC annealing temperature, 2 minutes' extension time. Table 2.8, primer pair; CDT_F6 and CDT_R4, 50ºC annealing temperature, 2 minutes' extension time) were performed, and the products were gel extracted and sent for Sanger DNA sequencing with additionally designed primers (Table 2.8, primers; CDT_F7 and CDT_R4).

For the final batch of RAPID samples an additional CDT LongAmp DNA Polymerase PCR (Table 2.8, primer pair; CDT_F6/CDT_026_R, 53ºC annealing temperature, 8 minutes' extension time) was optimised and carried out, which when Sanger DNA sequenced, did not omit the nucleotide sequence from the 5' and 3' ends. Although additional walking primers were designed and used with the original optimised CDT PCR, the conclusive set of

walking primers to sequence the entire PCR product for the final batch of RAPID samples were, for the full CDT gene locus; Table 2.8, primers; CDT_F, CDT_F2, CDT_F3, CDT_F4, CDT_F5, CDT_F6, CDT_R and CDT_026_R, and for the ghost CDT gene locus; Table 2.8, primers; CDT_F, CDT_F2, CDT_F6, CDT_R, CDT_R2 and CDT_026_R.

RAPID sample 01013 Week 12 Colony 19 required a further PCR (Table 2.8, primer pair; 01013_12_19_F/01013_12_19_R, 52°C annealing temperature, 1 minute extension time) to amplify the DNA sequence absent between CDT_F7 and CDT_R. The PCR primers were designed using *C. difficile* strain 630, instead of *C. difficile* strain R20291, as with all the other CDT primers. This region was Sanger DNA sequenced with the previously stated PCR primers.

2.10.4.3. NON-TOXIGENIC CDT RAPID STRAIN PCR

If no CDT PCR DNA band was observed with a RAPID isolate, a supplementary Phusion DNA Polymerase PCR (Table 2.8, primer pair; CDT_026_F/CDT_026_R, 55ºC annealing temperature, 1 minute extension time) was performed to assess whether the strain was non-toxigenic for binary toxin (Carter *et al.*, 2007).

2.10.5. RPOB HOTSPOT PCR ANALYSIS

Rifaximin resistant RAPID isolates required a Phusion DNA Polymerase PCR (Table 2.9, primer pair; rpoB_F/rpoB_R, 55ºC annealing temperature, 1 minute extension time) on the RpoB hotspot region to analyse any potential single nucleotide polymorphisms (SNPs).

If no SNPs were observed in the RpoB hotspot region, the entire *rpoB* gene was amplified (Table 2.9, primer pair; rpoB_F1/rpoB_R1, LongAmp DNA Polymerase, 53ºC annealing temperature, 4 minutes' extension time), and the PCR product sent for Sanger DNA sequencing with various walking primers (Table 2.9, primers; rpoB_F, rpoB_F1, rpoB_F2, rpoB_F3, rpoB_F4, rpoB_F5, rpoB_F6, rpoB_R, rpoB_R1 and rpoB_R2).

2.10.6. 16S rRNA GENE PCR ANALYSIS

A 16s rRNA PCR (Table 2.10, primer pair; 27_F/1492_R, Dream Taq Green Master Mix (2x) (Thermo Fisher Scientific), PCR program; 94ºC for 5 minutes, 94ºC for 30 seconds, 55ºC for 1 minute, 72ºC for 1 minute 30 seconds, 72ºC for 10 minutes and 15ºC hold [repeat x30]) was performed on specific RAPID isolates. The PCR product was gel extracted and sent for Sanger DNA sequencing with the PCR primers.

2.11. BIOINFORMATICS METHODS

2.11.1. GENOME VISUALISATION

Bacterial genomes were visualised using Artemis, which is available at http://www.sanger.ac.uk/.

2.11.2. BASIC LOCAL ALIGNMENT SEARCH TOOL

The Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi), a genomic database, was used to find regions of similarity between biological sequences. All nucleotide BLAST searches examined the nucleotide collection (nr/nt) database using Megablast, which is optimised for highly similar sequences. All protein BLAST searches scanned the non-redundant protein sequences (nr) database using the blastp algorithm. Identification of conserved regions within a protein sequence were completed using the CD-search, an interface which searches the Conserved Domain Database (CDD), located at https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi.

2.11.3. PREDICTION OF BACTERIAL PROMOTERS

The Prediction of Bacterial Promoters (BPROM) online program (Solovyev and Salamov, 2011) was used to predict potential transcription start positions in the *C. difficile* R20291 binary toxin gene locus.

2.11.4. PLASMID MAP DESIGN

Vector map design was carried out using the CLC Genomics Workbench (Qiagen, CLC Bio).

2.11.5. NUCLEOTIDE AND WHOLE GENOME SEQUENCE ANALYSIS

Nucleotide and whole genome sequence contig assembly and further analysis, including sequence alignment and basic variant detection was performed using the CLC Genomics Workbench.

2.11.6. PROTEIN SECONDARY STRUCTURE ANALYSIS

Analysis of the RpoB single nucleotide polymorphisms were performed using The PyMOL Molecular Graphics System (Version 1.8 Schrödinger, LLC.). PyMOL is a molecular visualization tool for rendering and animating 3D molecular structures.

2.12. DATA ANALYSIS

Microsoft Excel was used to record all raw data and perform initial calculations. Calculated data was then transferred to the GraphPad (Prism V7) program to prepare data in a graphical format and perform statistical analysis. Unless otherwise indicated, either a Two-Way ANOVA statistical test followed by a Dunnett's multiple comparison test, in which each test strain triplicate mean was compared against the control strain triplicate mean or a Two-Way ANOVA statistical test followed by a Tukey multiple comparison test, in which each strain triplicate mean was compared with the triplicate mean of every other strain, were performed. The P value accepted to indicate statistical significance was defined as ≤ 0.05.

CHAPTER 3. BINARY TOXIN REGULATION AND MECHANISM OF ACTION IN *CLOSTRIDIUM DIFFICILE*

3.1. INTRODUCTION

3.1.1. GENETIC TOOLS IN CLOSTRIDIA

Historically, exploitive knowledge and in-depth studies on the molecular basis of various significant genes underlying pathogenesis in *C. difficile* has been restricted due to the lack of reliable genetic tools. However, this has changed significantly over the last 10 years with the introduction and refined development of several manipulation techniques.

A methodology paper (O'Connor *et al.*, 2006) published in 2006 demonstrated the use of a shuttle vector to deliver gene constructs to the chromosome resulting in single-crossover integrational inactivation of the target gene. However, these mutants were highly unstable in the absence of antibiotic selective pressure and so reversion back to the wildtype strain was common. The following year, the universal ClosTron gene knock-out system for clostridia was described (Figure 3.1) (Heap *et al.*, 2007).

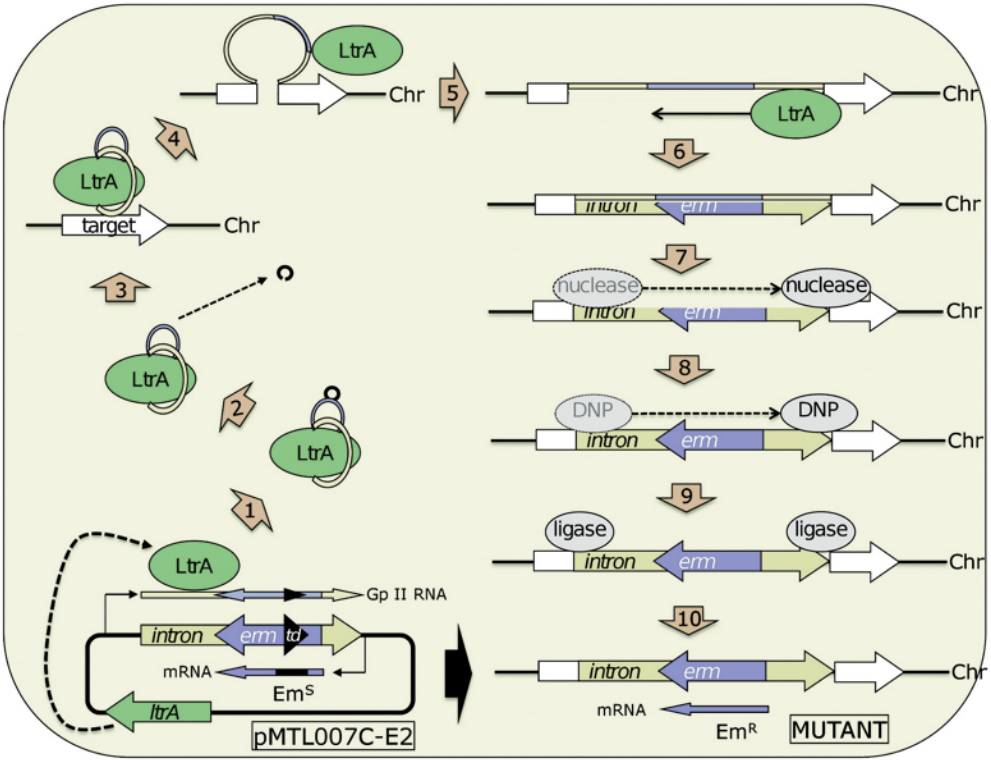


Figure 3.1 ClosTron Mutagenesis (Kuehne and Minton, 2012)

The ClosTron plasmid (pMTL007C-E2 in this instance) contains the group II intron with the retrotransposition-activated marker (RAM) erythromycin resistance (*ermB*) gene inserted. The *ermB* RAM is inactive at the start of mutagenesis due to the presence of the phage *td* group I intron. The *ltrA* gene is usually within the group II intron, however, it is positioned outside of this region on the plasmid. If the *ermB* gene is transcribed into mRNA, the transcript will contain the *td* group I intron. *td* can facilitate self-splicing from RNA transcripts, however, the process is orientation specific and so in this case the *ermB* gene remains inactive. If the opposite DNA strand with the group II intron is transcribed, the LtrA protein can bind forming a ribonuclear protein (RNP) complex (1). Due to the correct orientation, the *td* intron can promptly be spliced out (2). The RNP is then able to bind to the target gene on the chromosome (Chr) through a re-targeted region in the group II intron, designed using an online algorithm (http://www.clostron.com/) (Heap *et al.*, 2010) (3). LtrA inserts the RNA into the DNA target site (4, 5) and generates the complementary DNA strand via reverse transcriptase activity (6). Next, the RNA strand is degraded by the host nucleases (7) and the opposite DNA strand is produced by DNA polymerase (DNAP) (8). Lastly, the gaps are sealed by host ligases, completing the process (9). The created ClosTron mutant now contains the group II intron with a functional *ermB* gene incorporated into the target gene, which results in resistance to erythromycin (10).

The mutagenesis system was established using a mobile group II intron from the *Lactococcus lactis ltrB* gene. Selective antibiotic pressure by means of an erythromycin resistance (*ermB*) retrotransposition-activated marker (RAM)

gene allows for the acquisition of integrants (Heap *et al.*, 2007; Kuehne and Minton, 2012). Unlike the mutants previously mentioned in the single-crossover integration system (O'Connor *et al.*, 2006), ClosTron mutants are stable. They can also be generated as quickly as 10 to 14 days. Subsequently, numerous ClosTron mutants have been created in *C. difficile* (Heap *et al.*, 2007), including in an epidemic B1/NAP1/027 strain (Heap *et al.*, 2010), as well as in *Clostridium acetobutylicum*, *Clostridium botulinum*, *Clostridium sporogenes* (Heap *et al.*, 2007) and *Clostridium beijerinckii* (Heap *et al.*, 2010). The ClosTron mutagenesis system has also previously been used to successfully generate a *tcdA*$^{+}$ *tcdB*$^{+}$ *cdtA*$^{-}$ binary toxin knock-out mutant in the R20291 wildtype (WT) strain (Kuehne *et al.*, 2014). ClosTron technology is the method of choice when it is necessary to rapidly generate mutants with a disrupted target gene and expressing an altered phenotype, however, a major limitation is the potential observation of down-stream polar effects (Kuehne and Minton, 2012). Unfortunately, multiple intron insertions in sites other than the target gene have also been reported (Lehmann and Schmidt, 2003). Furthermore, the efficiency of integration decreases substantially when attempting to simultaneously introduce a 'cargo' DNA sequence greater than 1kb (Plante and Cousineau, 2006). Therefore, a more recently developed technique, based on allelic exchange, is employed to resolve these issues.

The allelic exchange system allows for the precise manipulation of the *C. difficile* genome by initially generating a strain with a *pyrE* gene deletion using allele-coupled exchange (ACE). The *pyrE* gene, which encodes for orotate phosphoribosyltransferase and is involved in pyrimidine biosynthesis, is made functionally inactive through utilisation of the pMTL-YN18 plasmid, which replaces the WT allele with a variant allele missing 50 nucleotides at the 5' end and 235bp at the 3' end of the gene (Figure 3.2) (Ng *et al.*, 2013).

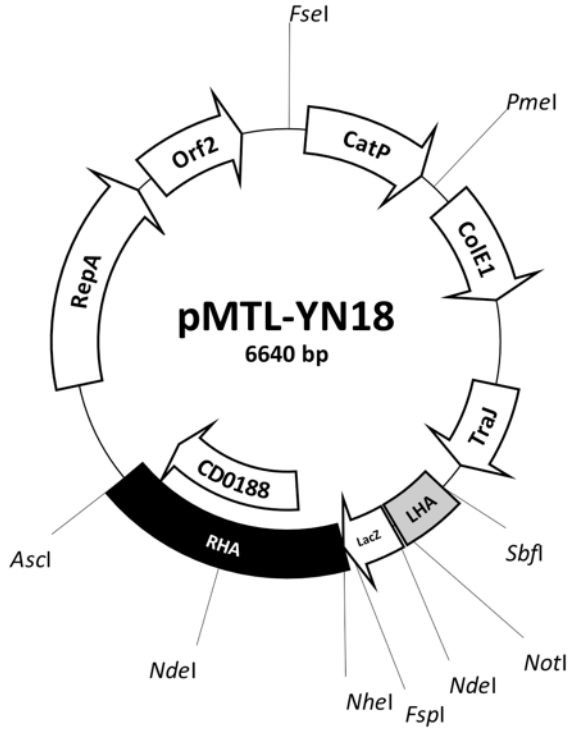


Figure 3.2 Allele-Coupled Exchange (ACE) Plasmid Vector pMTL-YN18 (Ng *et al.*, 2013)

The ACE pMTL-YN18 plasmid was specifically designed to create a *pyrE* deletion in the *Clostridium difficile* R20291 strain. The plasmid constituents are as follows: CatP, the *catP* gene of *Clostridium perfringens* providing chloramphenicol/thiamphenicol resistance; ColE1, the replication region of the *Escherichia coli* plasmid ColE1; TraJ, transfer function of the RP4 oriT region; LHA, the left-hand homology arm that consists of the R20291 *pyrE* gene, missing 50 nucleotides at the 5' end and 235bp at the 3' end; LacZ, the *lacZ* gene encoding the alpha fragment of the *E. coli* β-galactosidase (and also containing a multiple cloning site (MCS) taken from the pMTL20 vector (Chambers *et al.*, 1988)); RHA, the right-hand homology arm that comprises of the 1200bp region immediately downstream of the *pyrE* gene; and, RepA and Orf2, the *Clostridium botulinum* pBP1 plasmid replicating region.

This process generates a *C. difficile pyrE*$^{-}$ strain that requires the addition of uracil for growth (termed an auxotroph) and is resistant to 5-fluoroorotic acid (5-FOA), which is toxic to *pyrE* WT cells (Boeke *et al.*, 1987; Ng *et al.*, 2013). Allelic exchange can then be performed with this *pyrE* deletion background strain to accurately modify target genes, through the assistance of the pMTL-YN4 plasmid vector, which carries a non-homologous *pyrE* allele from *C. sporogenes* and allows for counter selection on media containing 5-FOA (Figure 3.3) (Ng *et al.*, 2013).

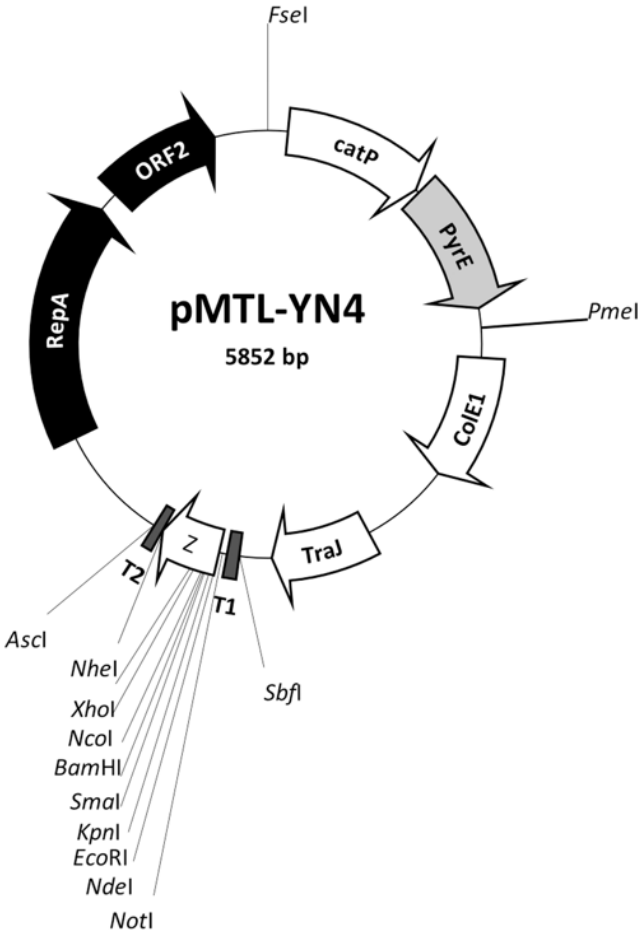


Figure 3.3 Allelic Exchange Plasmid Vector pMTL-YN4 (Ng *et al.*, 2013)

The allelic exchange pMTL-YN4 vector was specifically designed for use in *Clostridium difficile* strain R20291. The main pMTL-YN4 vector components comprise of: CatP, the *catP* gene of *Clostridium perfringens* conferring chloramphenicol/thiamphenicol resistance; PyrE, the *pyrE* gene of *Clostridium sporogenes*; ColE1, the replication region of the *Escherichia coli* plasmid ColE1; TraJ, transfer function of the RP4 oriT region; Z, the *lacZ* gene encoding the alpha fragment of the *E. coli* β-galactosidase (and also containing a multiple cloning site (MCS) taken from the pMTL20 vector (Chambers *et al.*, 1988)); T1, transcriptional terminator isolated from downstream of the *C. difficile* strain 630 CD0164 gene; T2, transcriptional terminator of the ferredoxin (*fdx*) gene of *Clostridium pasteurianum*; and, RepA and Orf2, the *Clostridium botulinum* pBP1 plasmid replicating region.

Those strains which have effectively undergone recombination will be isolated as double crossover mutants on media containing 5-FOA. However, those strains which have reverted to WT and/or are involved in unsuccessful plasmid excision will retain a functional *pyrE* gene and so not survive (Al-Hinai *et al.*, 2012).

After the in-frame deletion allelic exchange mutant has been generated, the WT *pyrE* gene can then be restored. This returns the strain to uracil prototrophy, which is essential for mutant characterisation in a clean, WT background. The process is achieved through ACE and the *pyrE* correction vector, pMTL-YN2. Moreover, alternative plasmid components permit concomitant complementation of the deleted target gene, either under the control of the native promoter at the suitable gene dosage (pMTL-YN2C) or overexpressed by the strong ferredoxin (*fdx*) gene promoter (pMTL-YN2X) (Figure 3.4) (Ng *et al.*, 2013).

The use of the allelic exchange system has been proved effective and reliable through the creation of *C. difficile* in-frame deletions in the *spo0A*, *cwp84*, and *mtlD* genes in strain 630Δ*erm* and *spo0A* and *cwp84* genes in R20291 (Ng *et al.*, 2013). In-frame deletions have also been successfully generated in *C. acetobutylicum* ATCC 824 *spo0A*, *cac824I*, *amyP* and *glgA* genes (Ehsaan *et al.*, 2016).

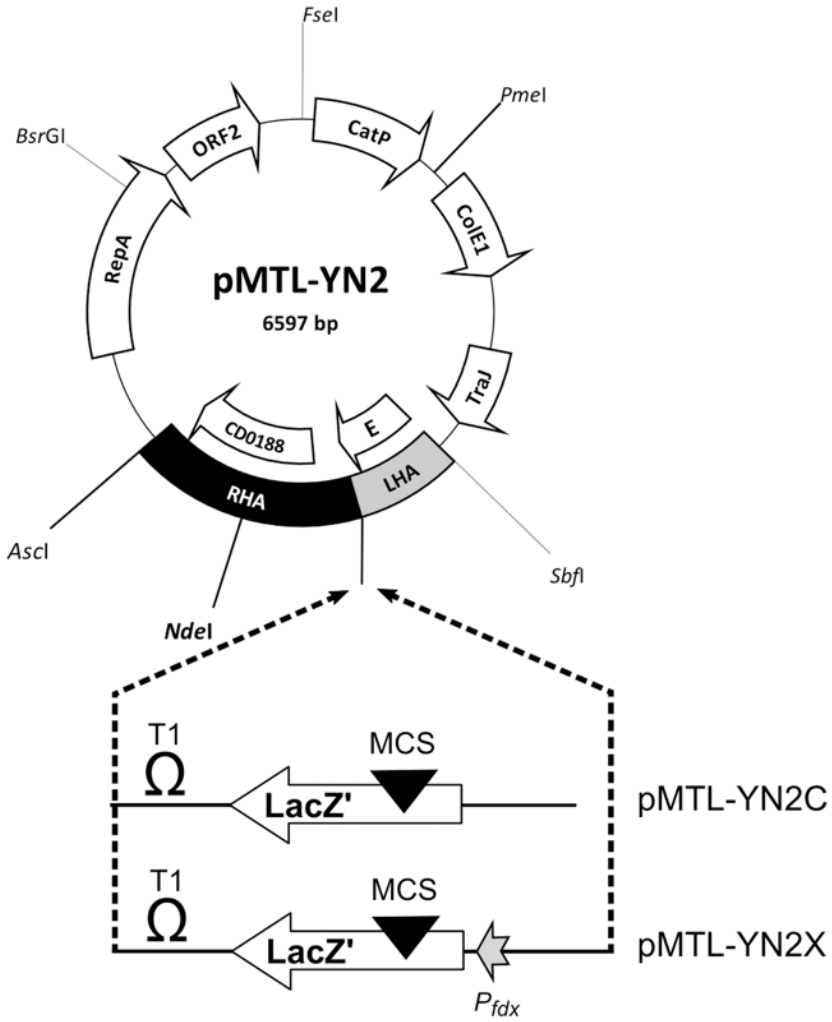


Figure 3.4 Allele-Coupled Exchange (ACE) Plasmid Vectors pMTL-YN2, YN2C and YN2X (Ng *et al.*, 2013)

The ACE plasmids pMTL-YN2, for *pyrE* correction, pMTL-YN2C, for complementation under the native promoter, and pMTL-YN2X, for complementation under the ferredoxin (*fdx*) promoter were specifically designed for use in *Clostridium difficile* strain R20291. The common plasmid constituents are as follows: CatP, the *catP* gene of *Clostridium perfringens* providing chloramphenicol/thiamphenicol resistance; ColE1, the replication region of the *Escherichia coli* plasmid ColE1; TraJ, transfer function of the RP4 oriT region; LHA, the left-hand homology arm that consists of the R20291 *pyrE* gene, missing 50 nucleotides at the 5' end and 235bp at the 3' end; RHA, the right-hand homology arm that comprises of the 1200bp region immediately downstream of the *pyrE* gene; and, RepA and Orf2, the *Clostridium botulinum* pBP1 plasmid replicating region. Plasmid pMTL-YN2C also contains an accompanying insert between the LHA and RHA consisting of: T1, transcriptional terminator of the *fdx* gene of *Clostridium pasteurianum*; LacZ, the *lacZ* gene encoding the alpha fragment of the *E. coli* β-galactosidase; and, a multiple cloning site (MCS) taken from the pMTL20 vector (Chambers *et al.*, 1988)). Plasmid pMTL-YN2X differs marginally from pMTL-YN2C by the addition of the *Clostridium sporogenes fdx* promoter region (P_{fdx}).

3.1.2. THE ROLE OF *CLOSTRIDIUM DIFFICILE* BINARY TOXIN

3.1.2.1. PATHOGENESIS

Interest in the *C. difficile* transferase (CDT) binary toxin was initially fuelled by the hypothesis that it played a complementary role to toxins A and B in *C. difficile* infection (CDI). An early study (Geric *et al.*, 2006) concluded that toxin A negative, toxin B negative, binary toxin positive strains were enterotoxic, caused pronounced fluid accumulation and could colonise the gastrointestinal tract, however, they were incapable of causing disease in hamsters. However, in more recent research, a study (Kuehne *et al.*, 2014) examining the importance in toxin A, toxin B and CDT in virulence discovered that when hamsters were challenged with a toxin A, toxin B ClosTron double mutant (and so only maintained a functional binary toxin gene locus), 3 out of the 9 animals died. They did not exhibit typical CDI symptoms, nonetheless, inflammation and haemorrhage in the small intestine was confirmed. In addition, the hamsters died at a significantly faster rate when given a toxin A and CDT producing mutant, then when given a strain producing only toxin A.

At the start of 2017, a paper (Secore *et al.*, 2017) was published indicating that inclusion of the CdtA and CdtB protein components in a *C. difficile* attenuated toxin A and B tetravalent vaccine improved vaccine efficiency in hamsters challenged with a B1/NAP1/027 strain.

3.1.2.2. COLONISATION

CDT has also been demonstrated to have a role in adherence. Recently published data (Schwan *et al.*, 2009; Schwan *et al.*, 2011; Schwan *et al.*, 2014; Schwan and Aktories, 2017) indicates that *C. difficile* binary toxin, like many other ADP-ribosylating toxins, can modify actin specifically at arginine position 177. This leads to actin polymerisation being blocked and the rearrangement of the actin cytoskeleton. The modification produces further consequences that affect other elements of the cytoskeleton such as the formation of microtubule-like protrusions, which have been well studied using human colon adenocarcinoma Caco-2 and HT29 cell lines, as well as in rat

primary intestinal epithelial cells (Schwan *et al.*, 2009; Schwan *et al.*, 2011; Schwan *et al.*, 2014). Septins are also known to be essential for protrusion formation and redistribute from the cytosol to the membrane, creating ring and funnel-shaped structures for the microtubule ends to interact with (Nolke *et al.*, 2016). Lastly, vesicle traffic is affected, with Rab-5 and Rab-11 associated vesicles observed within the protrusions. These vesicles, which contain extracellular matrix (ECM) proteins such as fibronectin and laminin, are rerouted to the apical membrane (Schwan *et al.*, 2014). Through this work, it has been illustrated that CDT is involved in pathogen colonisation. The bacteria become embedded in a network of microtubule-based protrusions and binding proteins accumulate at the membrane surface, which leads to increased adherence of the pathogen (Schwan and Aktories, 2017).

Although much research has been accomplished on the role of CDT in adherence, other colonisation and persistence factors, such as biofilm formation, have yet to be completely investigated. Biofilms are immobile, surface-associated microbial communities, with bacteria in these structures more resistant to environmental stress, such as the presence of antibiotics (Dapa and Unnikrishnan, 2013). Chronic or recurrent infections, including CDAD, typically form biofilms to persist in their environment and escape the host immune response. This manner of growth tends to lead to a reduced antibiotic treatment efficiency (Dapa and Unnikrishnan, 2013; Crowther *et al.*, 2014). One study (Semenyuk *et al.*, 2014) demonstrated that cells present in a biofilm are 100-fold more resistant to metronidazole then cells grown in liquid media. Biofilm formation could possibly be one of the factors contributing to CDI recurrence (Dapa and Unnikrishnan, 2013; Crowther *et al.*, 2014). Certain work (Dawson *et al.*, 2012; Dapa *et al.*, 2013) has reported how formation of a biofilm is controlled by multiple elements, including cell wall protein (CWP) 84, *spo0A*, flagella and a quorum-sensing regulator, *luxS*.

3.1.3. HORIZONTAL GENE TRANSFER

The *C. difficile* genome consists of approximately 11% of mobile genetic elements (MGEs), primarily in the form of conjugative transposons (CTns). These MGEs allow the organism to acquire many genes involved in virulence,

antibiotic resistance, host colonisation and the production of surface proteins (Sebaihia *et al.*, 2006).

The PaLoc has some key properties of an MGE, such as the fact it occupies the same position on the genome in all toxigenic strains, and in non-toxigenic strains a 115bp non-coding nucleotide sequence replaces the 19.6kb region (Braun *et al.*, 1996). The presence of TcdE and its similarity to bacteriophage holin proteins, as well as the observed regulatory effect of some bacteriophages on toxin production is also indicative (Govind *et al.*, 2009; Sekulovic *et al.*, 2011; Roberts *et al.*, 2014). However, it lacks origin of transfer (*oriT*) and recombinase genes (Mullany *et al.*, 2015). Nonetheless, a paper published in 2013 (Brouwer *et al.*, 2013) demonstrated horizontal gene transfer of the PaLoc from the *C. difficile* 630 strain to the non-toxigenic CD37 strain and non-toxigenic clinical isolate strains, OX904 and OX2157. Usually MGEs transfer as distinct regions with clear 3' and 5' ends, however, in this case the PaLoc transferred on DNA fragments ranging from 66kb to 272kb (Figure 3.5) (Brouwer *et al.*, 2013; Roberts *et al.*, 2014). Importantly, it was demonstrated through a cytotoxicity assay that one of the transconjugants could produce toxin B at a comparable concentration to the WT donor strain (Brouwer *et al.*, 2013).

The transfer frequency was exceptionally low (1 transconjugant isolated per 10^8 recipient cells), however, a transconjugant containing the PaLoc almost always contained a non-targeted CTn. The authors (Brouwer *et al.*, 2013) went on to state that the PaLoc and CTn transfer were connected. They hypothesised that this was due to the CTn, which contains an *oriT* gene, facilitating the transfer of large fragments of the genome, if the CTn was not excised. They termed this process a chromosomal transfer and recombination (CTaR) event (Mullany *et al.*, 2015).

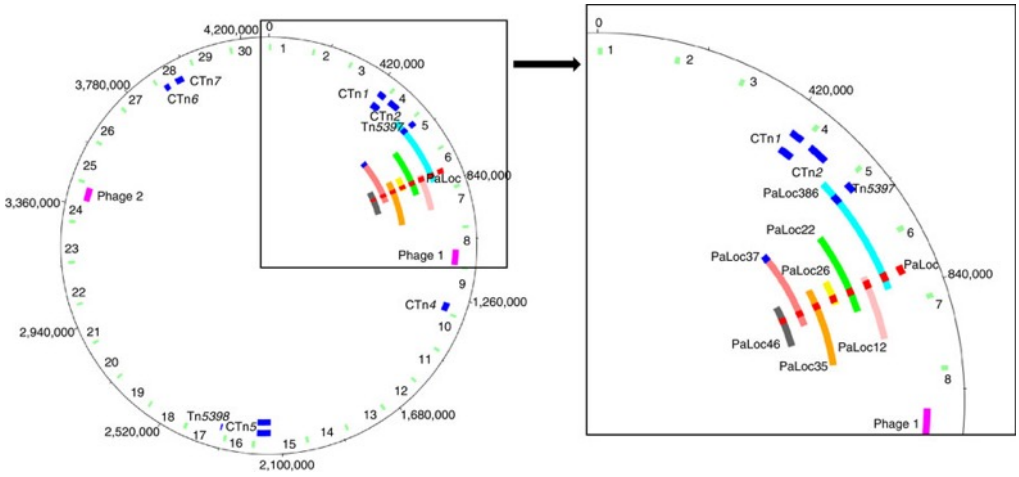


Figure 3.5 *Clostridium difficile* 630 Strain Pathogenicity Locus (PaLoc) Horizontal Gene Transfer (Brouwer *et al.*, 2013)

The outer circle represents the complete genome sequence of the *C. difficile* 630 strain. On the second circle, the green bands represent 10kb fragments used to differentiate the 630 donor strain from the CD37 recipient strain. The third circle comprises of blue regions for the sites of conjugative transposons (CTns), pink regions for the prophage positions and red for the PaLoc location. The fourth to the tenth circle signify the CD37 transconjugants containing the PaLoc on variable fragment sizes (66kb to 272kb). The right-hand panel illustrates the transconjugants in further detail (Mullany *et al.*, 2015).

3.1.4. AIMS OF THIS STUDY

The previous literature on *C. difficile* binary toxin confirms the need to clarify its precise role and significance in bacterial pathogenesis and colonisation. As the range of genetic tools has grown significantly over the last 10 years, so has the opportunity to characterise previously unexplored genes. The ClosTron mutagenesis system has been used in the past to make various knock-out mutants from the R20291 WT strain including R20291::*tcdA*; R20291::*tcdB*; and R20291::*cdtA* (Kuehne *et al.*, 2014). However, due to the potential of phenotypic polar effects, which cannot be disregarded, the work described in this chapter will focus on the construction of in-frame deletion knock-out mutants for CDT using the allelic exchange method.

The transcriptional linkage of the binary toxin gene locus was assessed. It was hypothesised that the *cdtR*, *cdtA* and *cdtB* genes of the binary toxin locus were transcriptionally linked, with the promoter upstream of the *cdtR* gene. This hypothesis was tested by using the Prediction of Bacterial Promoters

(BPROM) online program to predict potential promoters, as well as detecting RNA expression using reverse transcriptase (RT) PCR. Once the binary toxin locus transcriptional linkage was determined, the CDT mutants were further complemented under the control of the native promoters. The pathogenicity locus (PaLoc) was also deleted via allelic exchange in the generated CDT mutants so that the binary toxin could be evaluated without the presence of toxins A and B.

All the in-frame deletion mutants were characterised for their effect on virulence and colonisation factors, focusing on growth rates, CDT toxin production and biofilm formation. The hypothesis that bacterial growth followed a typical growth curve was tested by comparing the optical density (OD_{600}) measurements of the *C. difficile* mutants with the R20291 WT strain over a 24-hour period. Furthermore, it was hypothesised that all CDT in-frame deletion mutants would no longer produce binary toxin, as either the partial or no gene locus was present. This theory was investigated by measuring Vero cell cytotoxicity levels, as well as using the TaqMan Protein Assay to quantify the binary toxin protein. It was also argued that the loss of the binary toxin gene locus may upregulate the formation of biofilms. To test this idea, biofilm formation in the CDT in-frame deletion mutants was quantitatively measured by taking absorbance readings of the crystal violet stained biofilm matrix.

Finally, the hypothesised ability of the binary toxin gene locus to horizontally transfer from a toxigenic *C. difficile* strain to a non-toxigenic strain was investigated. This was assessed by using the filter mating and bacterial conjugation methods of transfer.

3.2. RESULTS

3.2.1. CONSTRUCTION OF THE CDT ALLELIC EXCHANGE KNOCK-OUT PLASMIDS

The allelic exchange system was used to make three different binary toxin knock-out mutants in the *C. difficile* R20291Δ*pyrE* strain; R20291Δ*cdtR*, R20291Δ*cdtA*Δ*cdtB* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*.

3.2.1.1. HOMOLOGY ARM CREATION

Firstly, primers were designed to amplify a 700bp region directly upstream and downstream of the target gene(s), termed left-hand and right-hand homology arms (LHA and RHA). Almost all homology arms were successfully generated on the first attempt (Figure 3.6, Figure 3.7 and Figure 3.8).

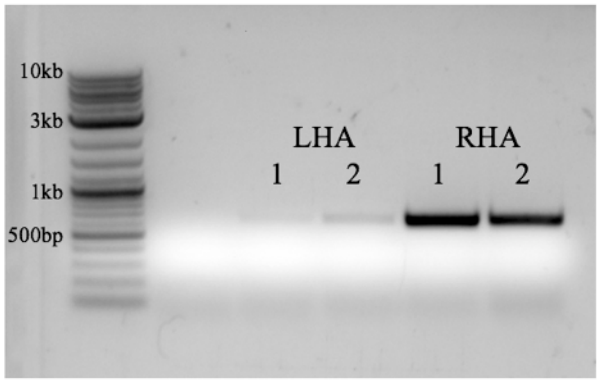


Figure 3.6 *Clostridium difficile* R20291 *cdtR* Homology Arm PCR

A 1% agarose electrophoresis gel to demonstrate the *cdtR* left-hand and right-hand homology arm PCR products. Each homology arm produced a DNA fragment of 700bp and were duplicated to generate a higher DNA yield when gel extracted. The negative gel is shown to highlight the lighter bands.

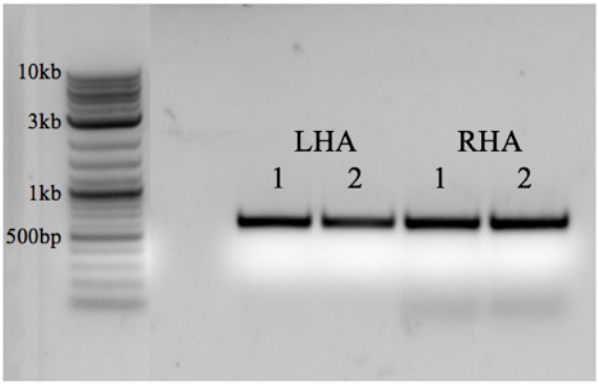


Figure 3.7 *Clostridium difficile* R20291 *cdtA* and *cdtB* Homology Arm PCR

A 1% agarose electrophoresis gel to demonstrate the *cdtA* and *cdtB* left-hand and right-hand homology arm PCR products. Each homology arm produced a DNA fragment of 700bp and were duplicated to generate a higher DNA yield when gel extracted. The negative gel is shown to highlight the lighter bands.

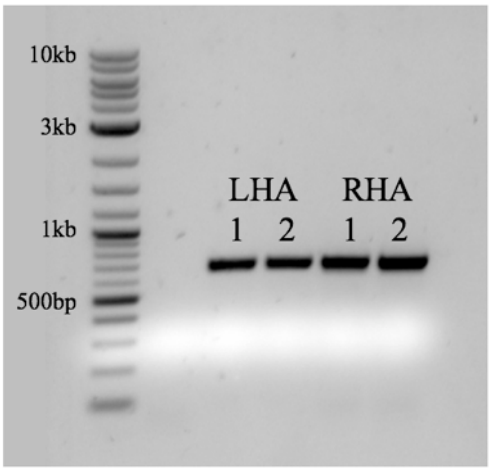


Figure 3.8 *Clostridium difficile* R20291 *cdtR*, *cdtA* and *cdtB* Homology Arm PCR

A 1% agarose electrophoresis gel to demonstrate the *cdtR*, *cdtA* and *cdtB* left-hand and right-hand homology arm PCR products. Each homology arm produced a DNA fragment of 700bp and were duplicated to generate a higher DNA yield when gel extracted. The negative gel is shown to highlight the lighter bands.

The *cdtR* LHA PCR required further optimisation before the DNA fragment was produced (Figure 3.9). Each homology arm was duplicated to generate a higher DNA yield when gel extracted.

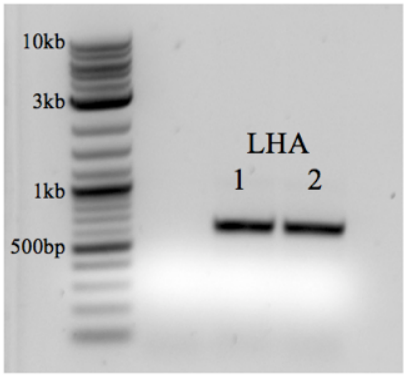


Figure 3.9 *Clostridium difficile* R20291 *cdtR* Left-Hand Homology Arm Optimised PCR

A 1% agarose electrophoresis gel to demonstrate the optimised *cdtR* left-hand homology arm PCR products. Each homology arm produced a DNA fragment of 700bp and were duplicated to generate a higher DNA yield when gel extracted. The negative gel is shown to highlight the lighter bands.

3.2.1.2. RECOMBINANT ALLELE CREATION

After the successful generation of the individual homology arms, a SOEing PCR was used to create a 1,400bp recombinant allele consisting of a 700bp left-hand homology arm and a corresponding 700bp right-hand homology arm (Figure 3.10, Figure 3.11 and Figure 3.12). There was initial difficulty in achieving the recombinant allele and so the SOEing PCR was optimised by gradient PCR. Sufficient PCR mixture was prepared to generate four recombinant allele DNA fragments, as it was observed in previous attempts that minor to moderate amounts of residual template DNA was still present. Although the SOEing PCRs were optimised, moderate amounts of residual template DNA was still evident for the *cdtA* and *cdtB* recombinant allele and the *cdtR*, *cdtA* and *cdtB* recombinant allele, with minor residual template DNA observed for the *cdtR* recombinant allele.

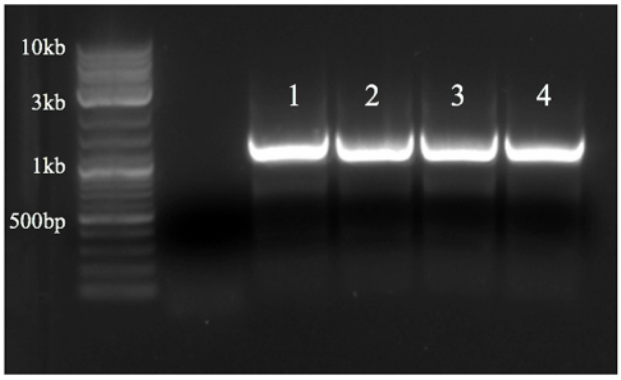


Figure 3.10 *Clostridium difficile* R20291 *cdtR* Recombinant Allele SOEing PCR

A 1% agarose electrophoresis gel to demonstrate the *cdtR* recombinant allele SOEing PCR products. Each recombinant allele produced a band of 1,400bp. Four DNA fragments were generated to produce a higher DNA yield when gel extracted. Minor amounts of residual template DNA were also observed at 700bp.

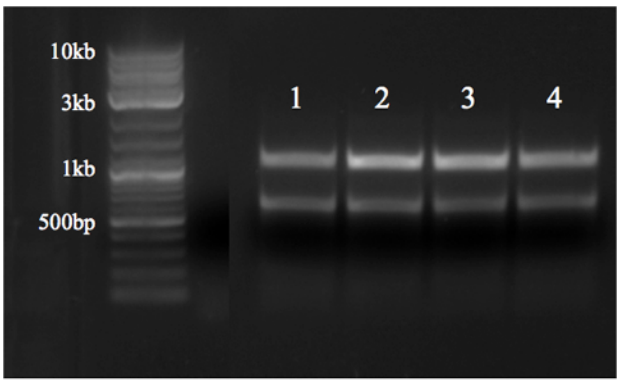


Figure 3.11 *Clostridium difficile* R20291 *cdtA* and *cdtB* Recombinant Allele SOEing PCR

A 1% agarose electrophoresis gel to demonstrate the *cdtA* and *cdtB* recombinant allele SOEing PCR products. Each recombinant allele produced a band of 1,400bp. Four DNA fragments were generated to produce a higher DNA yield when gel extracted. Moderate amounts of residual template DNA were also observed at 700bp.

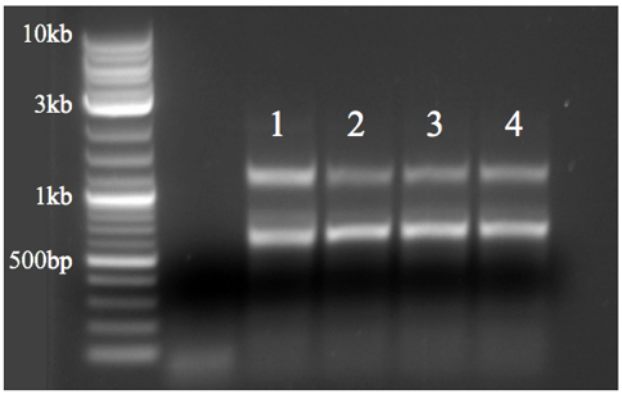


Figure 3.12 *Clostridium difficile* R20291 *cdtR*, *cdtA* and *cdtB* Recombinant Allele SOEing PCR

A 1% agarose electrophoresis gel to demonstrate the *cdtR*, *cdtA* and *cdtB* recombinant allele SOEing PCR products. Each recombinant allele produced a band of 1,400bp. Four DNA fragments were generated to produce a higher DNA yield when gel extracted. Moderate amounts of residual template DNA were also observed at 700bp.

3.2.1.3. KNOCK-OUT PLASMID CREATION

The recombinant alleles were inserted into the pMTL-YN4 vector between *Asc*I and *Sbf*I restriction sites, and confirmed by Sanger DNA sequencing. The following three knock-out plasmids were created in both *Escherichia coli* TOP10 and CA434; pMTL-YN4_*cdtR*, pMTL-YN4_*cdtA*_*cdtB* and pMTL-YN4_*cdtR*_*cdtA*_*cdtB* (Figure 3.13).

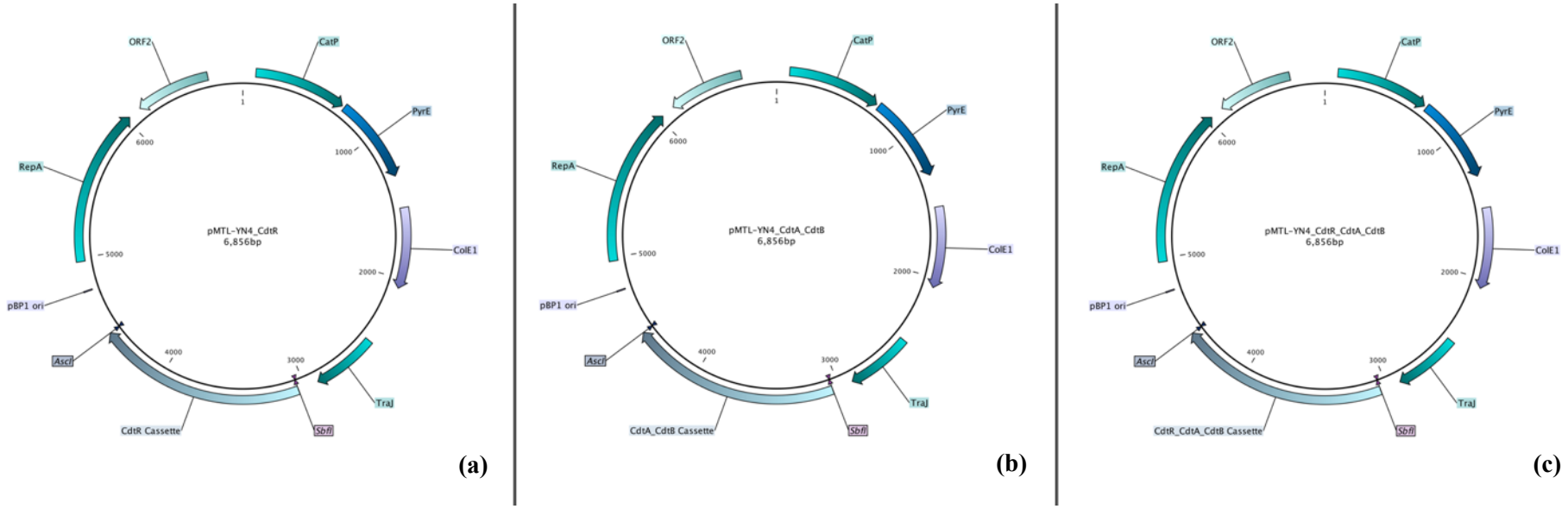


Figure 3.13 *Clostridium difficile* R20291 CDT Allelic Exchange Knock-Out Plasmid Maps

The vector maps illustrate the three knock-out plasmids that were created in the R20291 allelic exchange pMTL-YN4 vector; pMTL-YN4_*cdtR*, pMTL-YN4_*cdtA*_*cdtB* and pMTL-YN4_*cdtR*_*cdtA*_*cdtB*. The generated vector components comprise of: CatP, the *catP* gene of *Clostridium perfringens* conferring chloramphenicol/thiamphenicol resistance; PyrE, the *pyrE* gene of *Clostridium sporogenes*; ColE1, the replication region of the *Escherichia coli* plasmid ColE1; TraJ, transfer function of the RP4 oriT region; Insert between *Asc*I and *Sbf*I restriction sites, **(a)** *cdtR* cassette **(b)** *cdtA* and *cdtB* cassette and **(c)** *cdtR*, *cdtA* and *cdtB* cassette, and, RepA and Orf2, the *Clostridium botulinum* pBP1 plasmid replicating region.

3.2.2. CONSTRUCTION OF THE CDT ALLELIC EXCHANGE KNOCK-OUT STRAINS

3.2.2.1. ISOLATION OF SINGLE CROSS-OVER CLONES

The pMTL-YN4_*cdtR*, pMTL-YN4_*cdtA*_*cdtB* and pMTL-YN4_*cdtR*_*cdtA*_*cdtB* plasmids were transformed into *C. difficile* R20291Δ*pyrE* from *E. coli* CA434. The resulting transformants were isolated on selective BHIS agar containing *C. difficile* selective supplement and thiamphenicol. Single cross-over (SCO) clones were identified as faster growing colonies in amongst slower transconjugant growth. Six potential SCOs for each conjugation were screened by PCR to identify either LHA or RHA insertion direction, with several successful SCO clones obtained (Figure 3.14, Figure 3.15 and Figure 3.16). It was apparent that some of the SCOs were not pure as a PCR product was observed for both an SCO and for the WT, however, these colonies could still be used for subsequent isolation of double cross-over (DCO) clones.

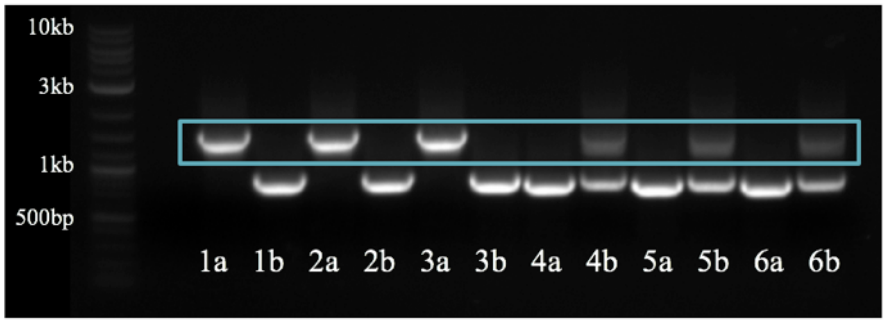


Figure 3.14 *Clostridium difficile* R20291Δ*pyrE* pMTL-YN4_*cdtR* Single Cross-Over PCR

A 1% agarose electrophoresis gel to demonstrate the R20291Δ*pyrE* pMTL-YN4_*cdtR* single cross-over (SCO) clone PCR products. (a) samples identify insertion direction via the left-hand homology arm (LHA) and (b) samples identify insertion direction via the right-hand homology arm (RHA). LHA insertion direction SCO clones generate a DNA fragment of 1,548bp. RHA insertion direction SCO clones create a DNA fragment of 1,590bp. Both LHA and RHA insertion direction SCOs were present as seen in the blue box. It was evident that certain clones were not pure and so a PCR product was observed for both an SCO and for the WT.

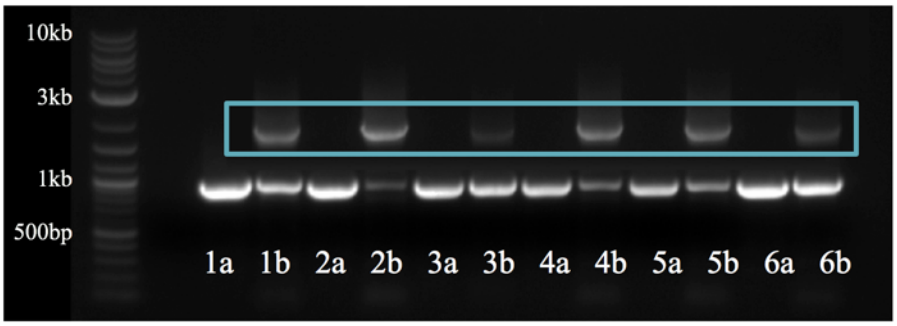


Figure 3.15 *Clostridium difficile* R20291Δ*pyrE* pMTL-YN4_*cdtA*_*cdtB* Single Cross-Over PCR

A 1% agarose electrophoresis gel to demonstrate the R20291Δ*pyrE* pMTL-YN4_*cdtA*_*cdtB* single cross-over (SCO) clone PCR products. (a) samples identify insertion direction via the left-hand homology arm (LHA) and (b) samples identify insertion direction via the right-hand homology arm (RHA). LHA insertion direction SCO clones generate a DNA fragment of 1,608bp. RHA insertion direction SCO clones create a DNA fragment of 1,620bp. Only RHA insertion direction SCOs were present as seen in the blue box. It was evident that the clones were not pure as a PCR product was observed for both an SCO and for the WT.

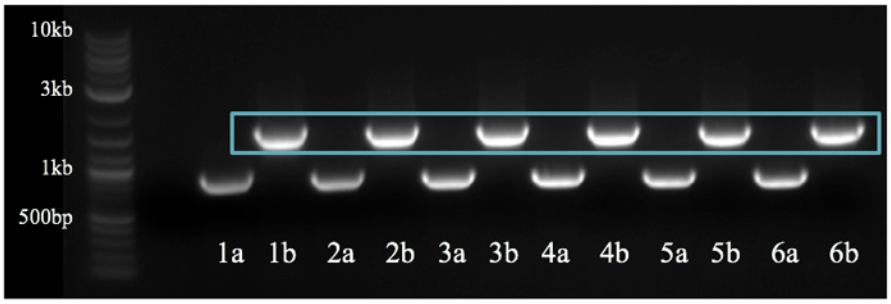


Figure 3.16 *Clostridium difficile* R20291Δ*pyrE* pMTL-YN4_*cdtR*_*cdtA*_*cdtB* Single Cross-Over PCR

A 1% agarose electrophoresis gel to demonstrate the R20291Δ*pyrE* pMTL-YN4_*cdtR*_*cdtA*_*cdtB* single cross-over (SCO) clone PCR products. (a) samples identify insertion direction via the left-hand homology arm (LHA) and (b) samples identify insertion direction via the right-hand homology arm (RHA). LHA insertion direction SCO clones generate a DNA fragment of 1,548bp. RHA insertion direction SCO clones create a DNA fragment of 1,620bp. Only RHA insertion direction SCOs were present as seen in the blue box.

3.2.2.2. ISOLATION OF DOUBLE CROSS-OVER CLONES

The SCO clones confirmed by PCR were then used to isolate DCO clones. To allow the second recombination event to occur, the SCOs were streaked onto

non-selective agar. The removal of selective pressure at that time resulted in the DCO, excision of the plasmid from the chromosome and subsequent plasmid loss from the cell. These potential DCO clones were selected for by plating onto a negative counter-selection medium. Media supplemented with 5-FOA will only allow DCO clones which have lost the plasmid, and so lack a functional *pyrE* gene, to grow. To ensure that the isolated DCOs were true clones, they were also restreaked onto media with and without thiamphenicol. True DCO clones were not able to grow on a thiamphenicol containing medium as they no longer carried the plasmid with the *catP* gene present. Those colonies which were resistant to 5-FOA and thiamphenicol are due to background growth which occurs because of the *pyrE* loss-of-function mutation.

For each DCO event, ten thiamphenicol sensitive clones were screened by PCR to verify whether the colony was a true recombinant clone or had reverted to the WT strain. Typically, a 50:50 mixture of DCOs and WT are expected. The R20291Δ*pyrE*Δ*cdtR* PCR demonstrated four DCO clones (Figure 3.17), the R20291Δ*pyrE*Δ*cdtA*Δ*cdtB* PCR revealed eight DCO clones (Figure 3.18) and the R20291Δ*pyrE*Δ*cdtR*Δ*cdtA*Δ*cdtB* PCR confirmed seven DCO clones (Figure 3.19), which were all confirmed by Sanger DNA sequencing.

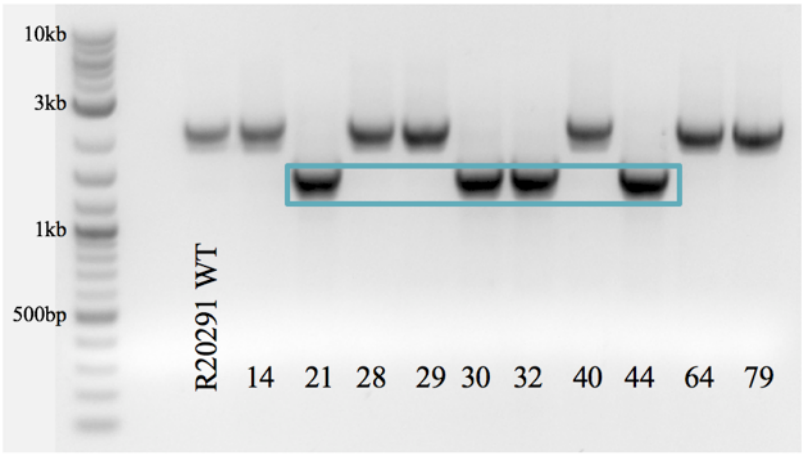


Figure 3.17 *Clostridium difficile* R20291Δ*pyrE*Δ*cdtR* Double Cross-Over PCR

A 1% agarose electrophoresis gel to demonstrate the R20291Δ*pyrE*Δ*cdtR* double cross-over (DCO) clone PCR products. True DCO clones generated a DNA fragment of 1,422bp. Reverted WT colonies created a DNA fragment of 2,169bp. A total of four DCOs were present as indicated in the blue box. The negative gel is shown to highlight the lighter bands.

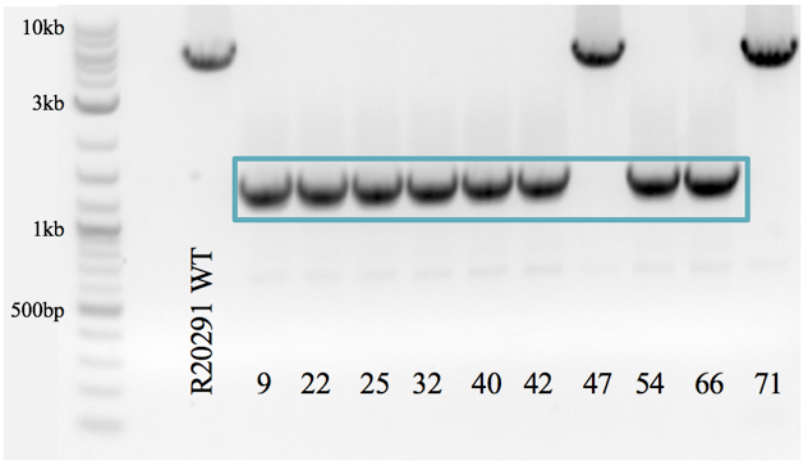


Figure 3.18 *Clostridium difficile* R20291Δ*pyrE*Δ*cdtA*Δ*cdtB* Double Cross-Over PCR

A 1% agarose electrophoresis gel to demonstrate the R20291Δ*pyrE*Δ*cdtA*Δ*cdtB* double cross-over (DCO) clone PCR products. True DCO clones generated a DNA fragment of 1,429bp. Reverted WT colonies created a DNA fragment of 5,504bp. A total of eight DCOs were present as indicated in the blue box. The negative gel is shown to highlight the lighter bands.

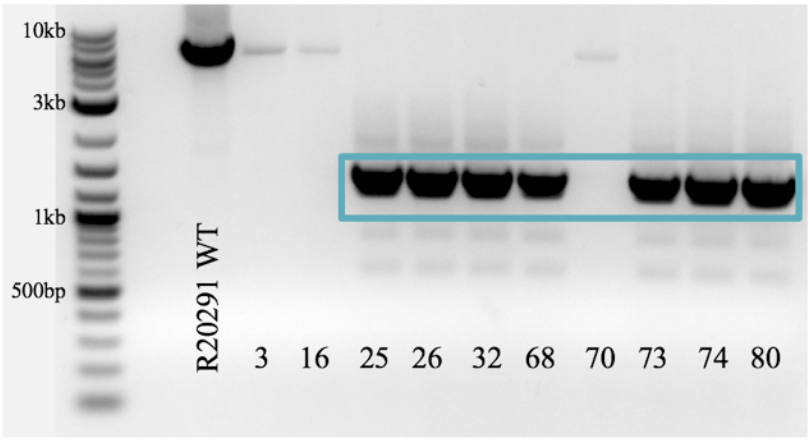


Figure 3.19 *Clostridium difficile* R20291Δ*pyrE*Δ*cdtR*Δ*cdtA*Δ*cdtB* Double Cross-Over PCR

A 1% agarose electrophoresis gel to demonstrate the R20291Δ*pyrE*Δ*cdtR*Δ*cdtA*Δ*cdtB* double cross-over (DCO) clone PCR products. True DCO clones generated a DNA fragment of 1,429bp. Reverted WT colonies created a DNA fragment of 6,640bp. A total of seven DCOs were present as indicated in the blue box. The negative gel is shown to highlight the lighter bands.

3.2.2.3. *pyrE* RESTORATION

Once the R20291Δ*pyrE*Δ*cdtR*, R20291Δ*pyrE*Δ*cdtA*Δ*cdtB* and R20291Δ*pyrE*Δ*cdtR*Δ*cdtA*Δ*cdtB* DCO clones were confirmed, it was necessary to convert the *pyrE* minus knock-out strains to prototrophy using the pMTL-YN2 ACE correction vector, as described in Chapter 2.8.1.4. This enabled all subsequent mutant characterisation to be analysed in a clean, WT background. In all cases, the thiamphenicol sensitive colonies screened by PCR contained the corrected *pyrE* gene (Figure 3.20, Figure 3.21 and Figure 3.22).

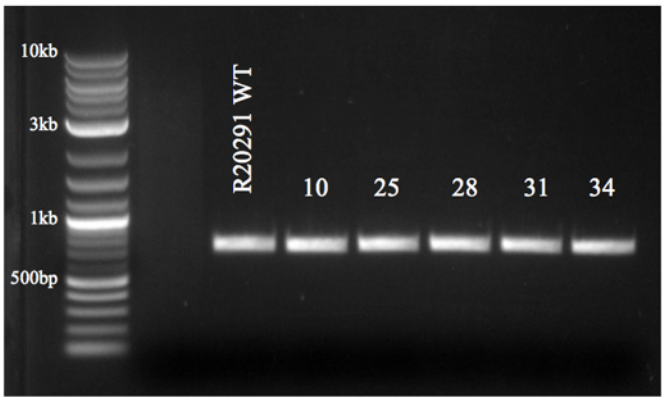


Figure 3.20 *Clostridium difficile* R20291Δ*cdtR pyrE* Correction PCR

A 1% agarose electrophoresis gel to demonstrate the *pyrE* restored R20291Δ*cdtR* PCR products. The corrected *pyrE* gene generated a DNA fragment of 724bp. All screened colonies contained the correct *pyrE* gene.

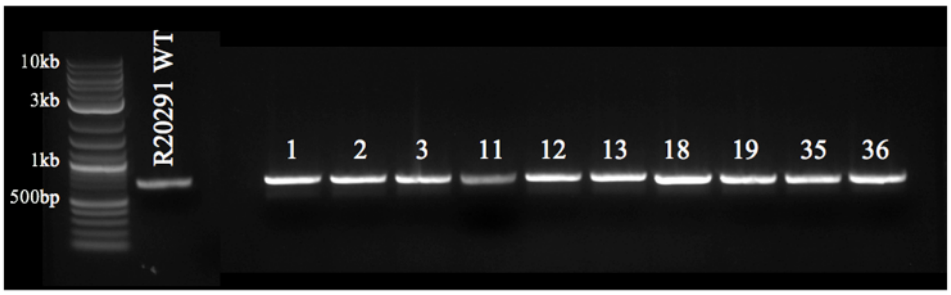


Figure 3.21 *Clostridium difficile* R20291Δ*cdtA*Δ*cdtB pyrE* Correction PCR

A 1% agarose electrophoresis gel to demonstrate the *pyrE* restored R20291Δ*cdtA*Δ*cdtB* PCR products. The corrected *pyrE* gene generated a DNA fragment of 724bp. All screened colonies contained the correct *pyrE* gene.

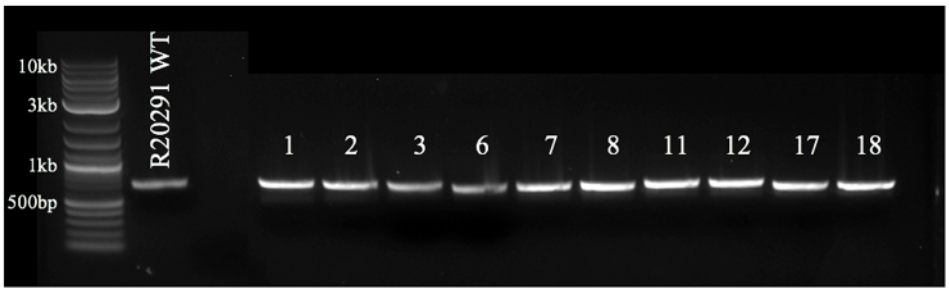


Figure 3.22 *Clostridium difficile* R20291Δ*cdtR*Δ*cdtA*Δ*cdtB pyrE* Correction PCR

A 1% agarose electrophoresis gel to demonstrate the *pyrE* restored R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* PCR products. The corrected *pyrE* gene generated a DNA fragment of 724bp. All screened colonies contained the correct *pyrE* gene.

After *pyrE* correction, the R20291Δ*cdtR*, R20291Δ*cdtA*Δ*cdtB* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* CDT in-frame deletion mutant strains were generated. Each CDT mutant strain was whole genome sequenced and compared against the R20291 whole genome reference by performing basic variant detection (ploidy of 1, minimum coverage of 1, minimum count of 1 and minimum frequency of 100%). This was to ensure that only the target genes had been deleted and no other mutations were present (data not shown).

3.2.3. CONSTRUCTION OF THE PALOC ALLELIC EXCHANGE KNOCK-OUT STRAINS

The allelic exchange system was employed to make three different control PaLoc knock-out mutants using the previously created CDT *pyrE* minus knock-out mutants and the *C. difficile* R20291Δ*pyrE* strain; R20291Δ*pyrE*ΔPaLoc, R20291ΔPaLocΔ*cdtA*Δ*cdtB* and R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB*. The pMTL-YN4_PaLoc ACE plasmid was initially generated by Terry Bilverstone (Bilverstone *et al.*, 2017).

3.2.3.1. ISOLATION OF SINGLE CROSS-OVER CLONES

The pMTL-YN4_PaLoc plasmid was transformed into *C. difficile* R20291Δ*pyrE*, R20291Δ*pyrE*Δ*cdtA*Δ*cdtB* or R20291Δ*pyrE*Δ*cdtR*Δ*cdtA*Δ*cdtB*, from *E. coli* CA434. SCO clones were identified, as previously described in

Chapter 2.8.1.1., and ten potential SCOs for each conjugation were screened by PCR to identify either LHA or RHA insertion direction (Figure 3.23, Figure 3.24 and Figure 3.25). For R20291Δ*pyrE* pMTL-YN4_PaLoc, all ten colonies screened were SCO clones inserting via the LHA. Regarding the R20291Δ*pyrE*Δ*cdtA*Δ*cdtB* pMTL-YN4_PaLoc and the R20291Δ*pyrE*Δ*cdtR*Δ*cdtA*Δ*cdtB* pMTL-YN4_PaLoc SCO clones, it was apparent that certain SCOs were not pure as a PCR product was observed for both a left-hand and right-hand SCO, however, only the pure SCO clones indicated in the blue boxes were used for subsequent isolation of DCO clones.

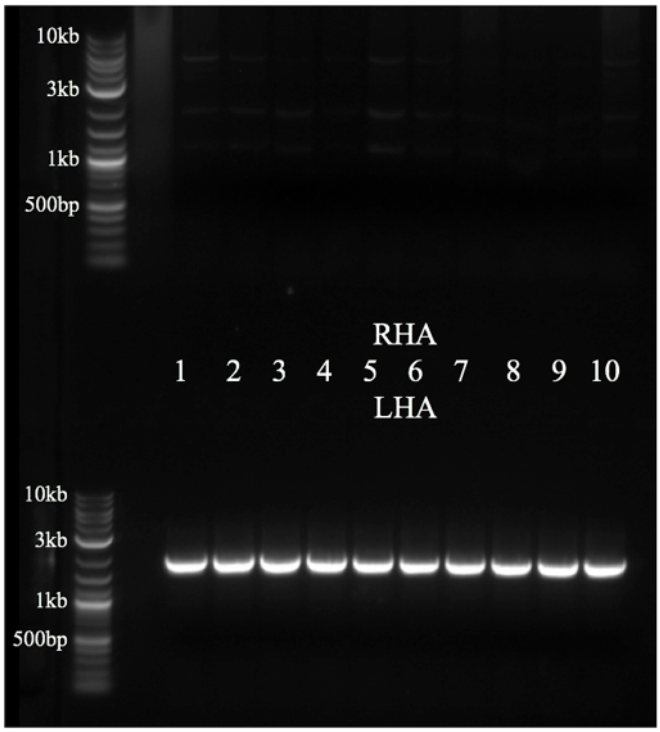


Figure 3.23 *Clostridium difficile* R20291Δ*pyrE* pMTL-YN4_PaLoc Single Cross-Over PCR

A 1% agarose electrophoresis gel to demonstrate the R20291Δ*pyrE* pMTL-YN4_PaLoc single cross-over (SCO) clone PCR products. The top agarose gel image identifies insertion direction via the right-hand homology arm (RHA) and the bottom image identifies insertion direction via the left-hand homology arm (LHA). LHA insertion direction SCO clones generate a DNA fragment of 2,012bp. RHA insertion direction SCO clones create a DNA fragment of 1,946bp. Only LHA insertion direction SCOs were present as illustrated.

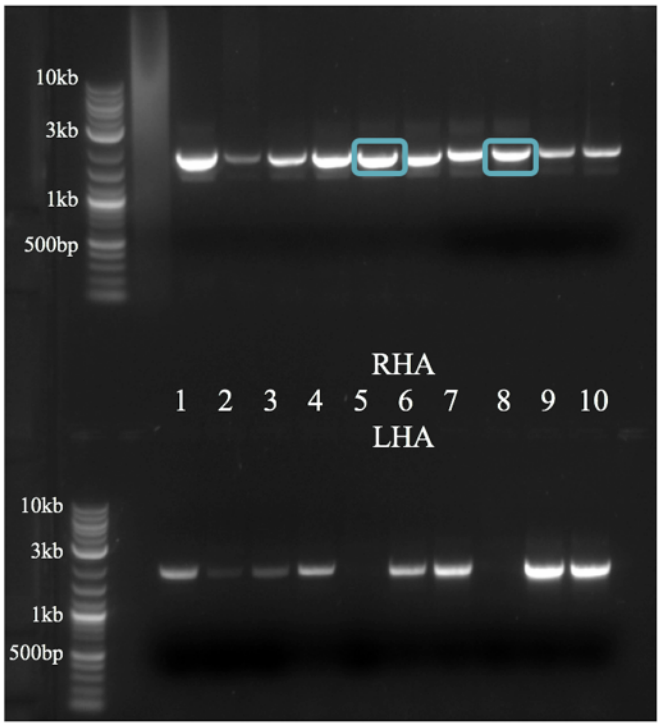


Figure 3.24 *Clostridium difficile* R20291Δ*pyrE*Δ*cdtA*Δ*cdtB* pMTL-YN4_PaLoc Single Cross-Over PCR

A 1% agarose electrophoresis gel to demonstrate the R20291Δ*pyrE*Δ*cdtA*Δ*cdtB* pMTL-YN4_PaLoc single cross-over (SCO) clone PCR products. The top agarose gel image identifies insertion direction via the right-hand homology arm (RHA) and the bottom image identifies insertion direction via the left-hand homology arm (LHA). LHA insertion direction SCO clones generate a DNA fragment of 2,012bp. RHA insertion direction SCO clones create a DNA fragment of 1,946bp. Both LHA and RHA insertion direction SCOs were present, however, only the pure SCO clones, as illustrated in the blue boxes, were used for subsequent DCO isolation.

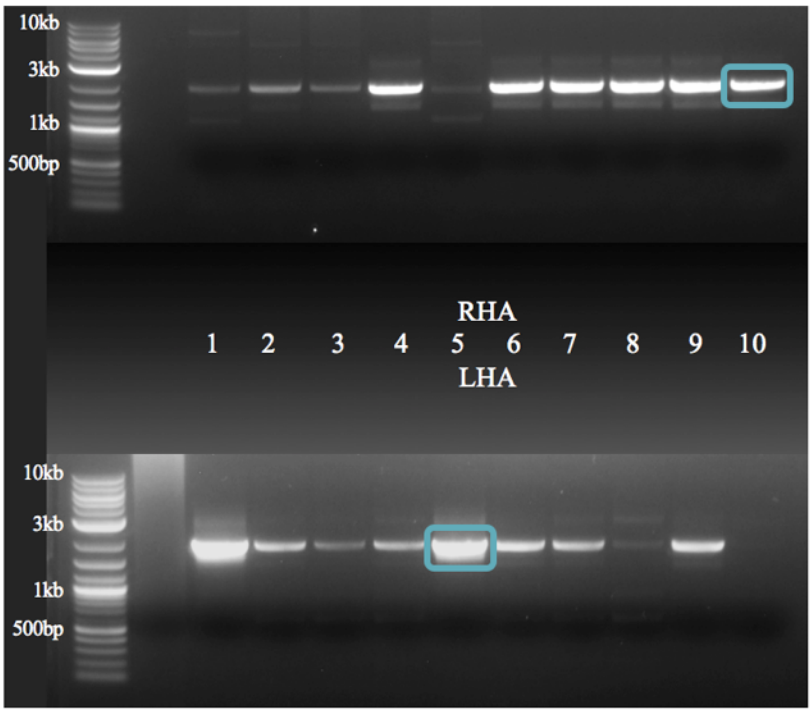


Figure 3.25 *Clostridium difficile* R20291Δ*pyrE*Δ*cdtR*Δ*cdtA*Δ*cdtB* pMTL-YN4_PaLoc Single Cross-Over PCR

A 1% agarose electrophoresis gel to demonstrate the R20291Δ*pyrE*Δ*cdtR*Δ*cdtA*Δ*cdtB* pMTL-YN4_PaLoc single cross-over (SCO) clone PCR products. The top agarose gel image identifies insertion direction via the right-hand homology arm (RHA) and the bottom image identifies insertion direction via the left-hand homology arm (LHA). LHA insertion direction SCO clones generate a DNA fragment of 2,012bp. RHA insertion direction SCO clones create a DNA fragment of 1,946bp. Both LHA and RHA insertion direction SCOs were present, however, only the pure SCO clones, as illustrated in the blue boxes, were used for subsequent DCO isolation.

3.2.3.2. ISOLATION OF DOUBLE CROSS-OVER CLONES

The SCO clones confirmed by PCR were then used to isolate DCO clones. To allow the second recombination event to occur, the SCOs were streaked onto non-selective agar, and potential DCOs were selected for by plating onto a negative counter-selection medium, as previously described in Chapter 2.8.1.2.

For the R20291Δ*pyrE*ΔPaLoc DCO event, three thiamphenicol sensitive clones were screened by PCR to verify whether the colony was a true recombinant clone or had reverted to the WT strain. In the case of the R20291Δ*pyrE*ΔPaLocΔ*cdtA*Δ*cdtB* and R20291Δ*pyrE*ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* DCO events, twelve clones were

screened. The R20291Δ*pyrE*ΔPaLoc PCR demonstrated one DCO clone (Figure 3.26), the R20291Δ*pyrE*ΔPaLocΔ*cdtA*Δ*cdtB* PCR revealed eight DCO clones (Figure 3.27) and the R20291Δ*pyrE*ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* PCR confirmed six DCO clones (Figure 3.28), which were all confirmed by Sanger DNA sequencing.

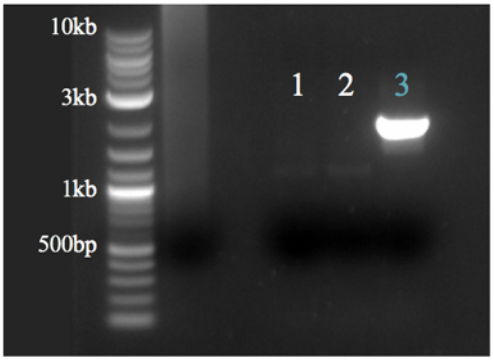


Figure 3.26 *Clostridium difficile* R20291Δ*pyrE*ΔPaLoc Double Cross-Over PCR

A 1% agarose electrophoresis gel to illustrate the R20291Δ*pyrE*ΔPaLoc double cross-over (DCO) clone PCR products. True DCO clones demonstrated a DNA fragment of 1,975bp. Reverted WT colonies produced no band as the PaLoc locus was too large to generate a PCR product. One DCO was present as indicated by the blue number font.

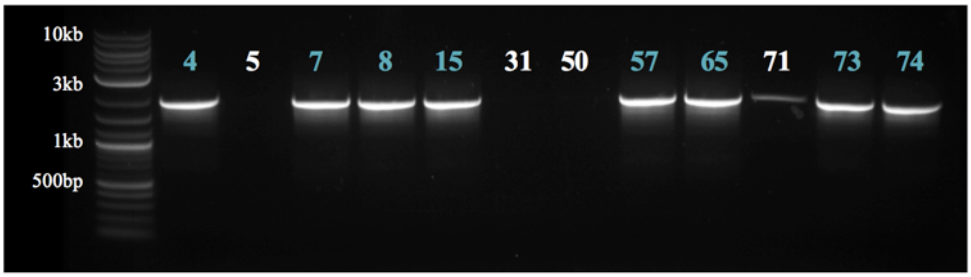


Figure 3.27 *Clostridium difficile* R20291Δ*pyrE*ΔPaLocΔ*cdtA*Δ*cdtB* Double Cross-Over PCR

A 1% agarose electrophoresis gel to illustrate the R20291Δ*pyrE*ΔPaLocΔ*cdtA*Δ*cdtB* double cross-over (DCO) clone PCR products. True DCO clones demonstrated a DNA fragment of 1,975bp. Reverted WT colonies produced no band as the PaLoc locus was too large to generate a PCR product. Eight DCOs were present as indicated by the blue number font.

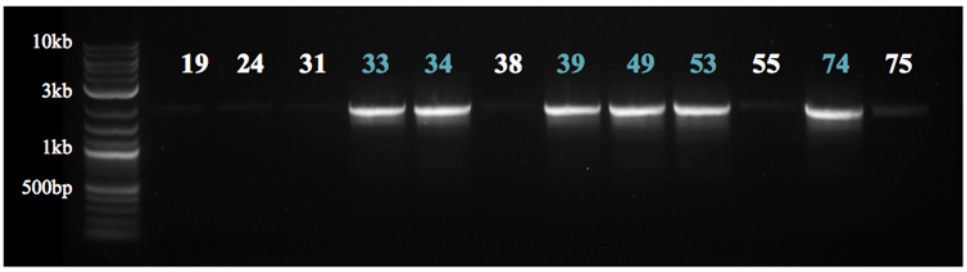


Figure 3.28 *Clostridium difficile* R20291Δ*pyrE*ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* Double Cross-Over PCR

A 1% agarose electrophoresis gel to illustrate the R20291Δ*pyrE*ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* double cross-over (DCO) clone PCR products. True DCO clones demonstrated a DNA fragment of 1,975bp. Reverted WT colonies produced no band as the PaLoc locus was too large to generate a PCR product. Six DCOs were present as indicated by the blue number font.

3.2.3.3. *pyrE* RESTORATION

Once the R20291Δ*pyrE*ΔPaLocΔ*cdtA*Δ*cdtB* and R20291Δ*pyrE*ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* DCO clones were confirmed, it was necessary to convert the *pyrE* minus knock-out strains to prototrophy, as previously described in Chapter 2.8.1.4. In both cases, the thiamphenicol sensitive colonies screened by PCR contained the corrected *pyrE* gene (Figure 3.29).

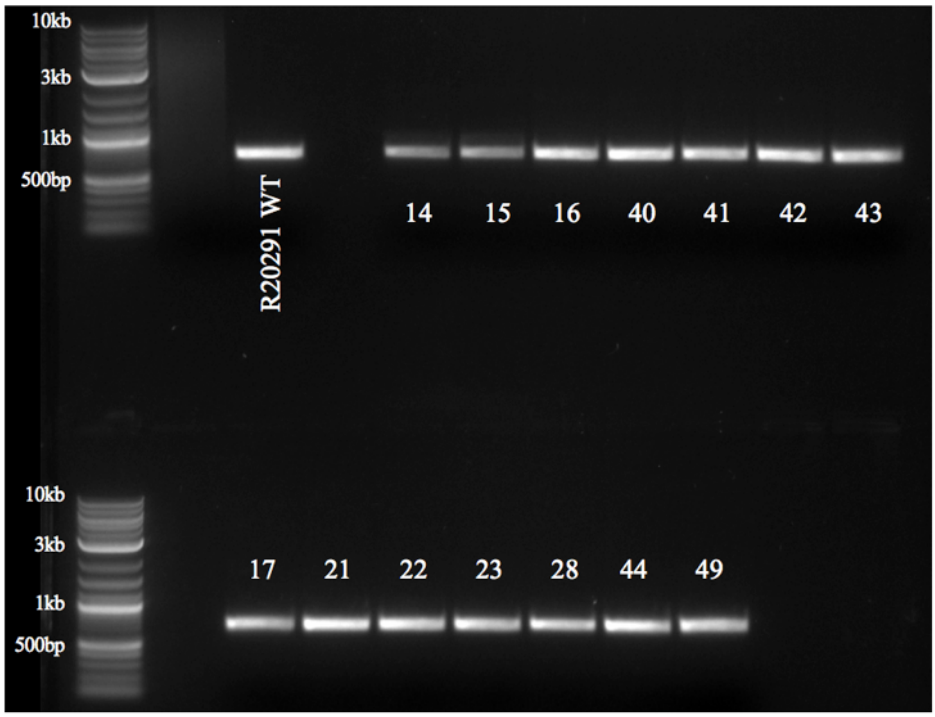


Figure 3.29 *Clostridium difficile* R20291ΔPaLocΔ*cdtA*Δ*cdtB* and R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB pyrE* Correction PCR

A 1% agarose electrophoresis gel to demonstrate the *pyrE* restored R20291ΔPaLocΔ*cdtA*Δ*cdtB* (top row image) and R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* (bottom row image) PCR products. The corrected *pyrE* gene generated a DNA fragment of 724bp. All screened colonies contained the correct *pyrE* gene.

After *pyrE* correction, the R20291ΔPaLocΔ*cdtA*Δ*cdtB* and R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* PaLoc and CDT in-frame deletion mutant control strains were generated. The R20291Δ*pyrE*ΔPaLoc mutant was created in duplicate by both myself and Terry Bilverstone, and so the *pyrE* restored version utilised in knock-out mutant characterisation was corrected by Terry Bilverstone. The R20291ΔPaLocΔ*cdtR* control strain was produced at an earlier point by Terry Bilverstone using the R20291Δ*pyrE*Δ*cdtR* knock-out mutant (Bilverstone *et al.*, 2017).

3.2.4. TRANSCRIPTIONAL LINKAGE OF THE CDT GENE LOCUS

RT PCR was utilised to determine whether the *C. difficile* R20291 binary toxin locus *cdtR*, *cdtA* and *cdtB* genes were transcriptionally linked. The

outcome of this investigation determined how the CDT knock-out mutant complement inserts were designed.



Figure 3.30 *Clostridium difficile* R20291 Binary Toxin Promoter Prediction

The figure illustrates the promoter position that is allocated to the first nucleotide of the transcript, also known as the transcription start site position (TSSP), as well as two conserved promoter sequences, 10 and 35 nucleotides upstream, and their respective scores. The linear discriminant function (LDF) score combines functional motif characteristics and the nucleotide sequence of these regions. **(a)** Predicted promoter upstream of the *cdtR* gene (yellow) **(b)** Predicted promoter upstream of the *cdtA* gene (blue). The boxes on the right-hand side of the figure describe features of the transcriptional factor binding sites, and their specific scores.

Initially, computer analysis was completed to predict potential promoters using the Prediction of Bacterial Promoters (BPROM) online program (Solovyev and Salamov, 2011). This algorithm calculates probable transcription start site positions (TSSP) of bacterial genes by sigma70 (σ^{70}) promoters. Members of this family are known to form parts of the RNA polymerase (RNAP) holoenzyme that directs the core RNAP to promoter features, positioned 10 and 35 nucleotides upstream of the TSSP (Paget and Helmann, 2003). The top scoring promoter results upstream of *cdtR* and *cdtA* are illustrated in Figure 3.30.

For the RT-PCR, two primer sets were designed within the CDT locus; one set which bridged the *cdtR* and *cdtA* genes, and another set which bridged the *cdtA* and *cdtB* genes (Figure 3.31).

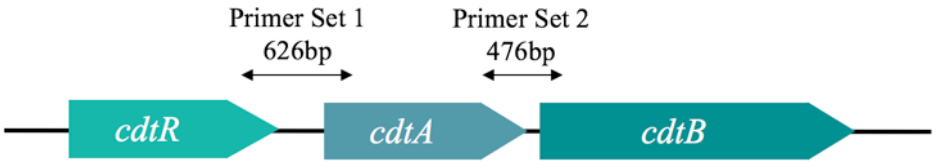


Figure 3.31 *Clostridium difficile* R20291 Binary Toxin Reverse Transcriptase PCR Primer Design

Figure to illustrate the two reverse transcriptase (RT) PCR primers sets designed within the CDT locus. Primer set 1 bridges the *cdtR* and *cdtA* genes and generates a PCR product of 626bp. Primer set 2 bridges the *cdtA* and *cdtB* genes and produces a PCR product of 476bp.

The RNA was extracted and purified, as previously described in Chapter 2.10.2., from *C. difficile* R20291 samples taken at 4, 8 and 24-hour time points. The amount of total RNA template for each PCR reaction was standardised so that 100ng/50µl reaction was achieved. A control PCR reaction was carried out using a Taq DNA polymerase instead of reverse transcriptase to ensure any amplification observed was not due to genomic DNA contamination. Each primer set was also tested with the R20291 strain acting as a positive control to confirm the PCR reaction conditions were optimised.

The results shown in Figure 3.32 demonstrated that the *C. difficile* binary toxin gene locus in R20291 is transcriptionally linked as RNA expression was qualitatively detected through the amplification of complementary DNA (cDNA) from the introns between *cdtR* and *cdtA*, and *cdtA* and *cdtB*. It also revealed that transcription occurs at both early and late exponential phase (4 and 8-hour time points), as well as in stationary phase (24-hour time point). The control Taq DNA polymerase PCR reaction demonstrated no detectible DNA contamination.

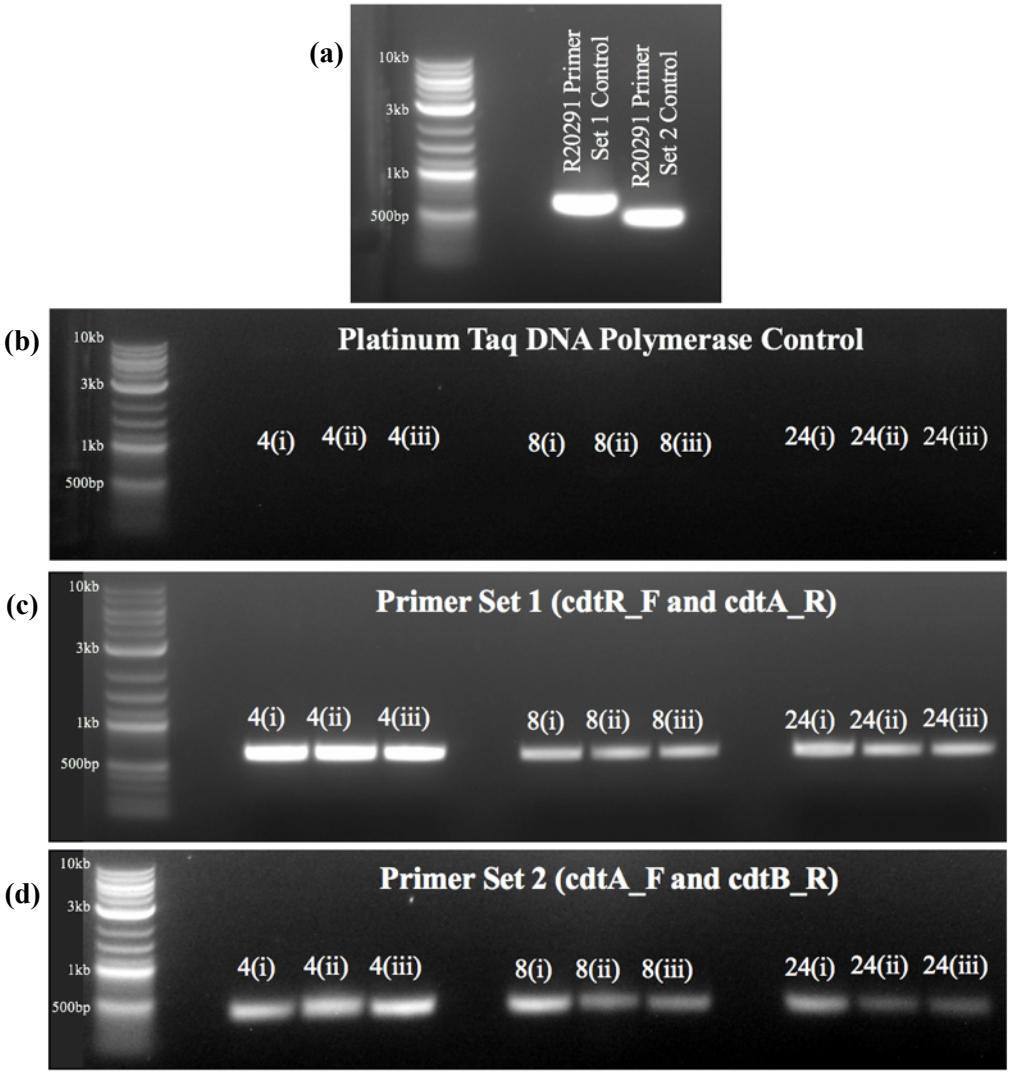


Figure 3.32 *Clostridium difficile* R20291 Binary Toxin Reverse Transcriptase PCR

The reverse transcriptase (RT) PCR demonstrated that the *C. difficile* R20291 binary toxin gene locus is transcriptionally linked, as observed in the **(c)** and **(d)** 1% agarose electrophoresis gels. Transcription was evident at both early and late exponential phase (4 and 8-hour time points), as well as in stationary phase (24-hour time point). The control Taq DNA polymerase PCR reaction to ensure any amplification observed was not due to genomic DNA contamination is seen in agarose gel **(b)**. Each primer set was also tested with R20291 to confirm the PCR reaction conditions were optimised **(a)**.

3.2.5. CONSTRUCTION OF THE CDT COMPLEMENTED PLASMIDS

The R20291Δ*cdtR*, R20291Δ*cdtA*Δ*cdtB* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* knock-out mutant strains were complemented. To complement the R20291Δ*cdtA*Δ*cdtB* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* strains, a two-step method

was designed. This was a necessary requirement to avoid the insertion of a whole bacterial toxin gene locus with a functional promoter into the *E. coli* host. A bacterial toxin gene that retains the native promoter can potentially be expressed in *E. coli*, creating a toxigenic recipient strain that poses a greater risk to human health. These scenarios usually demand national regulatory approval and a higher containment level (Health and Safety Executive, 2007; Health and Safety Executive, 2014).

For the first step in the two-step complementation method, a pMTL-YN18 ACE plasmid containing the *cdtA* (missing the first 700 nucleotides) and *cdtB* genes was produced. Then each complemented knock-out mutant had an allocated pMTL-YN2C complementation plasmid created for the second step of the process. The RT-PCR revealed that *cdtR*, *cdtA* and *cdtB* were transcriptionally linked, however, promoter prediction analysis determined that there was likely a promoter region upstream of *cdtA*. It was decided to create the R20291Δ*cdtA*Δ*cdtB***cdtA***cdtB* complemented strain using the intergenic spacer (IGS) region containing the predicted promoter instead of the promoter upstream of *cdtR*. This was to prevent additional cloning complications when complementing this mutant strain. Therefore, the pMTL-YN2C plasmid for the R20291Δ*cdtA*Δ*cdtB* strain contained the IGS region (between *cdtR* and *cdtA*) and the *cdtA* gene. A pMTL-YN2C plasmid containing the promoter region (PR), *cdtR* and *cdtA* genes was also generated to complement the R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* strain.

The three different vector inserts (*cdtA_cdtB*, IGS_*cdtA* and PR_*cdtR_cdtA*) were originally PCR amplified with specific restriction sites for ligation into the pMTL-YN18 or pMTL-YN2C plasmids. In this situation, the only successful ligation was for the IGS_*cdtA* insert (Figure 3.33) into the pMTL-YN2C plasmid via the *Not*I and *Asc*I restriction sites. The vector was subsequently transformed into *E. coli* TOP10 and 10 colonies were confirmed by control PCR using the primers for amplification of the insert (Figure 3.34). Following Sanger DNA sequencing verification, the plasmid was transformed into *E. coli* CA434.

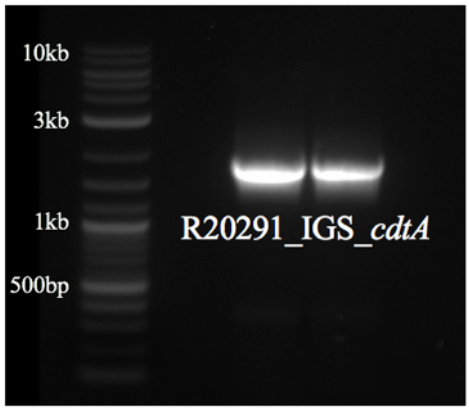


Figure 3.33 Complement IGS_*cdtA* Insert PCR

A 1% agarose electrophoresis gel to demonstrate the complement IGS_*cdtA* insert PCR product. The PCR product produced a DNA fragment of 1,797bp and was duplicated to generate a higher DNA yield when gel extracted.

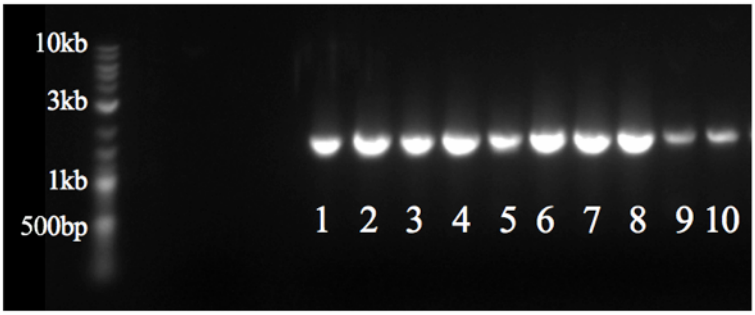


Figure 3.34 Complement pMTL-YN2C_IGS_*cdtA* Plasmid *E. coli* TOP10 Control PCR

A 1% agarose electrophoresis gel to demonstrate the control PCR product of the complement pMTL-YN2C_IGS_*cdtA* insert in *E. coli* TOP10. The insert PCR product produced a DNA fragment of 1,797bp. All ten colonies screened were confirmed by the presence of the insert at the correct band size.

After the unsuccessful attempt of plasmid creation for the other two vector inserts (*cdtA_cdtB* and PR_*cdtR_cdtA*), the Gibson Assembly method was employed. The primers were redesigned to fulfil the necessary requirements of creating a double-stranded fragment with a 15-80bp overlap. In this case, 16 nucleotides, as well as the restriction sites from the pMTL-YN18 or pMTL-YN2C vectors (*Not*I/*Nhe*I and *Not*I/*Asc*I, respectively), were used as the

plasmid overlapping fragments. The two inserts were amplified by PCR with the redesigned primers (Figure 3.35) and both plasmids were linearised by digestion with their corresponding restriction endonuclease enzymes. The specific insert and vector combination was added to a Gibson Assembly master mixture containing an exonuclease; to produce single-stranded 3' overhangs, a polymerase; to complete gaps in the annealed product, and a DNA ligase; to seal nicks. The resulting pMTL-YN18_*cdtA_cdtB* and pMTL-YN2C_PR_*cdtR_cdtA* plasmids were subsequently transformed into *E. coli* TOP10. Ten colonies from each transformation were screened for the correct insert by a control digest with the relevant restriction endonuclease enzymes. The control digest for the allelic exchange pMTL-YN18_*cdtA_cdtB* plasmid identified nine correct inserts (Figure 3.36). For the complement pMTL-YN2C_PR_*cdtR_cdtA* plasmid there were four confirmed inserts observed (Figure 3.37).

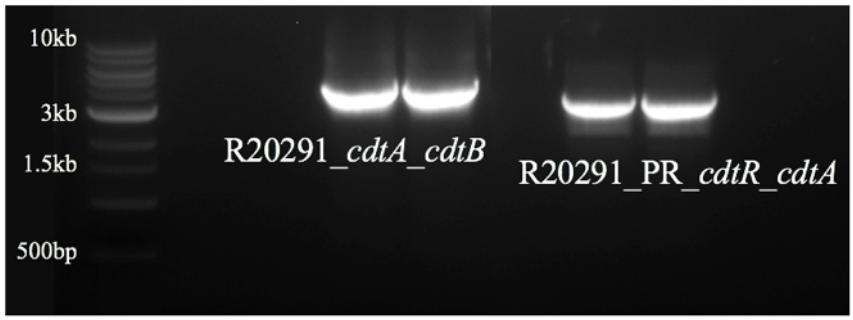


Figure 3.35 Allelic Exchange *cdtA_cdtB* and Complement PR_*cdtR_cdtA* Inserts PCR

A 1% agarose electrophoresis gel to demonstrate the allelic exchange *cdtA_cdtB* and complement PR_*cdtR_cdtA* PCR products. The allelic exchange *cdtA_cdtB* PCR product produced a DNA fragment of 3,419bp. The complement PR_*cdtR_cdtA* PCR product generated a DNA fragment of 3,276bp. Both inserts were duplicated to generate a higher DNA yield when gel extracted.

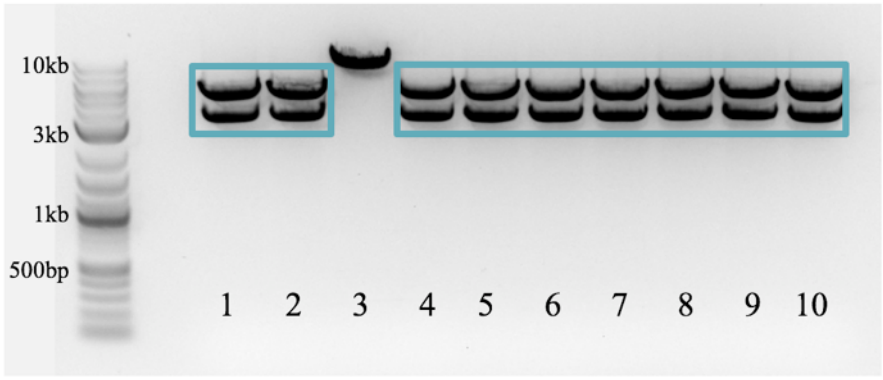


Figure 3.36 Allelic Exchange pMTL-YN18_*cdtA_cdtB* Plasmid *E. coli* TOP10 Control Digest

A 1% agarose electrophoresis gel to demonstrate the control digest product of the allelic exchange pMTL-YN18_*cdtA_cdtB* insert in *E. coli* TOP10. The digest products produced a DNA fragment of 3,419bp for the insert and 6,562bp for the empty plasmid. Nine colonies were confirmed by the presence of the insert and plasmid at the correct band sizes. The negative gel is shown to highlight the lighter bands.

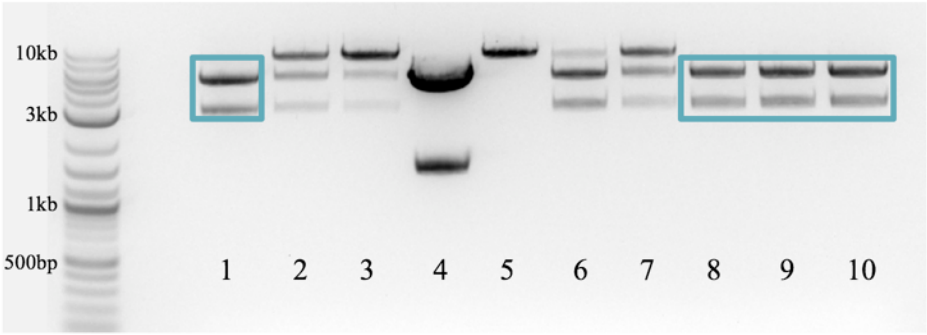


Figure 3.37 Complement pMTL-YN2C_PR_*cdtR_cdtA* Plasmid *E. coli* TOP10 Control Digest

A 1% agarose electrophoresis gel to demonstrate the control digest product of the complement pMTL-YN2C_PR_*cdtR_cdtA* insert in *E. coli* TOP10. The digest products produced a DNA fragment of 3,276bp for the insert and 5,354bp for the empty plasmid. Four colonies were confirmed by the presence of the insert and plasmid at the correct band sizes. The negative gel is shown to highlight the lighter bands.

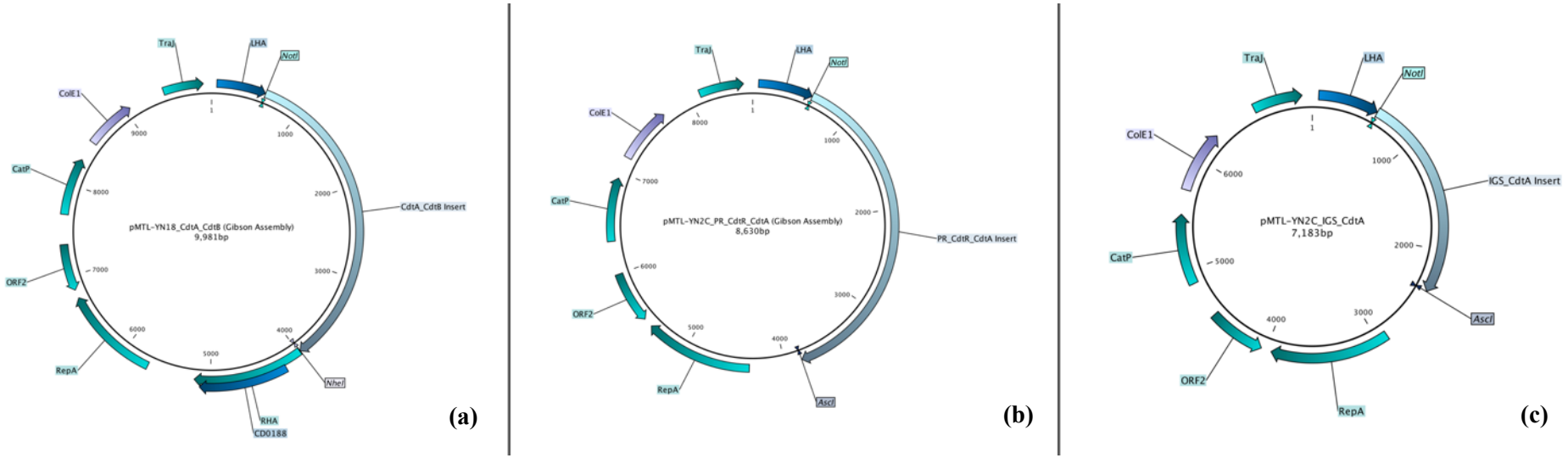


Figure 3.38 *Clostridium difficile* R20291 Allelic Exchange and Complement CDT Plasmid Maps for Complementation

The vector maps illustrate the complementation plasmids that were created in the R20291 allelic exchange pMTL-YN18 vector or the complement pMTL-YN2C vector; pMTL-YN18_*cdtA_cdtB*, pMTL-YN2C_PR_*cdtR_cdtA* and pMTL-YN2C_IGS_*cdtA*. The identical vector components of both pMTL-YN18 and pMTL-YN2C comprise of: CatP, the *catP* gene of *Clostridium perfringens* providing chloramphenicol/thiamphenicol resistance; ColE1, the replication region of the *Escherichia coli* plasmid ColE1; TraJ, transfer function of the RP4 oriT region; LHA, the left-hand homology arm that consists of the R20291 *pyrE* gene, missing 50 nucleotides at the 5' end and 235bp at the 3' end; and, RepA and Orf2, the *Clostridium botulinum* pBP1 plasmid replicating region. The pMTL-YN18 also contains a RHA, the right-hand homology arm that comprises of the 1200bp region immediately downstream of the *pyrE* gene. This was deleted in the two created pMTL-YN2C plasmids and part of the subsequent insert acted as a RHA. The plasmid inserts consisted of: **(a)** pMTL-YN18 *cdtA_cdtB* insert generated by Gibson Assembly and between *Not*I and *Nhe*I restriction sites; **(b)** pMTL-YN2C PR_*cdtR_cdtA* insert generated by Gibson Assembly and between *Not*I and *Asc*I restriction sites, and; **(c)** pMTL-YN2C IGS_*cdtA* insert between *Not*I and *Asc*I restriction sites.

3.2.6. CONSTRUCTION OF THE CDT COMPLEMENTED STRAINS

3.2.6.1. ISOLATION OF SINGLE CROSS-OVER CLONES

To create the R20291Δ*cdtA*Δ*cdtB***cdtA***cdtB* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* complemented strains, the first step of the two-step complementation process consisted of conjugating the allelic exchange pMTL-YN18_*cdtA*_*cdtB* plasmid from *E. coli* CA434 into the *pyrE* corrected R20291Δ*cdtA*Δ*cdtB* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* knock-out strains. One SCO clone was successfully isolated, as previously described in Chapter 2.8.1.1., for the R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* complemented strain (Figure 3.39).

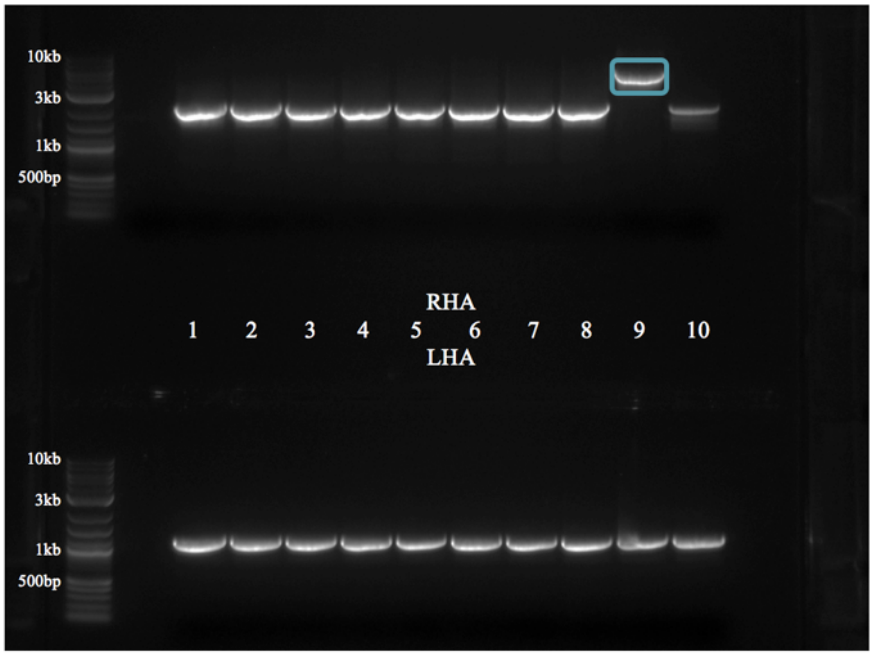


Figure 3.39 *Clostridium difficile* R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* pMTL-YN18_*cdtA*_*cdtB* Single Cross-Over PCR

A 1% agarose electrophoresis gel to demonstrate the R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* pMTL-YN18_*cdtA*_*cdtB* single cross-over (SCO) clone PCR products. The top agarose gel image identifies insertion direction via the right-hand homology arm (RHA) and the bottom image identifies insertion direction via the left-hand homology arm (LHA). LHA insertion direction SCO clones generate a DNA fragment of 3,902bp. RHA insertion direction SCO clones create a DNA fragment of 4,798bp. Only one RHA insertion direction SCO was present, as illustrated in the blue box. Although the SCO clone did not appear pure, it was still used for subsequent DCO isolation.

Although the SCO did not appear pure, it was still used for subsequent DCO isolation. Unfortunately, although several attempts were made, SCOs were not observed for the R20291Δ*cdtA*Δ*cdtB***cdtA***cdtB* complemented strain. No further optimisation was performed due to time constraints of this study and the need to characterise the generated mutant strains.

3.2.6.2. ISOLATION OF DOUBLE CROSS-OVER CLONES

The R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* pMTL-YN18_*cdtA*_*cdtB* SCO clone confirmed by PCR were then used to isolate a DCO clone, as previously described in Chapter 2.8.1.2. (Figure 3.40).

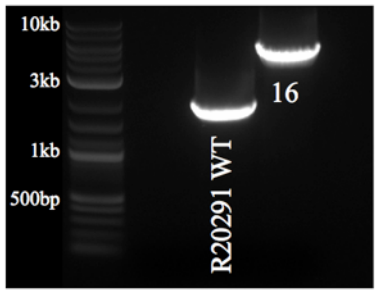


Figure 3.40 *Clostridium difficile* R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* pMTL-YN18_*cdtA*_*cdtB* Double Cross-Over PCR

A 1% agarose electrophoresis gel to illustrate the R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* pMTL-YN18_*cdtA*_*cdtB* double cross-over (DCO) clone PCR product. True DCO clones demonstrated a DNA fragment of 5,125bp. The R20291 WT strain and reverted WT colonies produced a PCR band visible at 1,738bp. One potential DCO clone was screened and found to be correct.

3.2.6.3. COMPLEMENTATION STRAIN GENERATION AND *pyrE* RESTORATION

Once the R20291Δ*pyrE*Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtA***cdtB* DCO clone (with the *cdtA* missing 700 nucleotides at the 5' end) was confirmed by Sanger DNA sequencing, the conjugation with the pMTL-YN2C_PR_*cdtR*_*cdtA* complementation plasmid from *E. coli* CA434 was performed. This allowed the strain to be converted to *pyrE* prototrophy, with the concomitant insertion of the PR_*cdtR*_*cdtA* fragment. Three transconjugant colonies were screened by PCR, with two containing the entire complemented CDT gene locus (Figure 3.41). All colonies screened for the restored *pyrE* gene were correct (Figure 3.43). Furthermore, the R20291R20291Δ*pyrE*Δ*cdtR* strain was also *pyrE* restored and simultaneously complemented with the pMTL-YN2C_PR_*cdtR* complementation plasmid. The pMTL-YN2C_PR_*cdtR* plasmid was generated earlier by Terry Bilverstone. Two of the three transconjugant colonies that were PCR screened contained the *cdtR* gene (Figure 3.41). All colonies screened for the restored *pyrE* gene were correct (Figure 3.42).

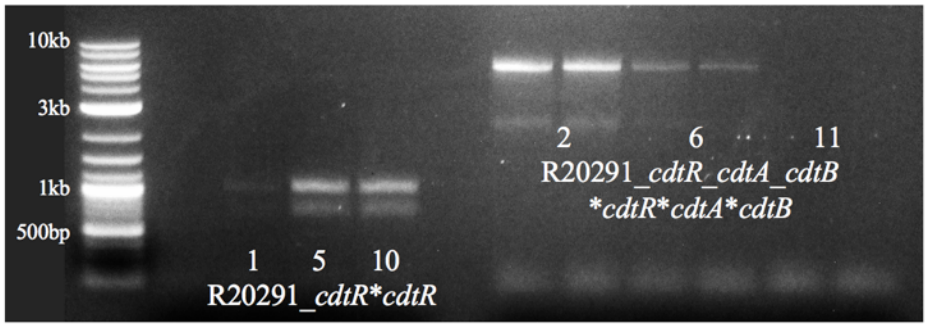


Figure 3.41 *Clostridium difficile* R20291Δ*cdtRcdtR and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB**cdtR*cdtA*cdtB Complementation PCR**

A 1% agarose electrophoresis gel to demonstrate the PCR products observed for the complementation of the R20291Δ*cdtR***cdtR* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* knock-out mutant strains. The R20291Δ*cdtR***cdtR* strain generated a DNA fragment of 1,044bp. The R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* produced a DNA band observed at 5,911bp and was duplicated to generate a higher DNA yield when gel extracted. Mild to moderate non-specific PCR binding was observed for both primer sets, however, this did not interfere with verification of the target DNA fragments.

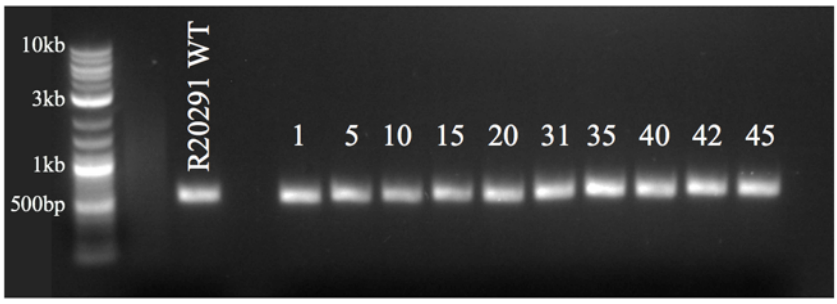


Figure 3.42 *Clostridium difficile* R20291Δ*cdtR*cdtR pyrE* Correction PCR**

A 1% agarose electrophoresis gel to demonstrate the *pyrE* restored R20291Δ*cdtR***cdtR* PCR products. The corrected *pyrE* gene generated a DNA fragment of 541bp. All screened colonies contained the correct *pyrE* gene.

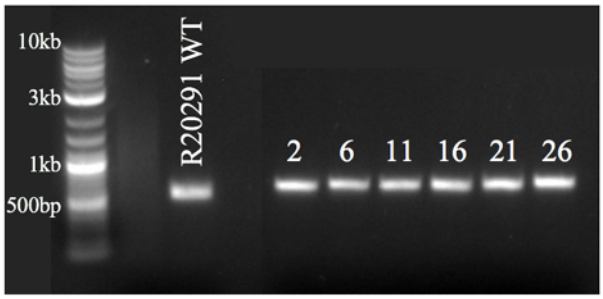


Figure 3.43 *Clostridium difficile* R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*cdtR***cdtA***cdtB pyrE* Correction PCR**

A 1% agarose electrophoresis gel to demonstrate the *pyrE* restored R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* PCR products. The corrected *pyrE* gene generated a DNA fragment of 541bp. All screened colonies contained the correct *pyrE* gene.

After complementation and *pyrE* correction, the R20291Δ*cdtR***cdtR* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* CDT complemented strains were generated.

3.2.7. CHARACTERISATION OF THE CDT AND PALOC ALLELIC EXCHANGE KNOCK-OUT STRAINS AND CDT COMPLEMENTED STRAINS

3.2.7.1. MEASUREMENT OF GROWTH RATES

Growth differences were assessed between the R20291 WT strain and the CDT knock-out mutant strains (R20291Δ*cdtR*, R20291Δ*cdtA*Δ*cdtB* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*), as well as the PaLoc knock-out control mutant strains (R20291ΔPaLoc, R20291ΔPaLocΔ*cdtR*, R20291ΔPaLocΔ*cdtA*Δ*cdtB* and R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB*), and the complemented strains (R20291Δ*cdtR***cdtR* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB*). All the strains were grown in BHIS broth and measured over a 24-hour period at OD_{600}.

Figure 3.44 demonstrates the growth for R20291ΔPaLoc, R20291Δ*cdtR*, R20291ΔPaLocΔ*cdtR* and R20291Δ*cdtR***cdtR* when compared to the R20291

WT strain. The phenotypic growth of R20291ΔPaLoc and R20291Δ*cdtR* were observed to be indistinguishable from the R20291 WT strain. However, the R20291ΔPaLocΔ*cdtR* and R20291Δ*cdtR***cdtR* strains appeared to reach higher exponential growth when compared to R20291 WT. No significant difference was determined between the R20291 WT and R20291ΔPaLoc and R20291Δ*cdtR*. A significant difference was detected between the R20291 WT and R20291ΔPaLocΔ*cdtR* ($p = 0.0437$) and R20291Δ*cdtR***cdtR* ($p = 0.0008$). Furthermore, when the R20291Δ*cdtR* and R20291Δ*cdtR***cdtR* strains were compared, they were found to be significantly different ($p = 0.0005$).

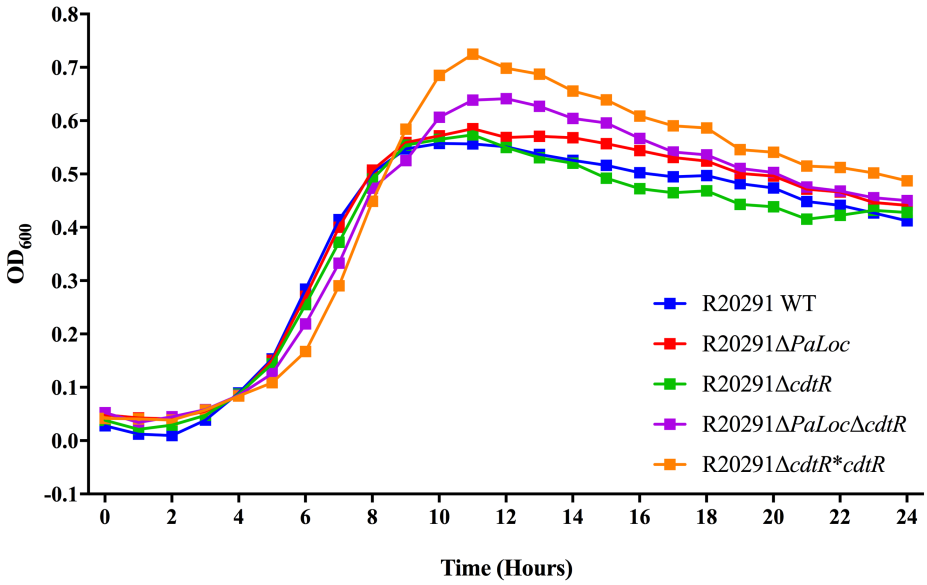


Figure 3.44 Growth Curve to Compare the *Clostridium difficile* Binary Toxin *cdtR* Mutant Strains

Growth curve to demonstrate the growth for R20291ΔPaLoc, R20291Δ*cdtR*, R20291ΔPaLocΔ*cdtR* and R20291Δ*cdtR***cdtR* when compared to the R20291 WT strain. Average of three biologically independent replicates. The growth (measured by optical density) of R20291ΔPaLoc and R20291Δ*cdtR* were observed to be indistinguishable from the R20291 WT strain. However, the R20291ΔPaLocΔ*cdtR* and R20291Δ*cdtR***cdtR* strains appeared to reach higher exponential growth when compared to R20291 WT. A significant difference was detected between the R20291 WT and R20291ΔPaLocΔ*cdtR* ($p = 0.0437$) and R20291Δ*cdtR***cdtR* ($p = 0.0008$). Furthermore, when the R20291Δ*cdtR* and R20291Δ*cdtR***cdtR* strains were compared, they were found to be significantly different ($p = 0.0005$).

Figure 3.45 demonstrates the growth for R20291ΔPaLoc, R20291Δ*cdtA*Δ*cdtB* and R20291ΔPaLocΔ*cdtA*Δ*cdtB* when compared to the R20291 WT strain. The phenotypic growth of R20291ΔPaLoc and R20291Δ*cdtA*Δ*cdtB* were observed to be indistinguishable from the R20291 WT strain. However, the R20291ΔPaLocΔ*cdtA*Δ*cdtB* strain appeared to reach higher exponential growth when compared to R20291 WT. No significant difference was determined between the R20291 WT and R20291ΔPaLoc. A significant difference was detected between the R20291 WT and R20291ΔPaLocΔ*cdtA*Δ*cdtB* ($p = 0.0015$). A significant difference was also observed between the R20291 WT and R20291Δ*cdtA*Δ*cdtB* ($p = 0.0211$), which was not visibly identified.

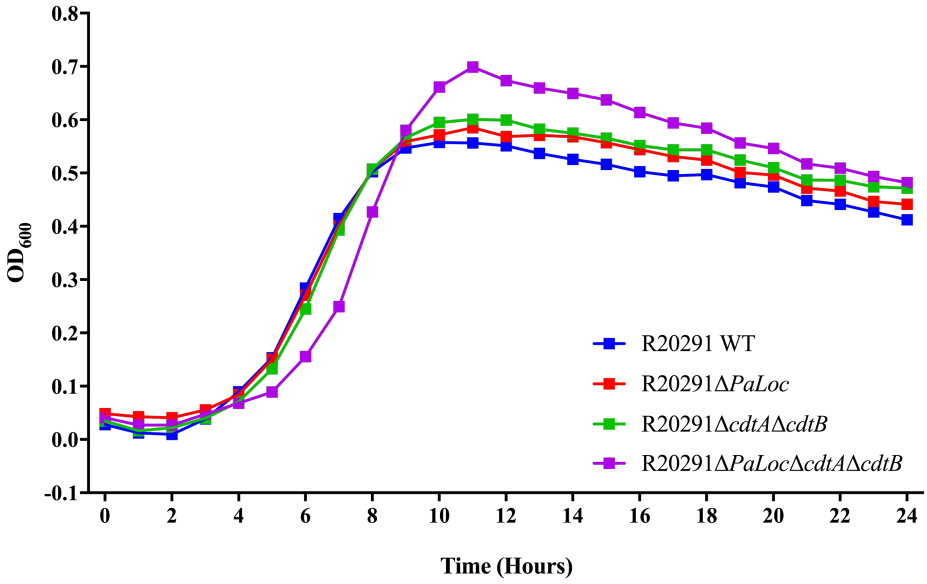


Figure 3.45 Growth Curve to Compare the *Clostridium difficile* Binary Toxin *cdtA* and *cdtB* Mutant Strains

Growth curve to demonstrate the growth for R20291ΔPaLoc, R20291Δ*cdtA*Δ*cdtB* and R20291ΔPaLocΔ*cdtA*Δ*cdtB* when compared to the R20291 WT strain. Average of three biologically independent replicates. The growth (measured by optical density) of R20291ΔPaLoc and R20291Δ*cdtA*Δ*cdtB* were observed to be indistinguishable from the R20291 WT strain. However, the R20291ΔPaLocΔ*cdtA*Δ*cdtB* strain appeared to reach higher exponential growth when compared to R20291 WT. A significant difference was detected between the R20291 WT and R20291ΔPaLocΔ*cdtA*Δ*cdtB* ($p = 0.0015$). A significant difference was also observed between the R20291 WT and R20291Δ*cdtA*Δ*cdtB* ($p = 0.0211$), which was not visibly identified.

Figure 3.46 demonstrates the growth for R20291ΔPaLoc, R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*, R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* when compared to the R20291 WT strain. The phenotypic growth of R20291ΔPaLoc, R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* were observed to be indistinguishable from the R20291 WT strain. However, the R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* strain appeared to reach higher exponential growth when compared to R20291 WT. No significant difference was determined between the R20291 WT and R20291ΔPaLoc. A significant difference was detected between the R20291 WT and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* ($p = 0.0050$). A significant difference was also observed between the R20291 WT and R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* ($p = 0.0167$) and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* ($p = 0.0037$), which was not visibly identified. Furthermore, when the R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* strains were compared, they were not found to be significantly different.

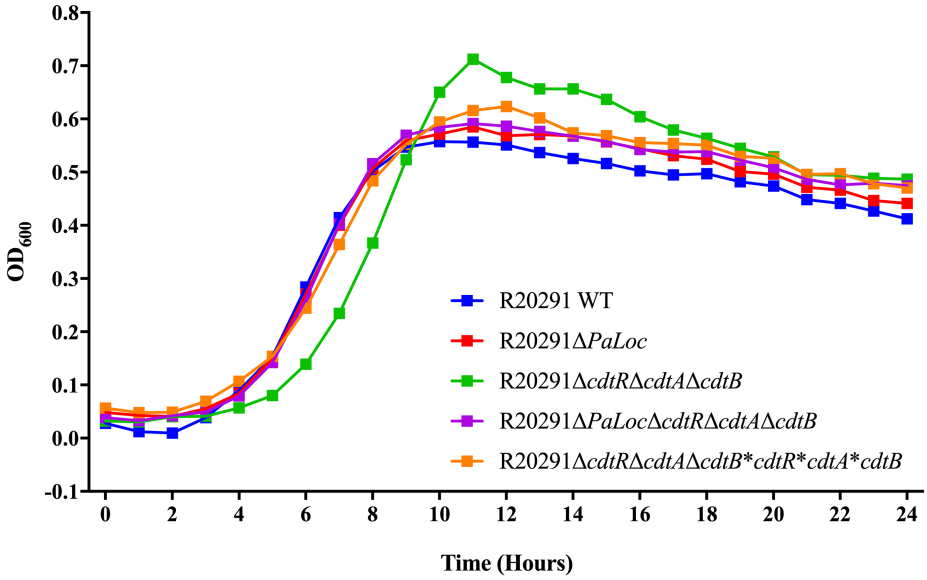


Figure 3.46 Growth Curve to Compare the *Clostridium difficile* Binary Toxin *cdtR*, *cdtA* and *cdtB* Mutant Strains

Growth curve to demonstrate the growth for R20291ΔPaLoc, R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*, R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* when compared to the R20291 WT strain. Average of three biologically independent replicates. The growth (measured by optical density) of R20291ΔPaLoc, R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* were observed to be indistinguishable from the R20291 WT strain. However, the R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* strain appeared to reach higher exponential growth when compared to R20291 WT. A significant difference was detected between the R20291 WT and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* ($p = 0.0050$). A significant difference was also observed between the R20291 WT and R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* ($p = 0.0167$) and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* ($p = 0.0037$), which was not visibly identified.

3.2.7.2. CYTOTOXICITY ASSAY

In vitro cell cytotoxicity assays are usually employed to determine the levels of *C. difficile* toxin A and B, however, in this case they were also used to detect trypsinised CDT binary toxin levels, as described in Chapter 2.9.4.3. Cytotoxicity levels were assessed between the R20291 WT strain and the CDT knock-out mutant strains (R20291Δ*cdtR*, R20291Δ*cdtA*Δ*cdtB* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*), as well as the PaLoc knock-out control mutant strains (R20291ΔPaLoc, R20291ΔPaLocΔ*cdtR*, R20291ΔPaLocΔ*cdtA*Δ*cdtB* and R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB*), and the complemented strains (R20291Δ*cdtR***cdtR* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB*). A

four-fold dilution of each strain 96-hour culture supernatant was applied to a vero cell monolayer and cell cytotoxicity was assessed by defining end-point titre after a 24-hour incubation (Figure 3.47).

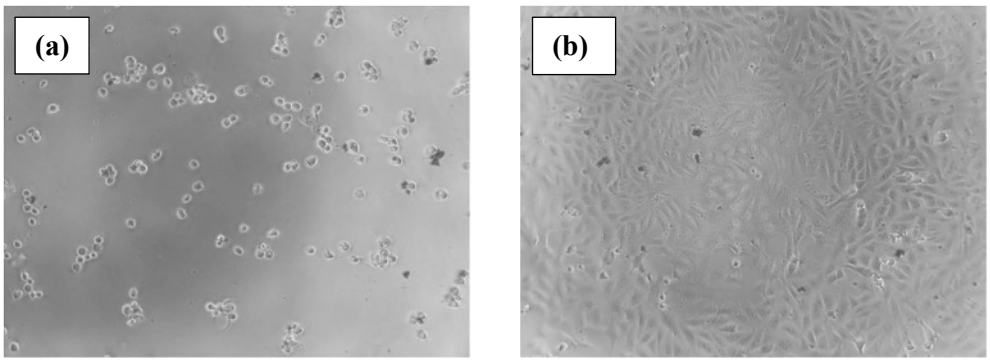


Figure 3.47 Representative Images of the Vero Cell Monolayer Cytotoxicity Assay

Representative images of the Vero cell monolayer treated with **(a)** a $1x10^1$ dilution of the R20291 WT positive control strain supernatant and **(b)** a $1x10^1$ dilution of the trypsinated phosphate buffered saline (PBS) negative control supernatant at 20x magnification.

Figure 3.48 demonstrates the Vero cell cytotoxicity levels for R20291ΔPaLoc, R20291Δ*cdtR*, R20291ΔPaLocΔ*cdtR* and R20291Δ*cdtR***cdtR* when compared to the R20291 WT strain. R20291 WT and R20291Δ*cdtR***cdtR* both demonstrated an end-point (1/toxin titre) of $1x10^6$. In contrast, R20291Δ*cdtR* showed a 3.5-log decrease and R20291ΔPaLoc a 5-log decrease. The R20291ΔPaLocΔ*cdtR* strain was comparable to the trypsinated PBS control, with no cytotoxicity observed. The statistical P value and significance data is detailed in Table 3.1.

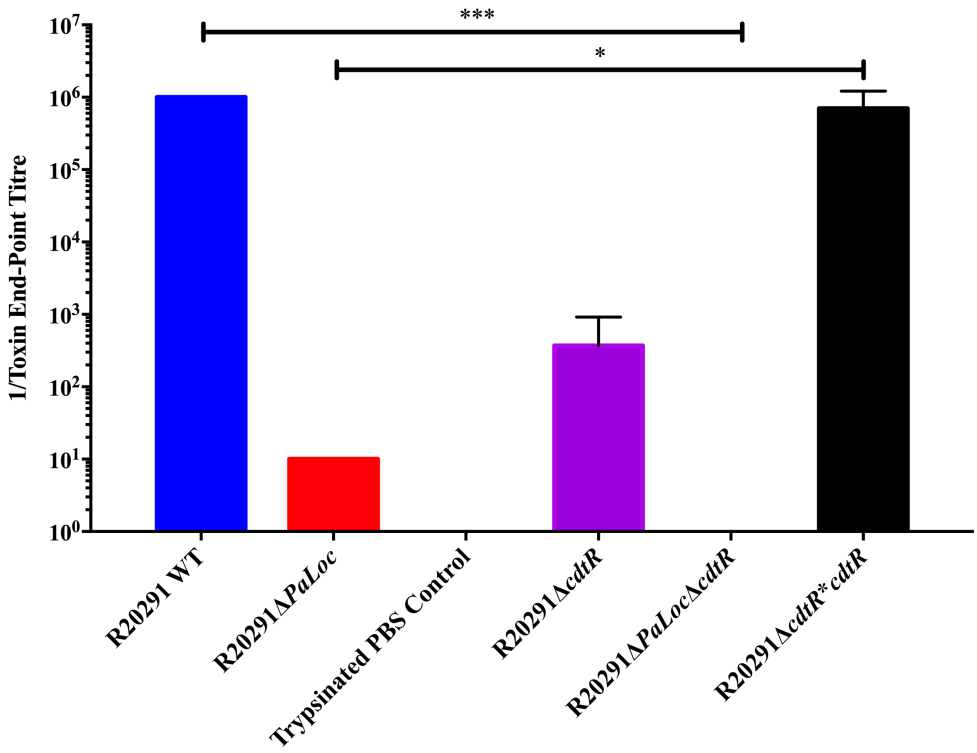


Figure 3.48 Vero Cell Cytotoxicity Assay to Compare the *Clostridium difficile* Binary Toxin *cdtR* Mutants at 96 Hours

Bar chart to demonstrate the Vero cell cytotoxicity levels for R20291ΔPaLoc, R20291Δ*cdtR*, R20291ΔPaLocΔ*cdtR* and R20291Δ*cdtR***cdtR* when compared to the R20291 WT strain. Average of three biologically independent replicates with error bars displaying the mean with standard deviation (SD). R20291 WT and R20291Δ*cdtR***cdtR* both demonstrated an end-point (1/toxin titre) of $1x10^6$. In contrast, R20291Δ*cdtR* showed a 3.5-log decrease and R20291ΔPaLoc a 5-log decrease. The R20291ΔPaLocΔ*cdtR* strain was comparable to the trypsinated PBS control, with no cytotoxicity observed. Key: * $p = \leq 0.05$ and *** $p = \leq 0.001$.

Table 3.1 Statistical Analysis to Compare the *Clostridium difficile* Binary Toxin *cdtR* Mutants in the Vero Cell Cytotoxicity Assay

Strain Comparison	P Value	Significant Difference
R20291 WT vs. R20291ΔPaLoc	p = 0.0010	Yes
R20291 WT vs. vs. Trypsinated PBS control	p = 0.0010	Yes
R20291 WT vs. R20291Δ*cdtR*	p = 0.0010	Yes
R20291 WT vs. R20291ΔPaLocΔ*cdtR*	p = 0.0010	Yes
R20291 WT vs. R20291Δ*cdtR*cdtR*	p = 0.5380	No
R20291ΔPaLoc vs. Trypsinated PBS control	p = >0.9999	No
R20291ΔPaLoc vs. R20291Δ*cdtR*	p = >0.9999	No
R20291ΔPaLoc vs. R20291ΔPaLocΔ*cdtR*	p = >0.9999	No
R20291Δ*cdtR* vs. Trypsinated PBS control	p = >0.9999	No
R20291Δ*cdtR* vs. R20291ΔPaLocΔ*cdtR*	p = >0.9999	No
R20291ΔPaLocΔ*cdtR* vs. Trypsinated PBS control	p = >0.9999	No
R20291Δ*cdtR*cdtR* vs. R20291ΔPaLoc	p = 0.0158	Yes
R20291Δ*cdtR*cdtR* vs. R20291Δ*cdtR*	p = 0.0159	Yes
R20291Δ*cdtR*cdtR* vs. R20291ΔPaLocΔ*cdtR*	p = 0.0158	Yes
R20291Δ*cdtR*cdtR* vs. Trypsinated PBS control	p = 0.0158	Yes

Figure 3.49 demonstrates the Vero cell cytotoxicity levels for R20291ΔPaLoc, R20291Δ*cdtA*Δ*cdtB* and R20291ΔPaLocΔ*cdtA*Δ*cdtB* when compared to the R20291 WT strain. R20291 WT demonstrated an end-point (1/toxin titre) of $1x10^6$. In contrast, R20291Δ*cdtA*Δ*cdtB* showed a 2.5-log decrease and R20291ΔPaLoc a 5-log decrease. The R20291ΔPaLocΔ*cdtA*Δ*cdtB* strain was comparable to the trypsinated PBS control, with no cytotoxicity observed. The statistical P value and significance data is detailed in Table 3.2.

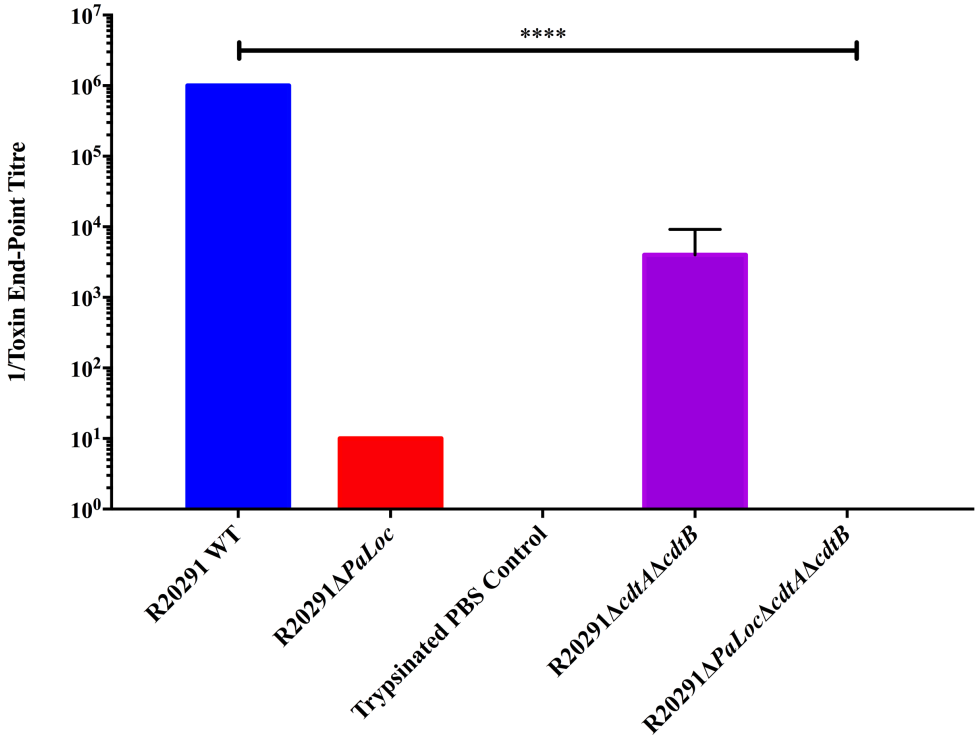


Figure 3.49 Vero Cell Cytotoxicity Assay to Compare the *Clostridium difficile* Binary Toxin *cdtA* and *cdtB* Mutants at 96 Hours

Bar chart to demonstrate the Vero cell cytotoxicity levels for R20291ΔPaLoc, R20291Δ*cdtA*Δ*cdtB* and R20291ΔPaLocΔ*cdtA*Δ*cdtB* when compared to the R20291 WT strain. Average of three biologically independent replicates with error bars displaying the mean with standard deviation (SD). R20291 WT demonstrated an end-point (1/toxin titre) of $1x10^6$. In contrast, R20291Δ*cdtA*Δ*cdtB* showed a 2.5-log decrease and R20291ΔPaLoc a 5-log decrease. The R20291ΔPaLocΔ*cdtA*Δ*cdtB* strain was comparable to the trypsinated PBS control, with no cytotoxicity observed. Key: **** $p = \leq 0.0001$.

Table 3.2 Statistical Analysis to Compare the *Clostridium difficile* Binary Toxin *cdtA* and *cdtB* Mutants in the Vero Cell Cytotoxicity Assay

Strain Comparison	P Value	Significant Difference
R20291 WT vs. R20291ΔPaLoc	p = <0.0001	Yes
R20291 WT vs. vs. Trypsinated PBS control	p = <0.0001	Yes
R20291 WT vs. R20291Δ*cdtA*Δ*cdtB*	p = <0.0001	Yes
R20291 WT vs. R20291ΔPaLocΔ*cdtA*Δ*cdtB*	p = <0.0001	Yes
R20291ΔPaLoc vs. Trypsinated PBS control	p = >0.9999	No
R20291ΔPaLoc vs. R20291Δ*cdtA*Δ*cdtB*	p = 0.2900	No
R20291ΔPaLoc vs. R20291ΔPaLocΔ*cdtA*Δ*cdtB*	p = >0.9999	No
R20291Δ*cdtA*Δ*cdtB* vs. Trypsinated PBS control	p = 0.2880	No
R20291Δ*cdtA*Δ*cdtB* vs. R20291ΔPaLocΔ*cdtA*Δ*cdtB*	p = 0.2882	No
R20291ΔPaLocΔ*cdtA*Δ*cdtB* vs. Trypsinated PBS control	p = >0.9999	No

Figure 3.50 demonstrates the Vero cell cytotoxicity levels for R20291ΔPaLoc, R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*, R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* when compared to the R20291 WT strain. R20291 WT demonstrated an end-point (1/toxin titre) of $1x10^6$ with a similarly comparable end-point for R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* at $1x10^{5.5}$. In contrast, R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* showed a 3.5-log decrease and R20291ΔPaLoc a 5-log decrease. The R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* strain was comparable to the trypsinated PBS control, with no cytotoxicity observed. The statistical P value and significance data is detailed in Table 3.3.

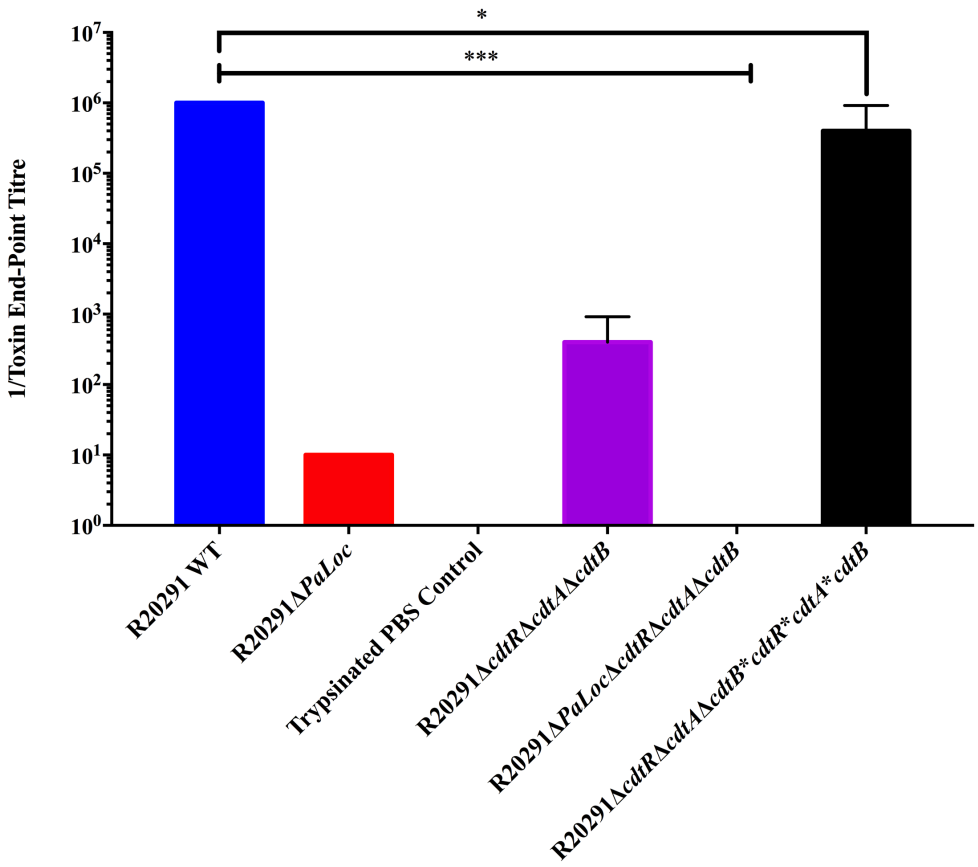


Figure 3.50 Vero Cell Cytotoxicity Assay to Compare the *Clostridium difficile* Binary Toxin *cdtR*, *cdtA* and *cdtB* Mutants at 96 Hours

Bar chart to demonstrate the Vero cell cytotoxicity levels for R20291ΔPaLoc, R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*, R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* when compared to the R20291 WT strain. Average of three biologically independent replicates with error bars displaying the mean with standard deviation (SD). R20291 WT demonstrated an end-point (1/toxin titre) of $1x10^6$ with a similarly comparable end-point for R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* at $1x10^{5.5}$. In contrast, R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* showed a 3.5-log decrease and R20291ΔPaLoc a 5-log decrease. The R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* strain was comparable to the trypsinated PBS control, with no cytotoxicity observed. Key: * $p = \leq 0.05$ and *** $p = \leq 0.001$.

Table 3.3 Statistical Analysis to Compare the *Clostridium difficile* Binary Toxin *cdtR*, *cdtA* and *cdtB* Mutants in the Vero Cell Cytotoxicity Assay

Strain Comparison	P Value	Significant Difference
R20291 WT vs. R20291ΔPaLoc	p = 0.0010	Yes
R20291 WT vs. vs. Trypsinated PBS control	p = 0.0010	Yes
R20291 WT vs. R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*	p = 0.0010	Yes
R20291 WT vs. R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB*	p = 0.0010	Yes
R20291 WT vs. R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*cdtR*cdtA*cdtB*	p = 0.0419	Yes
R20291ΔPaLoc vs. Trypsinated PBS control	p = >0.9999	No
R20291ΔPaLoc vs. R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*	p = >0.9999	No
R20291ΔPaLoc vs. R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB*	p = >0.9999	No
R20291ΔPaLoc vs. R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*cdtR*cdtA*cdtB*	p = 0.2619	No
R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* vs. Trypsinated PBS control	p = >0.9999	No
R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* vs. R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB*	p = >0.9999	No
R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* vs. R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*cdtR*cdtA*cdtB*	p = 0.2628	No
R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* vs. R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*cdtR*cdtA*cdtB*	p = 0.2619	No
R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* vs. Trypsinated PBS control	p = >0.9999	No
R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*cdtR*cdtA*cdtB* vs. Trypsinated PBS control	p = 0.2619	No

3.2.7.3. TAQMAN PROTEIN ASSAY

The TaqMan Protein Assay is a simple, rapid and effective method of quantifying proteins in cell and tissue lysate samples. When compared to other more traditional protein analysis methods, such as a Western blot, it requires 10 to 500-fold less sample input. A representative input sample range for mammalian cells is 5 to 500 cells, and 1 to 100ng of total protein for tissue lysate samples. This assay is based on the technology utilised by the proximity ligation assay (PLA) (Fredriksson *et al.*, 2002; Gullberg *et al.*, 2003), and involves quantitative (q) PCR-based detection of the DNA ligation bridge coupled to the antibody assay probe, which in turn is bound to the protein epitope in the sample lysate (Figure 3.51).

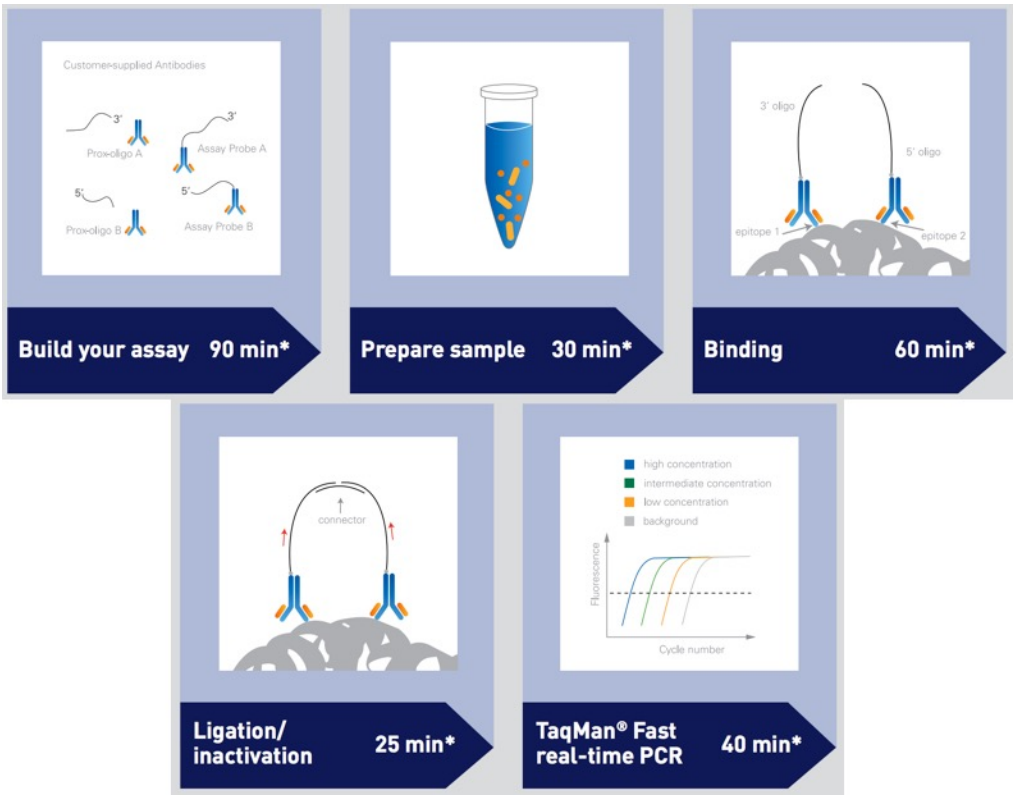


Figure 3.51 TaqMan Protein Assay Workflow (Applied Biosystems by Life Technologies, 2011b)

The TaqMan Protein Assay workflow consists of five steps: (1) The Assay Probes are created using a biotinylated antibody and streptavidin-linked oligonucleotides. (2) The cell samples are lysed and used directly in the assay without further protein purification. (3) The previously prepared sample lysates are combined with the two Assay Probes. The antibody binds to the protein epitopes bringing the probe oligonucleotides into proximity. (4) The probe oligonucleotides then bind to a connector oligonucleotide and are ligated by DNA ligase. Subsequent treatment by protease inactivates the ligase. (5) The oligonucleotide ligation product is amplified and detected by quantitative (q) PCR.

The TaqMan Protein Assay employs a user-supplied biotinylated antibody, which is initially used to prepare two Assay Probes with either a 5' or 3' streptavidin-linked Prox-Oligo, as described in Chapter 2.10.1.1. (Figure 3.52).

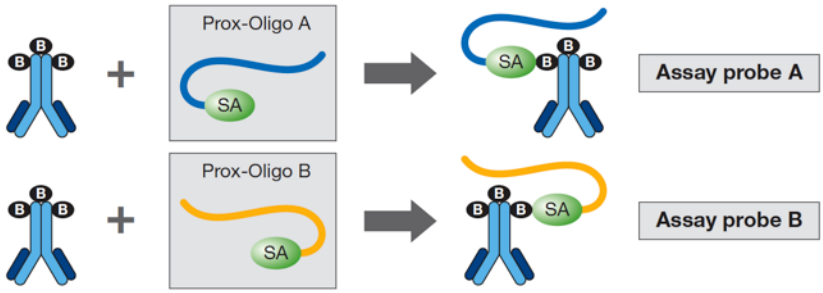


Figure 3.52 TaqMan Protein Assay Probe Development (Applied Biosystems by Life Technologies, 2011a)

A user-supplied biotinylated antibody is used to prepare two TaqMan Protein Assay Probes with either a 5' or 3' streptavidin-linked Prox-Oligo.

A Forced Proximity Probe (FPP) Test was performed to assess whether the biotinylated antibody can bind to the Prox-Oligos and is suitable for making proximity probes. The real-time qPCR data was analysed and crossing-point (C_P) or threshold cycle (C_T) values were obtained. This value is defined as the cycle at which the fluorescence reaches a defined threshold (Rodríguez-Lázaro and Hernández, 2013). A C_P/C_T value of ≥8.5 is a pass and demonstrates that the biotinylated antibody is suitable for use in the TaqMan Protein Assay. All four FPP sample replicates produced a C_P/C_T value of ≥8.5 (FPP 1: 12.74; FPP 2: 12.57; FPP 3: 12.84; and, FPP 4: 11.76) and are illustrated in Figure 3.53.

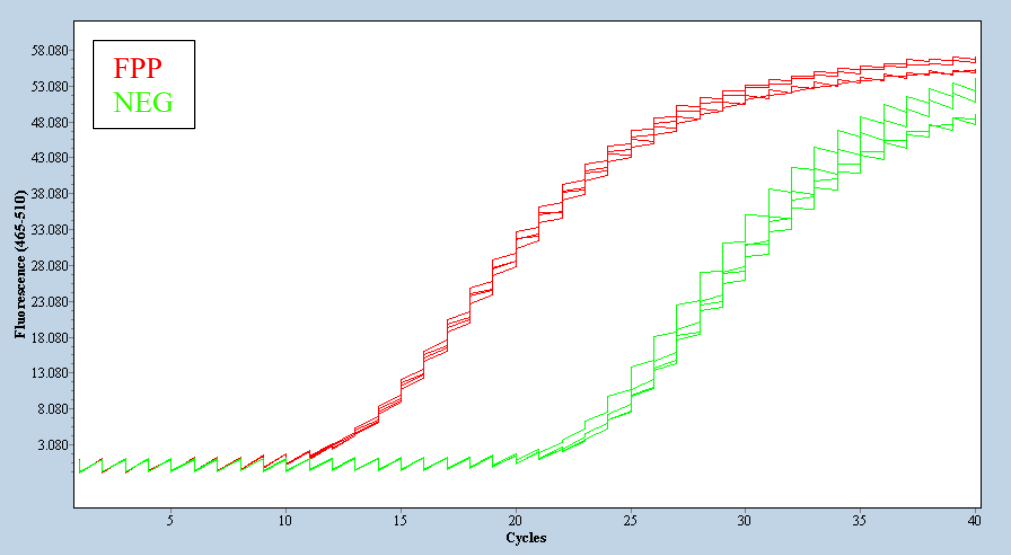


Figure 3.53 TaqMan Protein Assay Forced Proximity Probe Test

Graph to demonstrate the Forced Proximity Probe (FPP) Test, which was performed to assess whether the biotinylated antibody can bind to the Prox-Oligos and is suitable for making proximity probes. A quantitative (q) PCR C_P/C_T value of ≥8.5 is a pass and demonstrates that the biotinylated antibody is suitable for use in the TaqMan Protein Assay. All four FPP sample replicates produced a C_P/C_T value of ≥8.5 (FPP 1: 12.74; FPP 2: 12.57; FPP 3: 12.84; and, FPP 4: 11.76).

After the Assay Probes were successfully created, experiment optimisation was attempted using the complete binary toxin positive *C. difficile* R20291 WT strain as a positive control. Firstly, the strain was grown in TY and BHIS broth, with samples removed at 0, 24, 48, 72 and 96 hours, and processed as described in Chapter 2.10.1. Figure 3.54 illustrates the qPCR amplification curves of both growth media at the various time-points. All two-fold dilutions ($1x2^1$ to $1x2^{11}$ and the no protein control [NPC]) were analysed, however, the figure depicts just the $1x2^1$ dilution and the NPC due to a significant amplification drop-off for most samples after the initial dilution. In general, the TaqMan Protein Assay is deemed successful if the C_P/C_T value is ≥3 and produces a typical amplification curve. In this assay, the amplification profile appeared after approximately 19 cycles and C_P/C_T values were only obtained for the TY 24-hour time point $1x2^1$ and $1x2^5$ dilutions; 21.17 and 21.61, respectively.

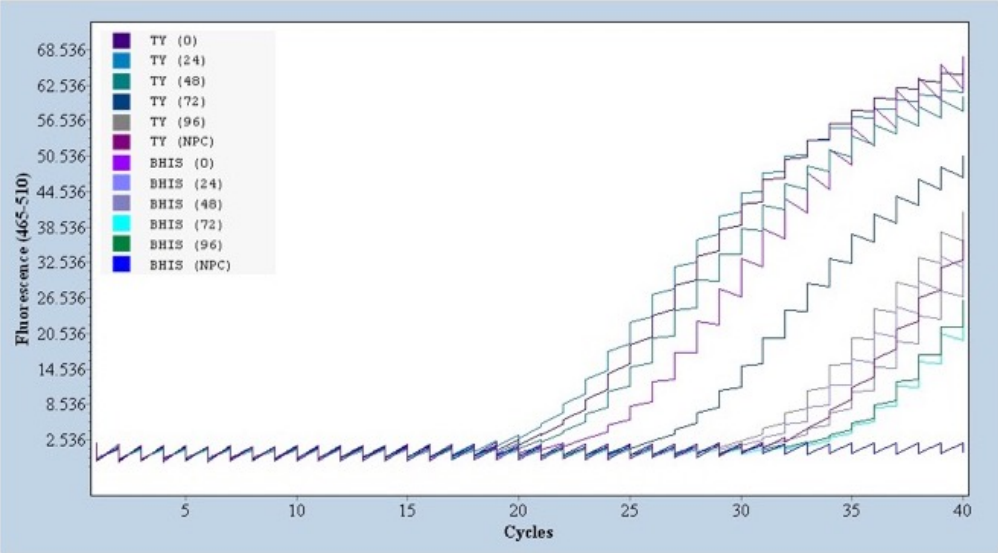


Figure 3.54 TaqMan Protein Assay Growth Media Optimisation

The graph illustrates the qPCR amplification curves of both TY and BHIS growth mediums at the 0, 24, 48, 72 and 96-hour time points. All two-fold dilutions ($1x2^{1}$ to $1x2^{11}$ and the no protein control [NPC]) were analysed, however, the figure depicts just the $1x2^{1}$ dilution and the NPC. The amplification profile appeared after approximately 19 cycles and C_P/C_T values were only obtained for the TY 24-hour time point $1x2^{1}$ and $1x2^{5}$ dilutions; 21.17 and 21.61, respectively.

Due to the growth media optimisation assay results, all subsequent strains were grown in TY medium. The next assay was completed to determine if earlier time-points (4, 8, 12, as well as 24 hours) produced an improved qPCR amplification curve. The *C. difficile* 630 WT strain, containing the partial ghost CDT gene locus, and the binary toxin negative CD37 strain were also investigated. Figure 3.55 illustrates the qPCR amplification curves of three control strains at the various time-points. All two-fold dilutions ($1x2^{1}$ to $1x2^{8}$) were analysed, however, the figure depicts just the $1x2^{1}$ dilution to avoid overcrowding of the results. The amplification profile appeared after approximately 24 cycles and C_P/C_T values were obtained for the R20291 8-hour time point and the R20291 12-hour time point (Table 3.4). Unfortunately, C_P/C_T values were also observed for the 630 WT intermediate strain at 4, 8, 12 and 24 hours, as well as the CD37 negative strain at 8 and 24 hours (Table 3.4).

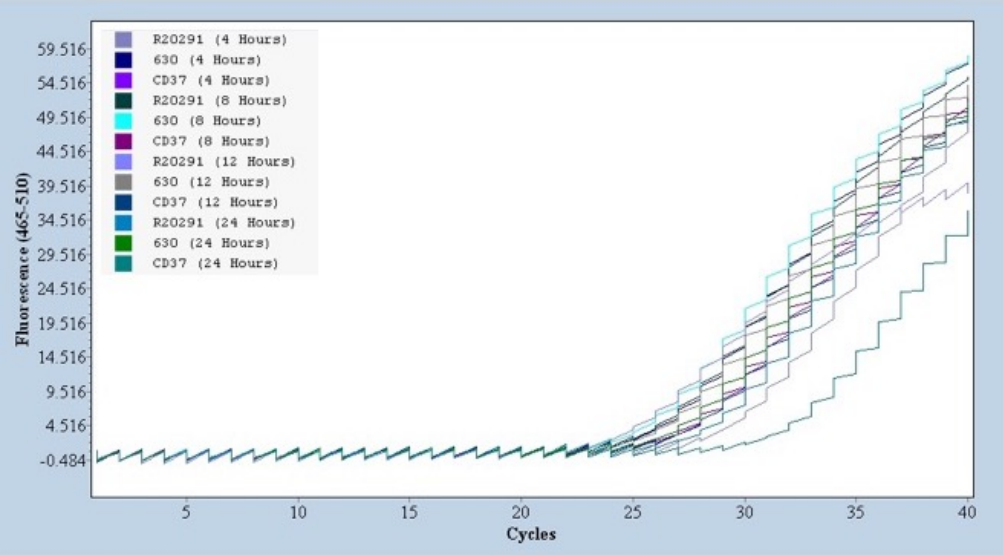


Figure 3.55 TaqMan Protein Assay Positive and Negative Control Optimisation

The graph illustrates the qPCR amplification curves of R20291 WT, 630 WT and CD37 control strains at 4, 8, 12 and 24-hour time points. All two-fold dilutions ($1x2^1$ to $1x2^8$) were analysed, however, the figure depicts just the $1x2^1$ dilution. The amplification profile appeared after approximately 24 cycles and C_P/C_T values were obtained for the R20291 8-hour time point: $1x2^2$ dilution C_P/C_T 31.87, $1x2^3$ dilution C_P/C_T 30.18, $1x2^4$ dilution C_P/C_T 31.80, $1x2^5$ dilution C_P/C_T 30.91, $1x2^6$ dilution C_P/C_T 29.72, and $1x2^8$ dilution C_P/C_T 30.23; and, the R20291 12-hour time point: $1x2^4$ dilution C_P/C_T 30.73 and $1x2^6$ dilution C_P/C_T 31.15. Unfortunately, C_P/C_T values were also observed for the 630 WT intermediate strain and CD37 negative strain. The 630 C_P/C_T values were as follows; 630 4-hour time point: $1x2^7$ dilution C_P/C_T 30.59; 630 8-hour time point: $1x2^2$ dilution C_P/C_T 31.07, $1x2^4$ dilution C_P/C_T 30.44 and $1x2^5$ dilution C_P/C_T 31.81; 630 12-hour time point: $1x2^5$ dilution C_P/C_T 33.25 and $1x2^6$ dilution C_P/C_T 31.95; and, 630 24-hour time point: $1x2^4$ dilution C_P/C_T 32.01 and $1x2^6$ dilution C_P/C_T 31.59. The CD37 C_P/C_T values were as follows; CD37 8-hour time point: $1x2^4$ dilution C_P/C_T 30.96; and, CD37 24-hour time point: $1x2^4$ dilution C_P/C_T 31.68.

Table 3.4 TaqMan Protein Assay C_P/C_T Values of the *Clostridium difficile* R20291, 630 and CD37 Strains Grown in TY Medium

Strains and Time Points	C_P/C_T Values (Two-Fold Dilutions $1x2^{-1}$ to $1x2^{-8}$)							
	$1x2^1$	$1x2^2$	$1x2^3$	$1x2^4$	$1x2^5$	$1x2^6$	$1x2^7$	$1x2^8$
R20291 8-hour		31.87	30.18	31.80	30.91	29.72		30.23
R20291 12-hour				30.73		31.15		
630 4-hour							30.59	
630 8-hour		31.07		30.44	31.81			
630 12-hour					33.25	31.95		
630 24-hour				32.01		31.59		
CD37 8-hour				30.96				
CD37 24-hour				31.68				

Although the previous assay indicated positive results at the 8 and 12-hour time points for the CDT positive R20291 WT control strain, two C_P/C_T values were also observed for the binary toxin negative CD37 control strain. To further optimise the assay, different sample lysate preparations were trialled. Previously, each sample had been frozen as pure culture at -20°C before the cells were lysed at a later point. In the next two assays, the 24-hour overnight starter culture as well as samples taken at 8, 12 and 24 hours were centrifuged, and the cell pellet frozen at -20°C until required. A sample taken at 96 hours was also centrifuged and the supernatant was filter-sterilised and frozen at -80°C. Additionally, the 24-hour overnight starter culture sample and samples taken at 8, 12 and 24 hours were lysed using lysozyme and the GenElute Bacterial Genomic DNA kit (Sigma), instead of using the TaqMan Protein Assay cell lysate reagents.

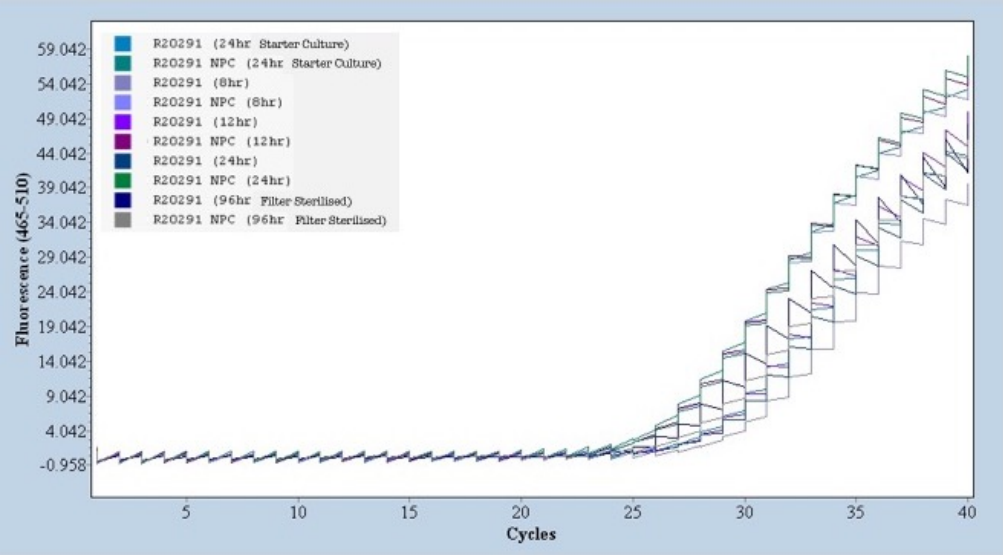


Figure 3.56 TaqMan Protein Assay Sample Lysate Preparation Optimisation (Centrifugation and Filter Sterilisation)

The graph illustrates the qPCR amplification curves of the centrifugation samples for the 24-hour overnight starter culture and the samples at 8, 12 and 24-hour time points, as well as the 96-hour time point filter-sterilised sample. All two-fold dilutions ($1x2^1$ to $1x2^{11}$ and the no protein control [NPC]) were analysed, however, the figure depicts just the $1x2^1$ dilution and the NPC. The amplification profile appeared after approximately 24 cycles and no positive C_P/C_T values were obtained for any samples.

Figure 3.56 illustrates the qPCR amplification curves of the centrifugation and filter-sterilised samples at the various time-points. All two-fold dilutions ($1x2^1$ to $1x2^{11}$ and the no protein control [NPC]) were analysed, however, the figure depicts just the $1x2^1$ dilution and the NPC due to a significant amplification drop-off for most samples after the initial dilution. The amplification profile appeared after approximately 24 cycles and no positive C_P/C_T values were obtained for any samples.

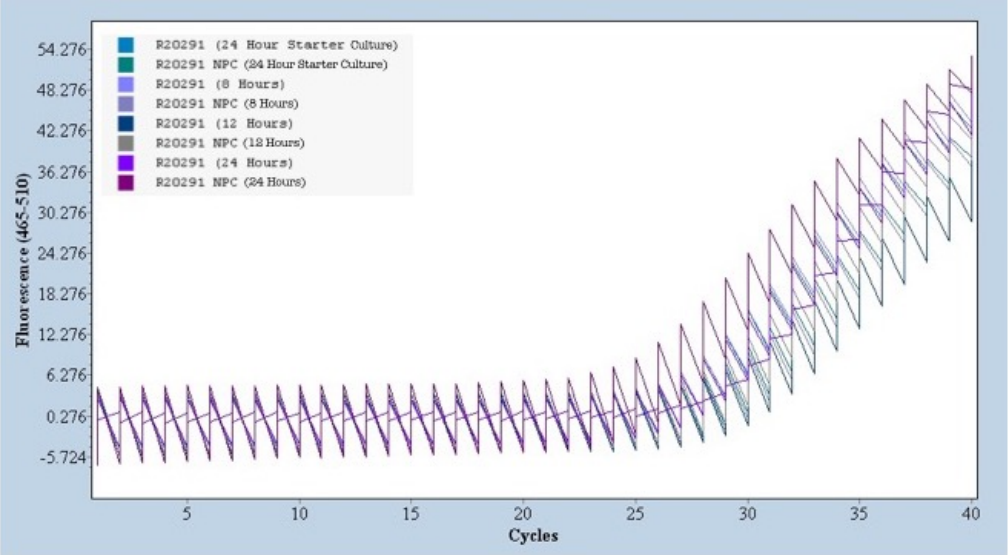


Figure 3.57 TaqMan Protein Assay Sample Lysate Preparation Optimisation (Lysozyme)

The graph illustrates the qPCR amplification curves of the lysozyme samples for the 24-hour overnight starter culture and the samples at 8, 12 and 24-hour time points. All two-fold dilutions ($1x2^{1}$ to $1x2^{11}$ and the no protein control [NPC]) were analysed, however, the figure depicts just the $1x2^{1}$ dilution and the NPC. The amplification profile appeared after approximately 24 cycles but is difficult to distinguish due to the high level of background florescence. The C_P/C_T values were obtained after dilution $1x2^{3}$ in which the background florescence had decreased. The C_P/C_T values were as follows R20291 24-hour overnight starter culture: $1x2^{3}$ dilution C_P/C_T 27.92, $1x2^{10}$ dilution C_P/C_T 31.66 and, $1x2^{11}$ dilution C_P/C_T 32.21; R20291 8-hour time point: $1x2^{5}$ dilution C_P/C_T 29.45; and, the R20291 12-hour time point: $1x2^{4}$ dilution C_P/C_T 30.86 and $1x2^{5}$ dilution C_P/C_T 29.70.

Figure 3.57 illustrates the qPCR amplification curves of the lysozyme samples at the various time-points. All two-fold dilutions ($1x2^{1}$ to $1x2^{11}$ and the no protein control [NPC]) were analysed, however, the figure depicts just the $1x2^{1}$ dilution and the NPC to avoid overcrowding of the results. The amplification profile appeared after approximately 24 cycles but is difficult to distinguish due to the high level of background florescence. The C_P/C_T values were obtained after dilution $1x2^{3}$ in which the background florescence had decreased. The C_P/C_T values were as follows R20291 24-hour overnight starter culture: $1x2^{3}$ dilution C_P/C_T 27.92, $1x2^{10}$ dilution C_P/C_T 31.66 and, $1x2^{11}$ dilution C_P/C_T 32.21; R20291 8-hour time point: $1x2^{5}$ dilution C_P/C_T 29.45; and, the R20291 12-hour time point: $1x2^{4}$ dilution C_P/C_T 30.86 and $1x2^{5}$ dilution C_P/C_T 29.70.

Unfortunately, the TaqMan Protein Assay was not further optimised or tested with the CDT allelic exchange knock-out strains, PaLoc allelic exchange knock-out strains and the CDT complemented strains due to time constraints.

3.2.7.4. BIOFILM ASSAY

Biofilm formation was assessed between the R20291 WT strain and the CDT knock-out mutant strains (R20291Δ*cdtR*, R20291Δ*cdtA*Δ*cdtB* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*), as well as the PaLoc knock-out control mutant strains (R20291ΔPaLoc, R20291ΔPaLocΔ*cdtR*, R20291ΔPaLocΔ*cdtA*Δ*cdtB* and R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB*), and the complemented strains (R20291Δ*cdtR***cdtR* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB*). All the strains were grown in BHIS broth supplemented with 0.1M glucose and the crystal violet-stained biofilms were measured after 24-hour and 96-hour periods, as described in Chapter 2.9.5.

Figure 3.58 demonstrates the biofilm formation for R20291ΔPaLoc, R20291Δ*cdtR*, R20291ΔPaLocΔ*cdtR* and R20291Δ*cdtR***cdtR* when compared to the R20291 WT strain. At the 24-hour time point, the R20291ΔPaLoc strain biofilm had the highest absorbance reading of 0.15nm, however, large error bars were observed for all samples. R20291 WT, R20291ΔPaLoc, R20291Δ*cdtR*, R20291ΔPaLocΔ*cdtR* and R20291Δ*cdtR***cdtR* absorbance readings were all comparable at the 96-hour time point. A noticeable decrease in absorbance was also demonstrated with readings taken at 24 hours, in contrast to 96 hours. No significant difference was determined for all strains at either the 24 or 96-hour time points. The statistical P value and significance data is detailed in Table 3.5.

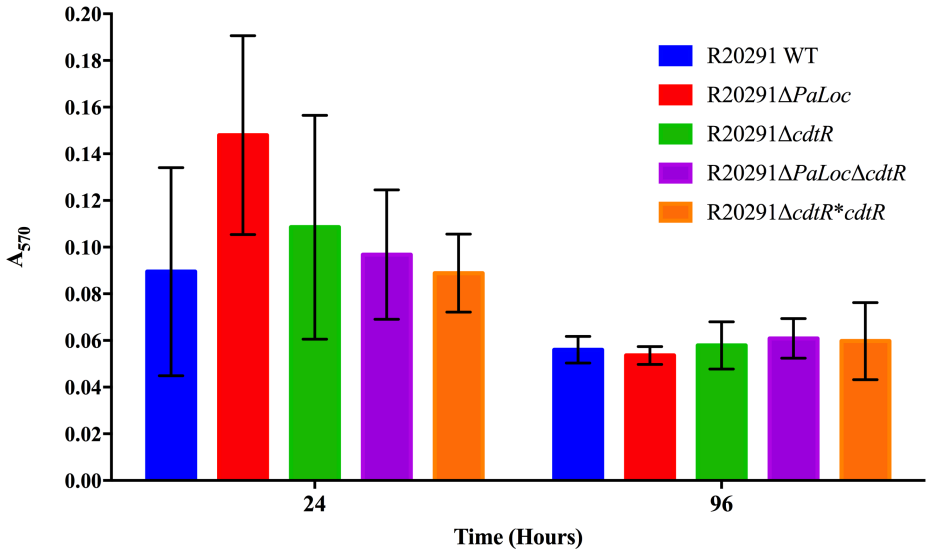


Figure 3.58 Biofilm Assay to Compare the *Clostridium difficile* Binary Toxin *cdtR* Mutants at 24 and 96 Hours

Bar chart to demonstrate the biofilm formation for R20291ΔPaLoc, R20291Δ*cdtR*, R20291ΔPaLocΔ*cdtR* and R20291Δ*cdtR***cdtR* when compared to the R20291 WT strain. Average of three biologically independent replicates with error bars displaying the mean with standard deviation (SD). At the 24-hour time point, the R20291ΔPaLoc strain biofilm had the highest absorbance reading of 0.15nm, however, large error bars were observed for all samples. R20291 WT, R20291ΔPaLoc, R20291Δ*cdtR*, R20291ΔPaLocΔ*cdtR* and R20291Δ*cdtR***cdtR* absorbance readings were all comparable at the 96-hour time point. A noticeable decrease in absorbance was also demonstrated with readings taken at 24 hours, in contrast to 96 hours.

Table 3.5 Statistical Analysis to Compare the *Clostridium difficile* Binary Toxin *cdtR* Mutants in the Biofilm Assay

Strain Comparison	P Value	Significant Difference
24-Hour Measurement		
R20291 WT vs. R20291ΔPaLoc	p = 0.1807	No
R20291 WT vs. R20291Δ*cdtR*	p = 0.9178	No
R20291 WT vs. R20291ΔPaLocΔ*cdtR*	p = 0.9974	No
R20291 WT vs. R20291Δ*cdtR*cdtR*	p = >0.9999	No
R20291ΔPaLoc vs. R20291Δ*cdtR*	p = 0.4878	No
R20291ΔPaLoc vs. R20291ΔPaLocΔ*cdtR*	p = 0.2714	No
R20291ΔPaLoc vs. R20291Δ*cdtR*cdtR*	p = 0.1741	No
R20291Δ*cdtR* vs. R20291ΔPaLocΔ*cdtR*	p = 0.9846	No
R20291Δ*cdtR* vs. R20291Δ*cdtR*cdtR*	p = 0.9085	No
R20291Δ*cdtR*cdtR* vs. R20291ΔPaLocΔ*cdtR*	p = 0.9964	No
96-Hour Measurement		
R20291 WT vs. R20291ΔPaLoc	p = >0.9999	No
R20291 WT vs. R20291Δ*cdtR*	p = >0.9999	No
R20291 WT vs. R20291ΔPaLocΔ*cdtR*	p = 0.9995	No
R20291 WT vs. R20291Δ*cdtR*cdtR*	p = 0.9998	No
R20291ΔPaLoc vs. R20291Δ*cdtR*	p = 0.9996	No
R20291ΔPaLoc vs. R20291ΔPaLocΔ*cdtR*	p = 0.9973	No
R20291ΔPaLoc vs. R20291Δ*cdtR*cdtR*	p = 0.9986	No
R20291Δ*cdtR* vs. R20291ΔPaLocΔ*cdtR*	p = >0.9999	No
R20291Δ*cdtR* vs. R20291Δ*cdtR*cdtR*	p = >0.9999	No
R20291Δ*cdtR*cdtR* vs. R20291ΔPaLocΔ*cdtR*	p = >0.9999	No

Figure 3.59 demonstrates the biofilm formation for R20291ΔPaLoc, R20291Δ*cdtA*Δ*cdtB* and R20291ΔPaLocΔ*cdtA*Δ*cdtB* when compared to the R20291 WT strain. At the 24-hour time point, both the R20291ΔPaLoc and R20291Δ*cdtA*Δ*cdtB* biofilms had the highest absorbance reading of approximately 0.15nm. The R20291ΔPaLocΔ*cdtA*Δ*cdtB* absorbance reading at the 96-hour time point was higher in comparison to the R20291 WT, R20291ΔPaLoc and R20291Δ*cdtA*Δ*cdtB* strains. However, large error bars were observed for all samples at both time points. Furthermore, a noticeable decrease in absorbance was also demonstrated for R20291 WT, R20291ΔPaLoc and R20291Δ*cdtA*Δ*cdtB* strains, when the 24-hour time point readings were compared to the readings taken at 96 hours. A significant difference was detected at the 24-hour time point between R20291Δ*cdtA*Δ*cdtB* and R20291ΔPaLocΔ*cdtA*Δ*cdtB* (p = 0.0332). No significant difference was observed for the remaining strains at either the 24 or 96-hour time points. The statistical P value and significance data is detailed in Table 3.6.

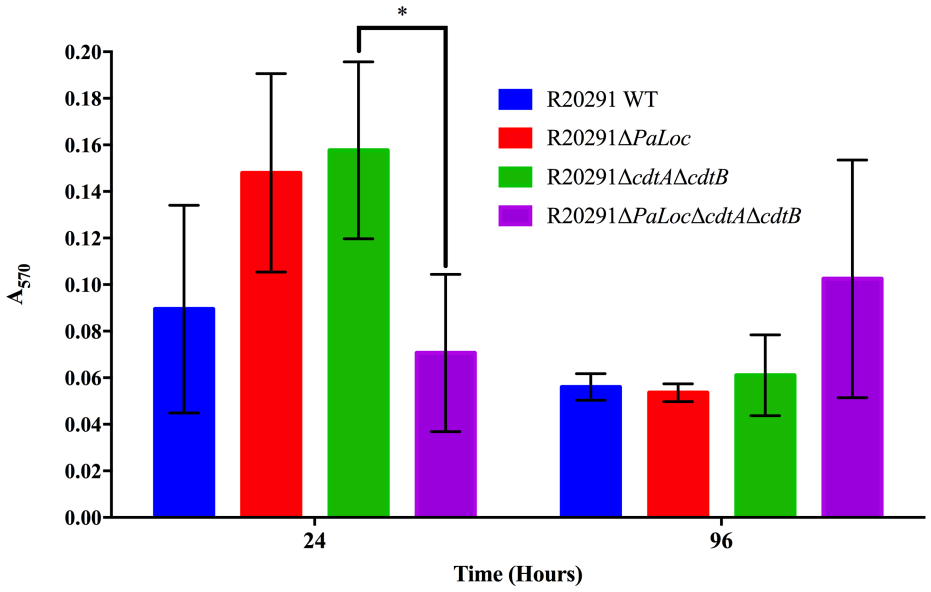


Figure 3.59 Biofilm Assay to Compare the *Clostridium difficile* Binary Toxin *cdtA* and *cdtB* Mutants at 24 and 96 Hours

Bar chart to demonstrate the biofilm formation for R20291ΔPaLoc, R20291Δ*cdtA*Δ*cdtB* and R20291ΔPaLocΔ*cdtA*Δ*cdtB* when compared to the R20291 WT strain. Average of three biologically independent replicates with error bars displaying the mean with standard deviation (SD). At the 24-hour time point, both the R20291ΔPaLoc and R20291Δ*cdtA*Δ*cdtB* biofilms had the highest absorbance reading of approximately 0.15nm. The R20291ΔPaLocΔ*cdtA*Δ*cdtB* absorbance reading at the 96-hour time point was higher in comparison to the R20291 WT, R20291ΔPaLoc and R20291Δ*cdtA*Δ*cdtB* strains. However, large error bars were observed for all samples at both time points. Furthermore, a noticeable decrease in absorbance was also demonstrated for R20291 WT, R20291ΔPaLoc and R20291Δ*cdtA*Δ*cdtB* strains, when the 24-hour time point readings were compared to the readings taken at 96 hours. A significant difference was detected at the 24-hour time point between R20291Δ*cdtA*Δ*cdtB* and R20291ΔPaLocΔ*cdtA*Δ*cdtB* ($p = 0.0332$). Key: * $p = \leq 0.05$.

Table 3.6 Statistical Analysis to Compare the *Clostridium difficile* Binary Toxin *cdtA* and *cdtB* Mutants in the Biofilm Assay

Strain Comparison	P Value	Significant Difference
24-Hour Measurement		
R20291 WT vs. R20291ΔPaLoc	p = 0.1455	No
R20291 WT vs. R20291Δ*cdtA*Δ*cdtB*	p = 0.0866	No
R20291 WT vs. R20291ΔPaLocΔ*cdtA*Δ*cdtB*	p = 0.8393	No
R20291ΔPaLoc vs. R20291Δ*cdtA*Δ*cdtB*	p = 0.9710	No
R20291ΔPaLoc vs. R20291ΔPaLocΔ*cdtA*Δ*cdtB*	p = 0.0542	No
R20291Δ*cdtA*Δ*cdtB* vs. R20291ΔPaLocΔ*cdtA*Δ*cdtB*	p = 0.0332	Yes
96-Hour Measurement		
R20291 WT vs. R20291ΔPaLoc	p = 0.9994	No
R20291 WT vs. R20291Δ*cdtA*Δ*cdtB*	p = 0.9958	No
R20291 WT vs. R20291ΔPaLocΔ*cdtA*Δ*cdtB*	p = 0.2722	No
R20291ΔPaLoc vs. R20291Δ*cdtA*Δ*cdtB*	p = 0.9860	No
R20291ΔPaLoc vs. R20291ΔPaLocΔ*cdtA*Δ*cdtB*	p = 0.2385	No
R20291Δ*cdtA*Δ*cdtB* vs. R20291ΔPaLocΔ*cdtA*Δ*cdtB*	p = 0.3502	No

Figure 3.60 demonstrates the biofilm formation for R20291ΔPaLoc, R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*, R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* when compared to the R20291 WT strain. At the 24-hour time point, both the R20291ΔPaLoc and R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* biofilms had the highest absorbance reading of approximately 0.15nm. The R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* absorbance reading at the 96-hour time point was higher in comparison to the R20291 WT, R20291ΔPaLoc and R20291Δ*cdtA*Δ*cdtB* strains at the identical time point. However, large error bars were observed for all samples at both time points. Furthermore, a noticeable decrease in absorbance was also demonstrated for the R20291 WT and R20291ΔPaLoc readings taken at 24 hours compared to 96 hours. A significant difference was detected at the 24-hour time point between R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* ($p = 0.0427$). No significant difference was observed for the remaining strains at either the 24 or 96-hour time points. The statistical P value and significance data is detailed in Table 3.7.

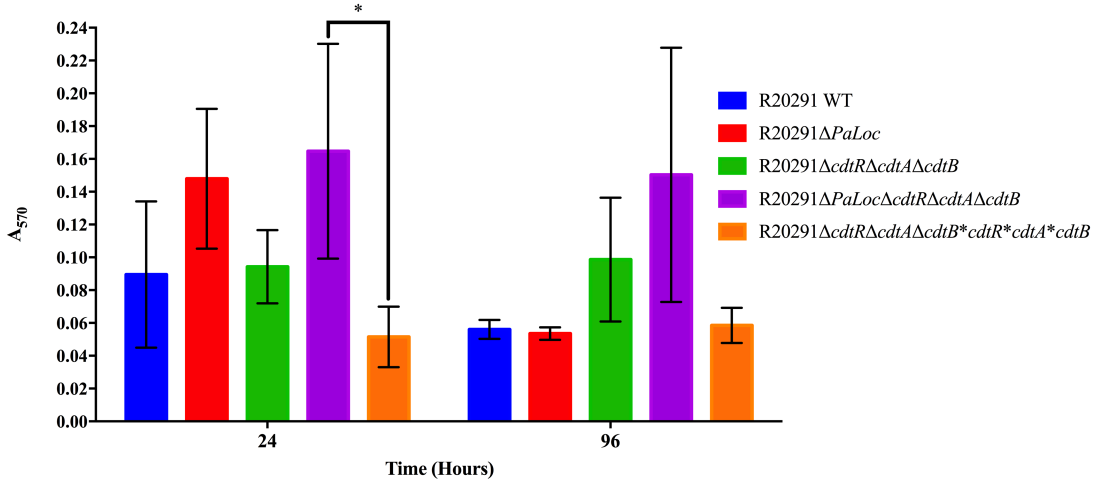


Figure 3.60 Biofilm Assay to Compare the *Clostridium difficile* Binary Toxin *cdtR*, *cdtA* and *cdtB* Mutants at 24 and 96 Hours

Bar chart to demonstrate the biofilm formation for R20291ΔPaLoc, R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*, R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* when compared to the R20291 WT strain. Average of three biologically independent replicates with error bars displaying the mean with standard deviation (SD). At the 24-hour time point, both the R20291ΔPaLoc and R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* biofilms had the highest absorbance reading of approximately 0.15nm. The R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* absorbance reading at the 96-hour time point was higher in comparison to the R20291 WT, R20291ΔPaLoc and R20291Δ*cdtA*Δ*cdtB* strains at the identical time point. However, large error bars were observed for all samples at both time points. Furthermore, a noticeable decrease in absorbance was also demonstrated for the R20291 WT and R20291ΔPaLoc readings taken at 24 hours compared to 96 hours. A significant difference was detected at the 24-hour time point between R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* ($p = 0.0427$). Key: * $p = \leq 0.05$.

Table 3.7 Statistical Analysis to Compare the *Clostridium difficile* Binary Toxin *cdtR*, *cdtA* and *cdtB* Mutants in the Biofilm Assay

Strain Comparison	P Value	Significant Difference
24-Hour Measurement		
R20291 WT vs. R20291ΔPaLoc	p = 0.4146	No
R20291 WT vs. R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*	p = 0.9999	No
R20291 WT vs. R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB*	p = 0.2166	No
R20291 WT vs. R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*cdtR***cdtA***cdtB***	p = 0.7525	No
R20291ΔPaLoc vs. R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*	p = 0.4877	No
R20291ΔPaLoc vs. R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB*	p = 0.9815	No
R20291ΔPaLoc vs. R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*cdtR***cdtA***cdtB***	p = 0.0881	No
R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* vs. R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB*	p = 0.2625	No
R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* vs. R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*cdtR***cdtA***cdtB***	p = 0.6723	No
R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* vs. R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*cdtR***cdtA***cdtB***	p = 0.0427	Yes
96-Hour Measurement		
R20291 WT vs. R20291ΔPaLoc	p = >0.9999	No
R20291 WT vs. R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*	p = 0.6747	No
R20291 WT vs. R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB*	p = 0.0966	No
R20291 WT vs. R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*cdtR***cdtA***cdtB***	p = >0.9999	No
R20291ΔPaLoc vs. R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*	p = 0.6303	No
R20291ΔPaLoc vs. R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB*	p = 0.0865	No
R20291ΔPaLoc vs. R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*cdtR***cdtA***cdtB***	p = 0.9998	No
R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* vs. R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB*	p = 0.5206	No
R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* vs. R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*cdtR***cdtA***cdtB***	p = 0.7173	No
R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* vs. R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*cdtR***cdtA***cdtB***	p = 0.1076	No

3.2.8. CDT HORIZONTAL GENE TRANSFER

The ability of the *C. difficile* binary toxin gene locus to horizontally transfer to a non-toxigenic CDT *C. difficile* strain was investigated. The *C. difficile* strain CD37 and R20291::CT*cdtB* ClosTron (which contains *ermB* inserted within *cdtB*) were initially assessed for their antibiotic sensitivity against tetracycline, erythromycin, lincomycin and rifaximin Table 3.8. It was determined from this antibiotic plate assay that erythromycin and rifaximin would be used for

the subsequent horizontal gene transfer assay. This enabled selection of potential CD37 colonies carrying the genetically marked CDT gene locus.

Table 3.8 CDT Horizontal Gene Transfer Strain Antibiotic Sensitivity

Antibiotic	CD37	R20291::CT*cdtB*
Lincomycin	✓ (Limited - Intermediate)	✓
Rifaximin	✓	×
Tetracycline	×	×
Erythromycin	×	✓

Key: × No Growth (Sensitive), ✓ Growth (Resistant)

Firstly, the transfer of DNA via the filter mating method was attempted, as previously described (Brouwer *et al.*, 2013). However, this procedure did not yield any transconjugant colonies and so the prevailing conjugation method (Heap *et al.*, 2009) with the more recent additional alterations (Kirk and Fagan, 2016) was used in its place. Using this technique, described in Chapter 2.4.6., two transconjugant colonies were isolated. Both isolates were screened by PCR for the binary toxin gene locus and demonstrated a DNA fragment at the correct band size (Figure 3.61), which was confirmed by Sanger DNA sequencing.

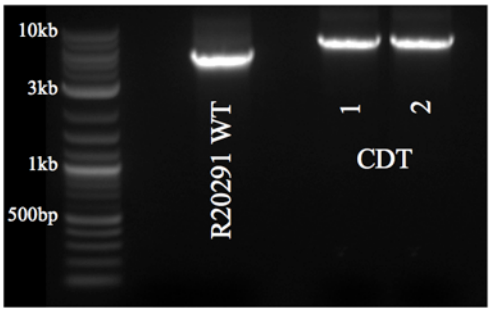


Figure 3.61 *Clostridium difficile* Binary Toxin Horizontal Gene Transfer Transconjugant PCR

A 1% agarose electrophoresis gel to demonstrate the DNA products generated from the *C. difficile* CDT horizontal gene transfer transconjugant screening PCR. The full binary toxin gene locus produced a DNA fragment of 5,128bp. The full binary toxin gene locus containing the ClosTron intron insertion generated a PCR band at 6,977bp.

To assess whether the colonies were true transconjugants or had resulted from spontaneous antibiotic resistance, several screening PCR reactions were conducted. A PCR to amplify *tcdA*, using previously published primers (Brouwer *et al.*, 2013), demonstrated the presence of the toxin A gene in both isolated transconjugants. An additional PCR, which assessed *sigK* organisation (whether it was disrupted or undisrupted due to the presence of *skin*^{Cd}) was also performed, using previously designed primer sets (Haraldsen and Sonenshein, 2003) (Figure 3.62). *C. difficile* R20291 is known to carry *skin*^{Cd} within the *sigK*, thereby disrupting the gene. The *skin*^{Cd} element is excised from the mother cell chromosome during sporulation and forms a circular molecule. On the contrary, strain CD37 is *skin*^{Cd} negative and so contains an undisrupted *sigK* gene.

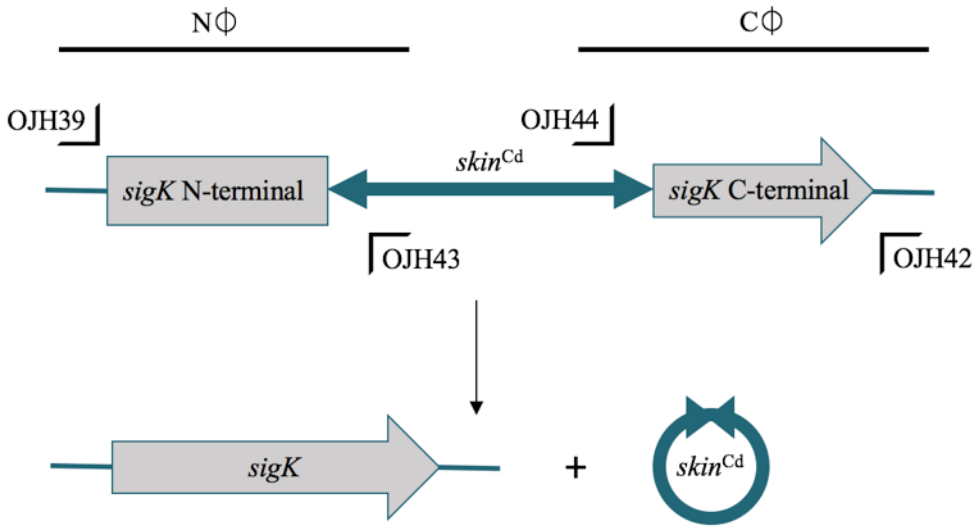


Figure 3.62 *Clostridium difficile* SigK Organisation PCR Primer Sets Adapted from (Haraldsen and Sonenshein, 2003)

Figure to illustrate *sigK* organisation. *C. difficile* R20291 is known to carry *skin*^{Cd} within the *sigK*, thereby disrupting the gene. The *skin*^{Cd} element is excised from the mother cell chromosome during sporulation and forms a circular molecule. On the contrary, strain CD37 lacks the *skin*^{Cd} element and so contains an undisrupted *sigK* gene. The NΦ primer set (OJH39 and OJH43) and CΦ primer set (OJH44 and OJH42) contain both the *sigK* sequence and *skin*^{Cd} sequence, and so if the *skin*^{Cd} is present, it would yield a 796 bp and 487 bp DNA fragment, respectively (Haraldsen and Sonenshein, 2003).

The PCR using the NΦ primer set was not successful, and no bands were observed for either potential transconjugant colony or the R20291 WT positive control strain. However, PCR bands were demonstrated for the PaLoc *tcdA* and CΦ primer set PCR reactions, illustrating that both transconjugant isolates were not correct, and were in fact spontaneous rifaximin resistant R20291::CT*cdtB* ClosTron colonies (Figure 3.63).

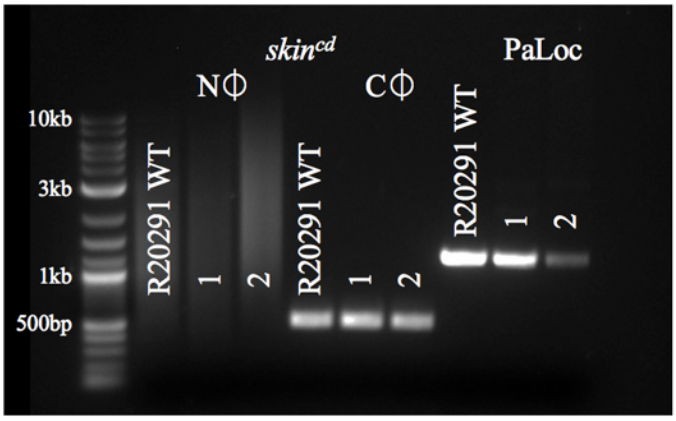


Figure 3.63 *Clostridium difficile* PaLoc and SigK Organisation Horizontal Gene Transfer Transconjugant PCR

A 1% agarose electrophoresis gel to demonstrate the DNA products generated from the *C. difficile* PaLoc *tcdA* and SigK organisation transconjugant screening PCR reactions. The PaLoc *tcdA* produced a PCR band of 1,155bp. The NΦ and CΦ primer sets yielded a 796 bp and 487 bp DNA fragment, respectively. The PCR using the NΦ primer set was not successful, and no bands were observed for either transconjugant colony or the R20291 WT positive control strain. PCR bands were demonstrated for both PaLoc *tcdA* and CΦ primer set PCR reactions for the transconjugant isolates.

One of the two potential transconjugants was also sent for ribotyping and was confirmed to be ribotype 027, for strain R20291, instead of ribotype 009, for strain CD37.

3.3. DISCUSSION

3.3.1. ALLELIC EXCHANGE STRAINS

The research described in this chapter set out to create CDT binary toxin allelic exchange knock-out strains in *C. difficile* R20291, PaLoc allelic exchange knock-out strains in *C. difficile* R20291 and the CDT knock-out strains, as well as, CDT complemented strains. The following four binary toxin allelic exchange in-frame deletion mutant strains are the first reported; R20291Δ*cdtA*Δ*cdtB*, R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*, R20291ΔPaLocΔ*cdtA*Δ*cdtB* and R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB*. Furthermore, the following two CDT complemented strains have never been described previously; R20291Δ*cdtR***cdtR* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB*. The allelic exchange method provided 'clean' knock-out strains to subsequently characterise, reducing the risk of potential down-stream polar effects (Kuehne and Minton, 2012) and multiple off-target insertions (Lehmann and Schmidt, 2003) observed in ClosTron mutants.

3.3.2. CDT TRANSCRIPTIONAL LINKAGE

Transcriptional linkage of the binary toxin gene locus was assessed in *C. difficile* R20291. The characterisation of CDT transcription was initially completed to aid with CDT complementation insert design. It was hypothesised that the *cdtR*, *cdtA* and *cdtB* genes of the binary toxin locus were transcriptionally linked, with the promoter upstream of the *cdtR* gene. *In silico* promoter sequence analysis revealed putative promoters upstream of the *cdtR* and *cdtA* genes. Several transcriptional elements were also identified, including the presence of conserved σ^{70} -10 and -35 site sequences. Fourteen transcriptional factor binding sites were identified in the promoter region directly upstream of *cdtR*. Twelve transcriptional factor binding sites were identified in the promoter region between the *cdtR* and *cdtA* genes. This analysis suggests that transcription of the CDT gene locus is regulated by a *E. coli* σ^{70}-like sigma factor (Metcalf and Weese, 2011). A putative promoter was not identified upstream of the *cdtB* gene, which correlates with the homologous *Clostridium perfringens* type E iota toxin Ib (Perelle *et al.*, 1993).

The RT-PCR results demonstrated that the *C. difficile* R20291 CDT gene locus is transcriptionally linked, with one mRNA transcript produced. It also revealed that transcription occurs at both early and late exponential phase, as well as in stationary phase. Furthermore, the results appear to show that expression was highest at the early exponential phase. It can be speculated that *in vivo* the CDT gene locus may be being transcribed almost continuously by vegetative cells of *C. difficile*.

Interestingly, although the transcriptional linkage assay indicated that one mRNA transcript is generated, it has been described in a recently published paper (Lyon *et al.*, 2016) that CdtA and CdtB proteins are still produced at decreased levels in a *cdtR* mutant strain, therefore, indicating that *cdtR* is not essential for CDT production *in vivo*. This discrepancy could be due to the secondary promoter identified upstream of the *cdtA* gene, which would allow the *cdtA* and *cdtB* genes to be transcribed without the presence of the *cdtR* gene. This information was used when designing the CDT complementation inserts.

3.3.3. ALLELIC EXCHANGE STRAIN CHARACTERISATION

3.3.3.1. GROWTH RATES AND CYTOTOXICITY ASSAY

All the generated allelic exchange knock-out strains, complemented strains and R20291 WT strain were assessed for growth and cytotoxicity. The hypothesis that bacterial growth of the binary toxin in-frame deletion mutants followed a typical growth curve was tested by comparing the optical density (OD_{600}) measurements with the R20291 WT strain over the course of 24 hours. Furthermore, it was hypothesised that all CDT in-frame deletion mutants would no longer produce binary toxin, as either the partial or no gene locus was present. This theory was investigated by measuring Vero cell cytotoxicity levels to quantify the binary toxin protein. Visible observations and statistical comparisons were made to determine any significant growth or cytotoxic effect differences.

There was no visibly observed or statistically significant difference in growth between the R20291 WT strain and the R20291ΔPaLoc in-frame deletion

strain. It has been previously reported that disruption of the *tcdC* gene (Bakker *et al.*, 2012) and the *tcdE* gene (Olling *et al.*, 2012) in the PaLoc gene locus does not affect growth kinetics, and both strains demonstrated similar growth profiles when compared to their equivalent WT strain. A 5-log decrease and statistically significant difference was observed between the R20291 WT strain and the R20291ΔPaLoc strain for cytotoxicity. The substantial decrease in toxin A and B cytotoxic effect when the PaLoc is deleted was anticipated. The remaining cytotoxicity observed was considered to be due to the binary toxin. Whether a binary toxin-producing, PaLoc negative *C. difficile* strain can cause disease *in vivo* is still debated. An early study (Geric *et al.*, 2006) concluded that toxin A negative, toxin B negative, binary toxin positive strains were enterotoxic, caused pronounced fluid accumulation and could colonise the gastrointestinal tract, however, they were incapable of causing disease in hamsters. However, more recent research (Kuehne *et al.*, 2014) discovered that when hamsters were challenged with a toxin A, toxin B ClosTron double mutant (and so only maintained a functional binary toxin gene locus), 3 out of the 9 animals died.

When the CDT gene locus was deleted in the ΔPaLoc strains to create R20291ΔPaLocΔ*cdtR*, R20291ΔPaLocΔ*cdtA*Δ*cdtB* and R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB*, the phenotypic growth was statistically significantly increased in all strains when compared to the R20291 WT strain (although R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* visibly appeared indistinguishable from the WT control). In recently published studies (Carlson *et al.*, 2013; Tschudin-Sutter *et al.*, 2016), ribotype 027 isolates have been shown to exhibit increased disease severity and high toxin production with a low growth rate. Therefore, it is not surprising that toxin negative in-frame deletion strains would display an increased growth rate. A statistically significant difference was also observed in cytotoxic effect when comparing the CDT ΔPaLoc knock-out strains with the R20291 WT strain. All three strains were comparable to the trypsinated PBS control, with no cytotoxicity observed, which supports the initial hypothesis. The evidence that no cytotoxic effect was demonstrated for the R20291ΔPaLocΔ*cdtR* knock-out strain opposes the data demonstrated in the (Lyon *et al.*, 2016) study.

However, in that research it was Western blot analysis that illustrated *cdtR* mutants were still able to produce CdtA and CdtB. It appears that although the *cdtA* and *cdtB* genes are transcribed and translated, the Vero cell cytotoxic effect is negligible (Bilverstone *et al.*, 2017). Whether this would be the case *in vivo* is yet to be investigated, however, it indicates that CDT production would be minimal, perhaps even undetectable.

With regards to the CDT knock-out strains, there was no visibly observed or statistically significant growth difference for the R20291Δ*cdtR* strain, whereas, both R20291Δ*cdtA*Δ*cdtB* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* strains displayed significantly increased growth, when compared to the R20291 WT strain (although R20291Δ*cdtA*Δ*cdtB* visibly appeared indistinguishable from the WT control). This result could again be explained by the finding that certain strains with increased toxin production exhibit a low growth rate (Carlson *et al.*, 2013; Tschudin-Sutter *et al.*, 2016), and the presence of the secondary promoter identified upstream of the *cdtA* gene. In the cytotoxicity assay, all the CDT knock-out strains displayed between a 2.5 and 3.5-log decrease and a statistically significant difference to the R20291 WT strain. Interestingly, the Vero cell cytotoxic effect was significantly decreased in these strains, despite the presence of the PaLoc. A previous study (Lyon *et al.*, 2016), recently demonstrated that toxin A and B cytotoxic activity was lower in supernatant from a *cdtR* mutant when compared to the WT strain. Other *in vivo* research (Kuehne *et al.*, 2014) has demonstrated that the presence of CDT in a toxin B mutant which continues to produce toxin A displayed increased virulence in a hamster model, with the animals dying at a significantly faster rate when compared to that of a toxin A positive, toxin B and CDT negative mutant strain. It appears that the *cdtA* and *cdtB* genes may also play a synergistic role with the production of toxin A and B.

The R20291Δ*cdtR*cdtR* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*cdtR*cdtA*cdtB* complemented strains both displayed a statistically significantly increased growth rate when compared to the R20291 WT strain (although R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*cdtR*cdtA*cdtB* visibly appeared indistinguishable from the WT control). However, only the growth of R20291Δ*cdtR*cdtR* was significantly increased when compared to the original CDT knock-out mutant

strain. The two CDT complemented strains both displayed cytotoxic activity comparable to the R20291 WT strain. However, only the R20291Δ*cdtR***cdtR* strain exhibited no significant difference. There was also a significant difference when the *cdtR* complemented strain was compared to the original knock-out mutant strain. In the complemented strains, the *cdtR* gene appears to be up-regulated at the *pyrE* gene locus, overexpressing the phenotype.

3.3.3.2. TAQMAN PROTEIN ASSAY

The hypothesis that all CDT in-frame deletion mutants would no longer produce binary toxin, as either the partial or no gene locus was present, was due to be investigated using the TaqMan Protein Assay. However, this method was not completely optimised or tested with the CDT allelic exchange knock-out strains, PaLoc allelic exchange knock-out strains and the CDT complemented strains due to time constraints. If time had not been a limiting factor, this assay would have demonstrated which strains were able to produce the CDT protein, and the amount of CDT produced at different time points. This perhaps may have identified those strains producing low levels, with an undetectable cytotoxic effect in the cytotoxicity assay, such as the R20291ΔPaLocΔ*cdtR* knock-out strain.

3.3.3.3. BIOFILM ASSAY

The biofilm assay was conducted at 24 and 96-hour time points for all the generated allelic exchange knock-out strains and complemented strains, as well as the R20291 WT control strain. It was hypothesised that the loss of the binary toxin gene locus may upregulate the formation of biofilms.

Almost all strains demonstrated less biofilm formation at 96 hours compared to 24 hours. This result has been demonstrated previously (Dapa *et al.*, 2013) where maximum biofilm formation for strain R20291 was observed at 24 hours, with uneven biofilms demonstrated by the 72-hour time point.

Statistical comparisons demonstrated a significant difference between R20291Δ*cdtA*Δ*cdtB* and R20291ΔPaLocΔ*cdtA*Δ*cdtB*, and R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* and

R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB*. R20291Δ*cdtA*Δ*cdtB* and R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* were also visually observed to have the highest absorbance readings of the crystal violet stained biofilm matrix, however, it is difficult to draw any conclusions as the replicate error bars were relatively large. This is thought to be due to the fragile biofilms lack of robustness at the PBS washing steps (Chapter 2.9.5). Nonetheless, there was no clear abolition of biofilm formation in any of the CDT knock-out mutant strains, and so the initial hypothesis cannot be accepted. This finding correlates with the outcome in the (Dapa *et al.*, 2013) paper, in which *cdtA* and *cdtB* ClosTron mutants displayed no significant defects in biofilm formation.

3.3.4. CDT HORIZONTAL GENE TRANSFER

It was hypothesised that the binary toxin gene locus could horizontally transfer from a toxigenic R20291 *C. difficile* strain to a non-toxigenic CD37 strain. The CDT horizontal gene transfer experiment was based on a study (Brouwer *et al.*, 2013) in which a genetically marked PaLoc was horizontally transferred from the 630 strain to the non-toxigenic CD37 strain. The genome of *C. difficile* 630 is well characterised and known to contain many MGEs, with CTn*1*, CTn*2* and Tn*5397* transposable elements in the vicinity of the PaLoc. The *C. difficile* R20291 genome is less well characterised and annotated. However, a search of the genome annotation list using the search term 'transposase', demonstrated the following genes with proximity to the CDT gene locus: CDR20291_2299; transposase, CDR20291_2501; transposase-like protein B, and CDR20291_2992; transposase IS4 family. The search terms 'conjugative transposon', 'phage' and 'plasmid' did not return any genes with proximity to the CDT gene locus. Although there were no annotated CTns in the vicinity of the CDT, this information did not indicate that horizontal gene transfer of the locus was not possible. In the (Brouwer *et al.*, 2013) study, the PaLoc was transferred to the CD37 strain independently of the CTn.

Unfortunately, the two transconjugant colonies isolated over three conjugation experiments were identified as spontaneous rifaximin resistant R20291::CT*cdtB* ClosTron mutants. Due to time restraints, no additional work was completed, and therefore the initial hypothesis cannot be accepted.

Further work could be attempted with a different ClosTron mutant, such as the R20291::CT*cdtA* strain which exhibits lincomycin resistance (Kuehne *et al.*, 2014). Moreover, a ClosTron could be created in the 630 strain, as a putative conjugative transposon (CD630_25200) is in proximity to the *cdtR* gene (CD630_26030). Although this strain contains the ghost CDT gene locus, it may provide an understanding into the mechanism.

A very recent paper (Riedel *et al.*, 2017), identified a *C. difficile* bacteriophage, designated phiSemix9P1, carrying a full and functional CDT gene locus. This is the first reported evidence that the binary toxin can be associated with putative genome MGEs, and so CDT horizontal gene transfer, and the biological impact of that, certainly needs to be investigated further.

CHAPTER 4. *CLOSTRIDIUM DIFFICILE* VIRULENCE FACTORS ASSOCIATED WITH PATIENT CLINICAL OUTCOME

4.1. INTRODUCTION

4.1.1. RIFAXIMIN FOR PREVENTING RELAPSE OF *CLOSTRIDIUM DIFFICILE* ASSOCIATED DIARRHOEA CLINICAL STUDY

The Rifaximin for Preventing Relapse of *Clostridium difficile* Associated Diarrhoea (RAPID) clinical study (trial number NCT01670149) was conducted with the Nottingham Digestive Diseases Centre Biomedical Research Unit (NDDCBRU) at the Queen's Medical Centre (QMC). The trial involved a randomised group of patients receiving either metronidazole or vancomycin treatment, and then a course of 'follow on' Rifaximin or a placebo control for the prevention of relapse of *C. difficile* associated diarrhoea (CDAD). The central hypothesis was that rifaximin is able to reduce relapse by suppressing *C. difficile* growth, while also allowing regrowth of normal microbiota leading to the restoration of colonisation resistance (Spiller *et al.*, 2011). A relapse was defined in the trial as "the onset of >3 loose bowel movements per day for at least two days combined with a positive toxin assay (glutamate dehydrogenase [GDH] positive and toxin enzyme immunoassay [EIA] positive), with or without a positive *C. difficile* culture" (Spiller *et al.*, 2012).

The overall objective of the trial was to advance understanding of the long-term consequences for patients with gastrointestinal infections, in the hope of developing better targeted treatment options. The clinical study was multi-disciplinary with research areas including gastro-duodenal and *Helicobacter pylori*-induced disease, intestinal and *C. difficile* toxicity and recurrence, as well as post-*Campylobacter jejuni* infection dysfunction, and finally, liver with a focus on post-hepatitis C disease (Spiller *et al.*, 2011).

The key aims for the research area involving *Clostridium difficile* infection (CDI) were to study the factors determining virulence, such as toxin production, sporulation and germination and correlate these findings to patient clinical outcome. *C. difficile* strains in patients that relapsed and those that did not were also characterised with the expectation of relating strain phenotype to

relapse rate and host influences (Spiller *et al.*, 2011). The *C. difficile* microbiology investigations were conducted by a team of post-doctorate researchers and PhD students at the NDDCBRU and Synthetic Biology Research Centre (SBRC) in the Centre for Biomolecular Sciences.

4.1.2. VIRULENCE FACTORS ASSOCIATED WITH PATIENT CLINIAL OUTCOME

4.1.2.1. BINARY TOXIN

C. difficile transferase (CDT) binary toxin has been linked to patient CDI disease severity and clinical outcome on numerous occasions in more recent years. Previous research (Barbut *et al.*, 2005) has illustrated that CDT-producing strains were associated with abdominal pain and liquid stools, indicating the correlation between CDT and severe acute disease. A Korean study (Kim *et al.*, 2013) also concluded that mucoid stools, as well as leucocytosis, correlated with binary toxin-positive strains. The association of binary toxin with leucocytosis was further corroborated by studies conducted in Belgium (Pilate *et al.*, 2016) and the U.K. (Goldenberg and French, 2011). Moreover, an inpatient retrospective study from 2000 to 2004 (Barbut *et al.*, 2007), reported that binary toxin-producing strains were associated with severe diarrhoea and greater case-fatality incidence. Two studies (Bacci *et al.*, 2011; Goldenberg and French, 2011), which compared 30-day case fatality rates for patients with CDI, discovered that patients with strains containing binary toxin had a significantly higher fatality rate than patients with CDT negative strains. In a U.S. hospital outbreak, 87.5% of the severe cases reported were attributed to strains carrying the binary toxin, compared to only 61.0% of the non-severe cases (McEllistrem *et al.*, 2005).

There have also been incidences of toxin A negative, toxin B negative, CDT positive strains, such as ribotype 033, causing clinically symptomatic disease, with some patients requiring metronidazole treatment (Stubbs *et al.*, 2000; Androga *et al.*, 2015; Eckert *et al.*, 2015).

Additionally, a research team (Stewart *et al.*, 2013; Stewart *et al.*, 2014) examining markers for CDAD and relapse, suggested that the presence of

binary toxin, as well as *tcdC* single nucleotide polymorphisms (SNPs), strongly predict recurrent CDI episodes. Conversely, another paper (Goldenberg and French, 2011) reported a lack of association and concluded that these features alone did not predict clinical virulence. These opposing associations may be due to the number of samples evaluated (73 (Stewart *et al.*, 2014) compared to 207 (Goldenberg and French, 2011)) or the strain ribotypes endemic to the study area.

In contrast to the research supporting CDT being linked to poor patient outcome, there are a handful of studies which found no such correlation between binary toxin and mortality (Hensgens and Kuijper, 2013), recurrence, treatment failure (Reigadas *et al.*, 2016) and disease severity (Tschudin-Sutter *et al.*, 2016). The fact that there are such conflicting opinions on whether *C. difficile* binary toxin is important in clinical disease, suggests that further study is necessary to determine the significance.

4.1.2.2. SPORULATION

It is well documented that *C. difficile* spores can lead to relapse or reinfection through either reactivation of the original strain in the patient's gastrointestinal tract or acquisition of a new strain from the environment (Shields *et al.*, 2015; Sim *et al.*, 2017). The fact that spores can be transmitted through the faecal-oral route and remain on surfaces within hospital and long-term care facilities could lead to potential outbreaks within that setting (Shields *et al.*, 2015). However, several studies (Figueroa *et al.*, 2012; Oka *et al.*, 2012; Sim *et al.*, 2017) have identified that endogenous carriage is the more likely scenario for patient relapse.

In mice, a spore supershedder state has been demonstrated (Lawley *et al.*, 2009). These immunocompetent animals become colonised asymptomatically by *C. difficile*, creating a persistent carrier state. At this stage, the mice shed small amounts of spores into their environment, however, other uninfected mice did not become ill. Upon completion of antibiotic treatment, the carrier mice converted into a contagious supershedder state. Transmission to immunocompromised mice then resulted in a severe gastrointestinal disease

and death in several cases. The authors concluded that *C. difficile* supershedders promote spore-mediated transmission. An additional study (Lawley *et al.*, 2012) by the same research group also highlighted that those supershedding strains were able to outcompete other ribotypes, persist in an infectious state in the host and maintain an enhanced transmission rate.

Originally, it was thought that one of the reasons why B1/NAP1/027 strains were unique and deemed hyper-virulent was because of increased sporulation (Akerlund *et al.*, 2008; Merrigan *et al.*, 2010). One study (Carlson *et al.*, 2013) even demonstrated a correlation between increased spore production of a B1/NAP1/027 strain with severe CDI, as determined by available clinical meta-data. However, the authors of another study (Burns *et al.*, 2010b) disputed previous claims and demonstrated that the diverse sporulation characteristics of *C. difficile* were not linked to specific ribotypes. Another more recent study (Aitken *et al.*, 2015) also went on to demonstrate that, in an endemic setting at least, ribotype 027 has virulent but not hyper-virulent phenotypes when compared to other ribotypes.

Research on the association between patient clinical outcome, strain ribotype and sporulation phenotype has been investigated in more recent years. Unfortunately, a number of studies (Sirard *et al.*, 2011; Oka *et al.*, 2012; Plaza-Garrido *et al.*, 2015; Reigadas *et al.*, 2017) have found no such correlation with single infection cases, relapse, reinfection or poor outcome. One study (Sirard *et al.*, 2011) also demonstrated that non-B1/NAP1/027 clinical strains actually had greater sporulating capacities compared to the ribotype 027 clinical strains, as they produced spores to a higher extent and at a faster rate. This sporulation data, however, could not be connected to CDI and disease severity.

Investigation into sporulation characteristics for clinical strains has not previously provided any association with patient outcome. However, it is still necessary to characterise the strains from the RAPID clinical study, as it is possible that an endemic strain associated with the QMC, or an individual patient may have a unique sporulation phenotype. In one instance, a paper (Cheng *et al.*, 2011) detailing a study performed in Hong Kong, discovered

that the predominant ribotype in that healthcare region, 002, had an increased sporulation frequency.

4.1.2.3. RIFAXIMIN AND FIDAXOMICIN ANTIBIOTIC RESISTANCE

The rising number of microorganisms with increased antibiotic resistance is a major global health concern. *C. difficile* has remained largely susceptible to the most common antimicrobial treatments available (Huang *et al.*, 2009). However, there were growing reports of decreased response rates and high recurrence rates associated with metronidazole and vancomycin (Shah *et al.*, 2010). These valid concerns led to the development of more recent antibiotics. Both rifaximin and fidaxomicin have been proposed as treatment, particularly for those patients at risk of recurrent episodes (Shah *et al.*, 2010; Mullane, 2014). Previous clinical studies (Johnson *et al.*, 2009; Garey *et al.*, 2011; Rubin *et al.*, 2011; Mattila *et al.*, 2013) have demonstrated how effective and safe rifaximin is as a first-line CDAD treatment or as a chaser regimen, with no recurrence in the majority of patients. Equally, Fidaxomicin has been shown to perform with identical efficiency compared to vancomycin but outperform regarding sustained cure rates (Louie *et al.*, 2011; Cornely *et al.*, 2012; Cornely *et al.*, 2014). The two antibiotics have the added benefit that they have a minimal effect on the commensal gastrointestinal flora (Mullane, 2014; Ponziani *et al.*, 2016).

RNA polymerase (RNAP) is comprised of five subunits and is an essential enzyme for transcription in bacteria (Chopra, 2007). There is remarkable conservation, both in DNA sequence and protein structure, between the domains of different bacterial species (Werner and Grohmann, 2011). Rifaximin and fidaxomicin are both bacteria transcriptional inhibitors and bind to RNAP, however, they act on different steps in the transcription initiation pathway (Figure 4.1) (Babakhani *et al.*, 2014). Fidaxomicin binds to the σ subunit of RNAP in the DNA template-RNAP closed complex prior to the formation of the open promotor DNA-RNAP complex where transcription is initiated (Venugopal and Johnson, 2012; Zhanel *et al.*, 2015; Taylor *et al.*, 2017). Rifaximin prohibits extension of the RNA transcripts further down the transcription pathway by binding to the β subunit of RNAP (RpoB). This

prevents formation of either the first or second phosphodiester bond in elongating RNA chains (Chopra, 2007; Babakhani *et al.*, 2014).

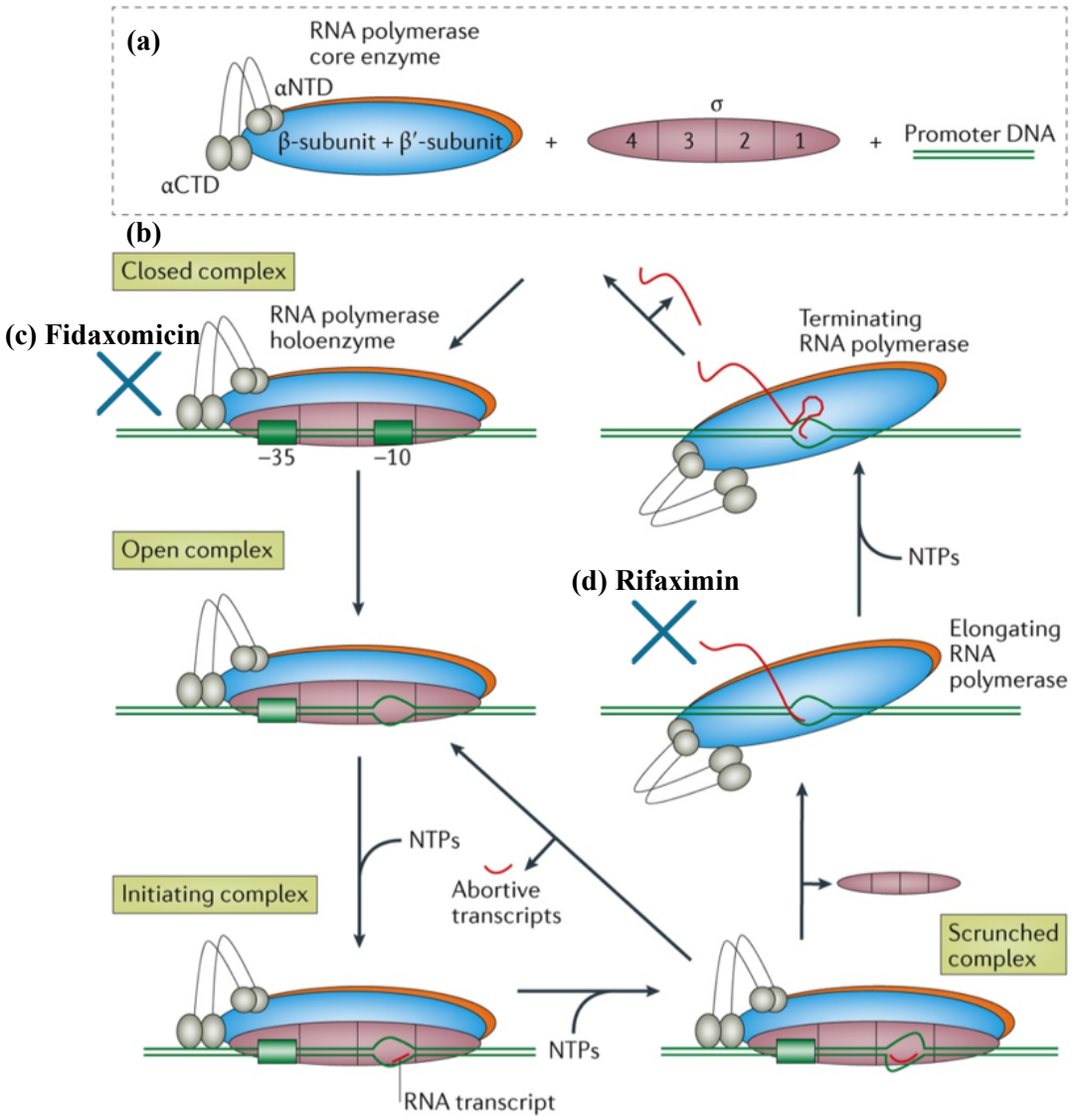


Figure 4.1 The Bacterial Transcription Pathway (Browning and Busby, 2016)

Figure 4.1 The Bacterial Transcription Pathway (Browning and Busby, 2016)
Legend (a) The RNA polymerase holoenzyme which comprises of the RNA polymerase (RNAP) enzyme and a sigma factor. **(b)** This binds to the promoter DNA template to form the closed complex. The DNA then unwinds in the transcription start site region to form the open complex. The RNA transcript is synthesised by the addition of nucleoside triphosphates (NTPs) which leads to a transition from the open complex to the initiating complex. The process of the template DNA strand being drawn into the initiating complex is termed 'scrunching'. At this step, either small aborted RNA fragments are made (due to the RNAP remaining at the promoter), or the RNAP enters elongation, which results in sigma factor release and RNA transcript elongation, facilitated by NTPs and elongation factors. The RNA transcript is released from the complex when the RNAP meets a transcriptional terminator and the enzyme separates from the DNA template. **(c)** Fidaxomicin acts on the closed complex by binding to the σ subunit of the RNAP before formation of the open complex. **(d)** Rifaximin binds to the RNAP β subunit to prevent RNA transcript elongation.

To date, resistance to fidaxomicin has only been observed in two clinical isolates (Goldstein *et al.*, 2011; Peng *et al.*, 2017a). However, unfortunately, rifaximin antibiotic resistance has been previously described on a number of occasions (Marchese *et al.*, 2000; Hecht *et al.*, 2007; O'Connor *et al.*, 2008; Carman *et al.*, 2012; Freeman *et al.*, 2015b) (please refer to Chapter 1.2.8 and 1.2.9.1 for further discussion on fidaxomicin and rifaximin resistance).

4.1.3. AIM OF THIS STUDY

Much of *C. difficile* research is conducted *in vitro*, either on a molecular level or through the application of model systems, such as with cell culture or by implementation of a gut model. In *in vivo* scenarios, the well-established hamster infection or humanised mouse models can be employed. However, there are particular aspects of CDI that are best studied with clinical isolates (Kuehne *et al.*, 2013). The RAPID clinical study provided a unique opportunity to access a large number of patient samples. These isolates could be completely characterised in terms of growth, toxin production (TcdA, TcdB and CDT), sporulation, germination and antibiotic resistance, with conclusions drawn from the unblinded trial data.

This chapter will focus on my research responsibility within the RAPID clinical study. My initial role was to ascertain the potential importance of CDT and investigate the hypothesis that the presence of the full binary toxin gene

locus in patient isolates is associated with recurrent episodes, specifically relapse. This was achieved through assessing the frequency of the CDT gene locus, via PCR and gel electrophoresis, and using Sanger DNA sequencing to identify clinically important SNPs in these samples.

In addition to this, sporulation rates were assessed for all RAPID isolates, using a sporulation assay. It was hypothesised that there may be individual patients that have strains with unique sporulation profiles, which could be correlated with relapse. A ClosTron mutant strain, which was generated to knock-out a putative spore envelope protein gene, was investigated alongside the RAPID strains. The hypothesis that this sporulation gene would negatively affect sporulation rate was tested.

As the primary objective for the NDDCBRU in the RAPID clinical trial was to assess whether rifaximin would reduce the relapse rate of CDAD, it seemed prudent to evaluate the sensitivity of the patient isolates to rifaximin, using an antibiotic resistance plate assay. Rifaximin resistance has been observed in clinical isolates (previously discussed in Chapter 1.2.9.1), and so it was hypothesised that emergence of resistance in the RAPID strains would be observed in at least one patient. If rifaximin resistance was demonstrated, it would be necessary to determine if this related to patient outcome. Fidaxomicin antibiotic resistance (previously discussed in Chapter 1.2.8) was also investigated, as this antibiotic similarly inhibits bacterial transcription, but it was hypothesised that neither fidaxomicin resistance nor cross-resistance with rifaximin would be observed in the RAPID clinical isolates.

4.2. RESULTS

4.2.1. RAPID CLINICAL STUDY PARTICIPANT DATA

Originally 180 patients were expected to enrol onto the RAPID clinical trial (Spiller *et al.*, 2012). At completion of the trial in July 2016, a total of 151 patients were enrolled from twenty different hospital sites (Table 4.1).

However, due to patient and hospital site nonfulfillment, 58 stool samples from 29 patients collected from nine hospital sites were analysed. Of the 29 patients, 7 were known to have relapsed during the 12-week sample collection period.

Table 4.1 RAPID Clinical Trial Hospital Sample Collection Sites

RAPID Number	Hospital Site
01/02	Nottingham University Hospitals (Queen's Medical Centre and City Hospital)
03	King's Mill Hospital, Sutton-in-Ashfield
04	Royal Derby Hospital
05	Northern General Hospital, Sheffield
06	Chesterfield Royal Hospital
07	Royal Shrewsbury Hospital
08/09	County Durham and Darlington NHS Foundation Trust
10	Sunderland Royal Hospital
11	Kettering General Hospital
12	Dorset County Hospital
13	James Cook Hospital, Middleborough
14	North Cumbria University Hospitals
15	Hampshire Hospitals NHS Foundation Trust
17	University Hospital Southampton NHS Foundation Trust
18	New Cross Hospital, Wolverhampton
19	Doncaster Royal Infirmary
20	Russells Hall Hospital, Dudley
21	Stepping Hill Hospital, Stockport
22	Queens Hospital, Romford
23	King's College Hospital, London

After specialist culture of the RAPID stool samples (Chapter 2.4.7.), in-house ribotyping on specific isolates was completed by Dr Emma Stevenson (Stevenson *et al.*, 2016). The isolates were tested in three separate groups, as the patient samples arrived at the NDDCBRU laboratory over a period of 3

years. Unfortunately, due to time limitations, the last series of samples were not ribotyped. The unblinded participant data was released after completion of the clinical trial. This included whether the patient was given a course of 'follow on' Rifaximin or a placebo control for the prevention of relapse of CDAD. Both ribotyping results and patient treatment data for each RAPID isolate is shown in Table 4.2.

Table 4.2 RAPID Isolate Ribotype and Unblinded Trial Patient Treatment Data

RAPID Isolate	Ribotype	Treatment	RAPID Isolates	Ribotype	Treatment
01006_-1_C10	014	Rifaximin	**11007_-1_C2**	014	Placebo
01006_0_C1	014		**11007_-1_C5**	029	
01006_12_C11	014		**11007_0_C14**	014	
01011_-1_C20	215	Rifaximin	**11007_RL_C1**	014	
01013_-1_C17	002	Rifaximin	**12001_0_C5**	070	Rifaximin
01013_12_C19	014		**12001_RL_C14**	070	
01015_-1_C1	023	Placebo	**12004_0_C2**	020	Placebo
01015_4_C1	023		**12004_RL_C14**	020	
01016_-1_C1	026	Rifaximin	**12006_12_C1**	Not Applicable	Placebo
01016_12_C1	026		**13002_-1_C13**	015	Rifaximin
01017_-1_C3	005	Rifaximin	**13007_0_C1**	Not Ribotyped	Rifaximin
01017_4_C4	005		**13007_4_C3**	Not Applicable	
01018_-1_C17	026	Placebo	**15003_-1_C1**	Not Ribotyped	Placebo
01018_-1_C20	015		**15003_12_C1**	Not Ribotyped	
01023_-1_C2	011	Placebo	**15003_RL_C1**	Not Ribotyped	
01023_0_C1	011		**17001_-1_C1**	Not Ribotyped	Placebo
01026_-1_C3	002	Placebo	**17001_0_C1**	Not Ribotyped	
01027_-1_C1	078	Rifaximin	**17001_4_C1**	Not Ribotyped	
01028_-1_C12	023	Placebo	**17002_-1_C1**	Not Ribotyped	Rifaximin
01028_0_C3	023		**17002_0_C1**	Not Ribotyped	
01029_-1_C19	014	Placebo	**17002_RL1_C1**	Not Ribotyped	

01036_-1_C4	002	Placebo	**17002_RL2_C1**	Not Ribotyped	
02001_4_C9	010	Placebo	**17002_RL3_C1**	Not Ribotyped	
03001_4_C2	349	Placebo	**18001_-1_C4**	020	Rifaximin
03001_12_C1	349		**18001_0_C6**	020	
03008_0_C2	002	Rifaximin	**18001_RL_C5**	020	
03008_4_C2	002		**21008_0_C1**	Not Ribotyped	Placebo
03010_4_C3	365	Placebo	**21008_4_C1**	Not Ribotyped	
11001_4_C1	029	Placebo			
11001_RL_C3	029				

Table 4.2 RAPID Isolate Ribotype and Unblinded Trial Patient Treatment Data Legend - Key: -1; Diagnostic sample, 0; Sample after successful metronidazole or vancomycin treatment, 4; Week 4 check-up sample, 12; Week 12 check-up sample, RL; Relapse sample, C; Colony number picked.

4.2.2. CLINICAL ISOLATE BINARY TOXIN GENE LOCUS

4.2.2.1. BINARY TOXIN GENE LOCUS STRAIN ALIGNMENT

Initially, the binary toxin CDT locus was compared between a number of *C. difficile* strains, with available genome sequences, so as to be able to design primers in conserved regions for use with the RAPID isolates. The results of a Basic Local Alignment Search Tool (BLAST) database search compared the binary toxin of *C. difficile* R20291, including 1kb upstream and downstream of the locus, with other *C. difficile* strains which exhibited either a complete or partial ghost gene locus (Figure 4.2). *C. difficile* strains CD196 and 2007855 generated 100% identify and query cover when related to R20291. The M120 and BI-1 isolates both produced results with 100% query cover but with 99% and 98% identities, respectively. There were numerous *C. difficile* strains identified, including K744, AE70, A765, AE16, AE978, C421, C959, AD667, CD98 and AM478, which displayed between 97% and 100% identities but with only an 82% to 83% query cover. The first 166 to 182 nucleotides at the 5' end were not conserved, as well as around 1kb immediately after the *cdtB* gene at the 3' end. The two ghost binary toxin gene locus isolates recognised through the database comparison search were strains 630 and BI-9. They

revealed a 96% identity compared to R20291, with a 73% query cover. The 5' and 3' ends were conserved as opposed to the strains stated previously, however, 2kb in the centre of the gene locus was absent as expected.

Once the search data had been obtained, the resulting sequences were aligned, and a forward and reverse primer designed in the regions of similarity (Figure 4.2). The primers were generated in such a way that a PCR product would be produced for both the full binary toxin sequence, as well as the locus containing the *cdtA* and *cdtB* pseudogenes.

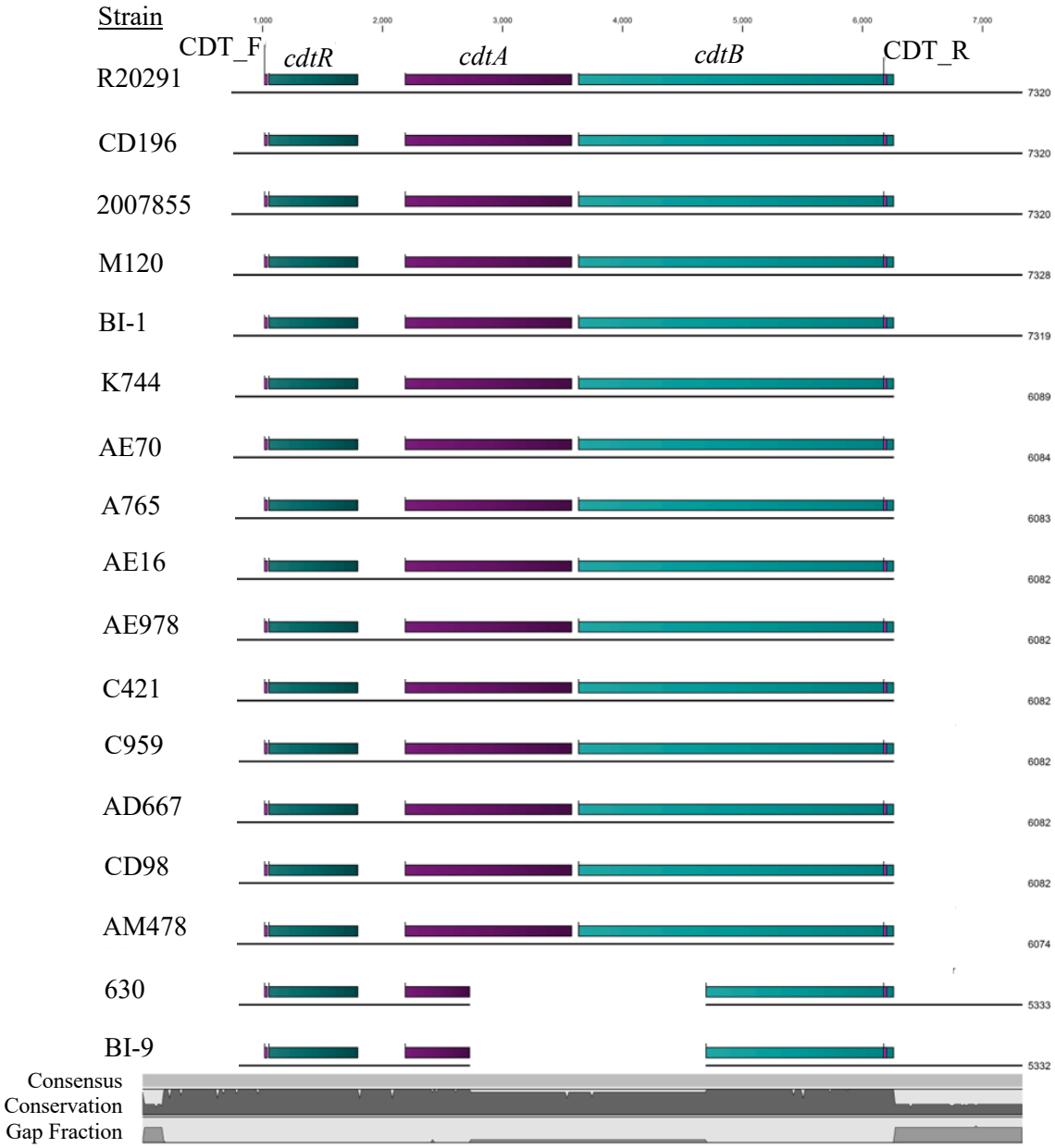


Figure 4.2 *Clostridium difficile* Binary Toxin Gene Locus Alignment

The alignment demonstrates the conserved regions of the CDT gene locus for *C. difficile* strain R20291, including 1kb upstream and downstream of the locus, when compared to other *C. difficile* strains with either a complete or partial ghost binary toxin gene locus using the Basic Local Alignment Search Tool (BLAST) database. The individual genes; *cdtR* (teal), *cdtA* (purple) and *cdtB* (turquoise), are labelled, along with the CDT_F forward primer (purple marker, located at the start of *cdtR*) and CDT_R reverse primer (purple marker, located at the end of *cdtB*) subsequently designed in conserved regions between the various *C. difficile* isolates.

4.2.2.2. BINARY TOXIN GENE LOCUS CONTROL PCR ANALYSIS

After designing the primers to be used to amplify the complete and partial CDT gene locus, a control PCR was performed to optimise the annealing temperature and extension time for a variety of *C. difficile* strains available in the SBRC laboratory collection. The full binary toxin locus generated a PCR product at 5,128bp, with the ghost locus fragment observed at 3,128bp due to the *cdtA* and *cdtB* 2kb deletion (Figure 4.3).

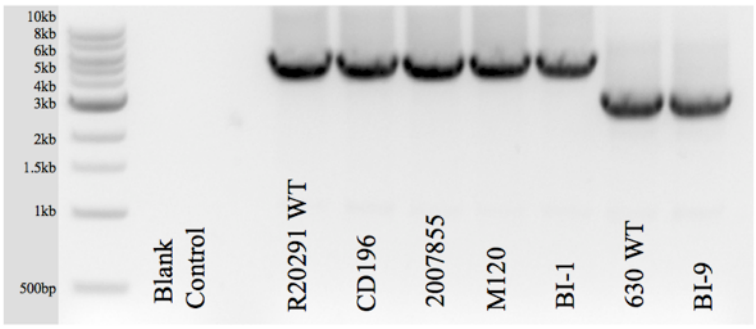


Figure 4.3 *Clostridium difficile* Binary Toxin Control PCR

A 1% agarose electrophoresis gel to demonstrate the PCR products generated from the *C. difficile* CDT control PCR using the primers designed from the Basic Local Alignment Search Tool (BLAST) data. The full binary toxin gene locus produced a DNA fragment of 5,128bp. A 3,128bp band was observed for the ghost CDT locus, due to the 2kb deletion present at the 3' end of *cdtA* and 5' end of *cdtB*. The negative gel is shown to highlight the lighter bands.

4.2.2.3. BINARY TOXIN GENE LOCUS RAPID ISOLATE PCR ANALYSIS

Once the CDT PCR primers had been optimised with different *C. difficile* control strains, the patient RAPID sample strains, that had been previously isolated through specialist stool culture (Chapter 2.4.7.), were genomic DNA extracted and tested. Due to the patient samples arriving at the NDDCBRU laboratory over a period of 3 years, the isolates were tested in three separate groups. Initially, when ribotypes were available, those isolates which were known to contain the complete CDT gene locus (in this case, ribotype 023 and 078) were investigated first (Figure 4.4). The CDT PCR demonstrated that each of the five samples examined contained the full binary toxin gene locus with a DNA fragment of 5,128bp.

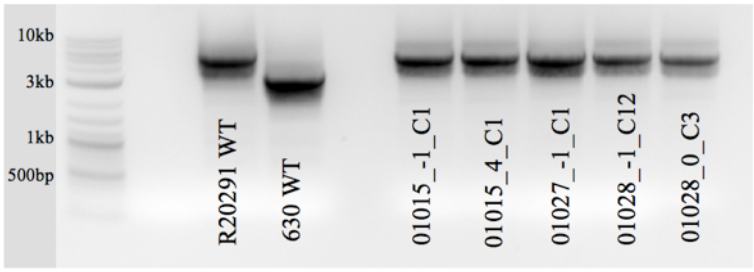


Figure 4.4 RAPID *Clostridium difficile* Binary Toxin Positive Isolates PCR

A 1% agarose electrophoresis gel to demonstrate the binary toxin PCR products generated from the RAPID *C. difficile* CDT positive isolates. The full binary toxin gene locus produced a DNA fragment of 5,128bp. A 3,128bp band was observed for the 630 wildtype (WT) ghost CDT locus, due to the 2kb deletion present at the 3' end of *cdtA* and 5' end of *cdtB*. Key: -1; Diagnostic sample, 0; Sample after successful metronidazole or vancomycin treatment, 4; Week 4 check-up sample, C; Colony number picked. The negative gel is shown to highlight the lighter bands.

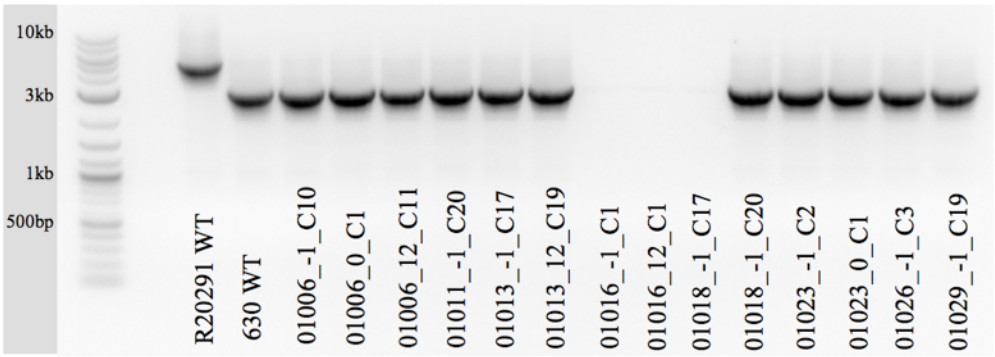


Figure 4.5 CDT PCR for the First Group of RAPID *Clostridium difficile* Binary Toxin Isolates

A 1% agarose electrophoresis gel to demonstrate the binary toxin PCR products generated from the first group of RAPID *C. difficile* CDT isolates. The ghost binary toxin gene locus produced a DNA fragment of 3,128bp, due to the 2kb deletion present at the 3' end of *cdtA* and 5' end of *cdtB*. Three isolates displayed no band. A 5,128bp band was observed for the R20291 wildtype (WT) full CDT gene locus. Key: -1; Diagnostic sample, 0; Sample after successful metronidazole or vancomycin treatment, 12; Week 12 check-up sample, C; Colony number picked. The negative gel is shown to highlight the lighter bands.

The additional RAPID *C. difficile* isolates in the first group of samples, which had a ribotype that either was acknowledged to contain a partial binary toxin gene locus or was undetermined in the literature, were tested subsequently

(Figure 4.5). All but three samples illustrated a DNA fragment band at 3,128bp for the partial ghost CDT gene locus. The three isolates which displayed no band on the agarose electrophoresis gel were verified with a non-toxigenic PCR described in Chapter 4.2.2.4.

The second group of RAPID *C. difficile* isolates were assessed for strains which carry the full CDT gene locus by ribotyping. On this occasion, there were no previously identified ribotypes and so all samples were tested simultaneously (Figure 4.6). Almost all samples demonstrated a DNA fragment band at 3,128bp for the ghost binary toxin gene locus. Four isolates exhibited no band on the agarose electrophoresis gel and so were verified with a non-toxigenic PCR described in Chapter 4.2.2.4.

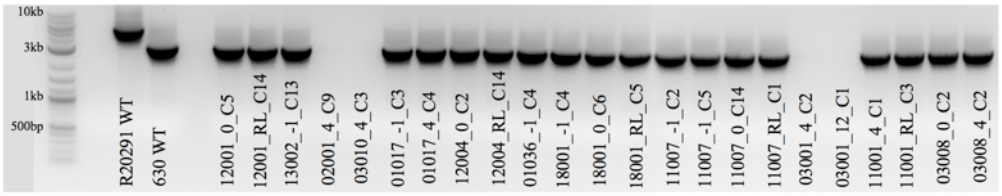


Figure 4.6 CDT PCR for the Second Group of RAPID *Clostridium difficile* Binary Toxin Isolates

A 1% agarose electrophoresis gel to demonstrate the binary toxin PCR products generated from the second group of RAPID *C. difficile* CDT isolates. The ghost binary toxin gene locus produced a DNA fragment of 3,128bp, due to the 2kb deletion present at the 3' end of *cdtA* and 5' end of *cdtB*. Four isolates displayed no band. A 5,128bp band was observed for the R20291 wildtype (WT) full CDT gene locus. Key: -1; Diagnostic sample, 0; Sample after successful metronidazole or vancomycin treatment, 4; Week 4 check-up sample, 12; Week 12 check-up sample, RL; Relapse sample, C; Colony number picked. The negative gel is shown to highlight the lighter bands.

Ribotyping was not completed on the third and final group of RAPID *C. difficile* isolates during the time of this study and so all samples were tested together (Figure 4.7). The CDT PCR revealed ten isolates which demonstrated a 3,128bp DNA band fragment for the ghost binary toxin gene locus. There were four samples where a 5,128bp band for the complete CDT gene locus was observed. Two isolates exhibited no band on the agarose electrophoresis

gel and so were verified with a non-toxigenic PCR described in Chapter 4.2.2.4.

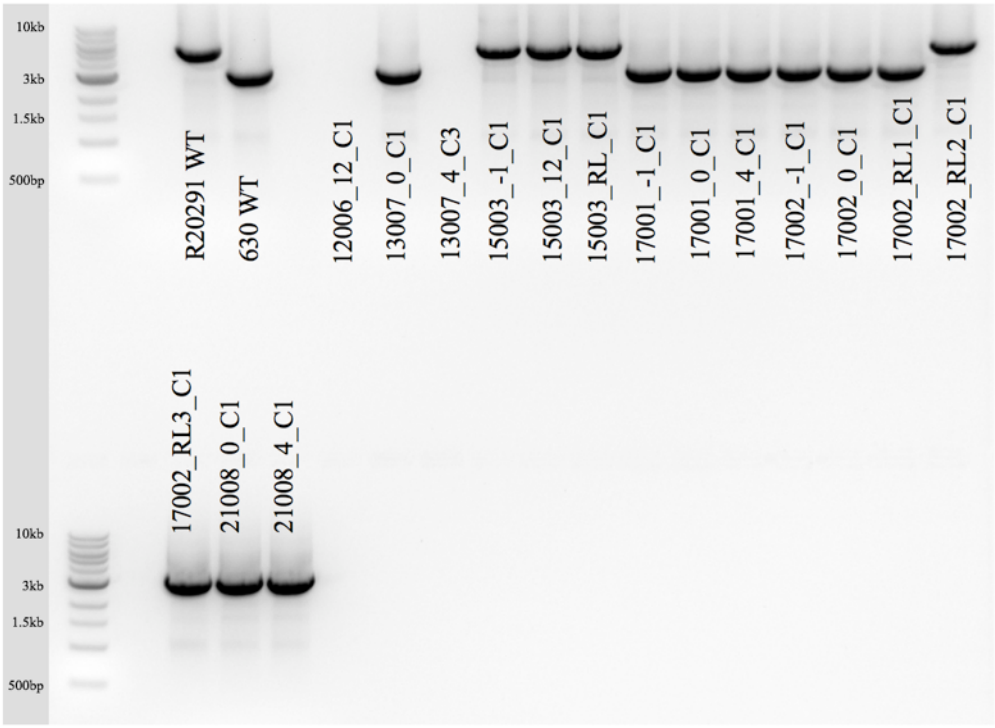


Figure 4.7 CDT PCR for the Third Group of RAPID *Clostridium difficile* Binary Toxin Isolates

A 1% agarose electrophoresis gel to demonstrate the binary toxin PCR products generated from the second group of RAPID *C. difficile* CDT isolates. The ghost binary toxin gene locus produced a DNA fragment of 3,128bp, due to the 2kb deletion present at the 3' end of *cdtA* and 5' end of *cdtB*. A 5,128bp band was observed for the full CDT gene locus. Two isolates produced no DNA fragment band. Key: -1; Diagnostic sample, 0; Sample after successful metronidazole or vancomycin treatment, 4; Week 4 check-up sample, 12; Week 12 check-up sample, RL; Relapse sample, C; Colony number picked. The negative gel is shown to highlight the lighter bands.

4.2.2.4. NON-TOXIGENIC RAPID ISOLATE PCR ANALYSIS

Several RAPID *C. difficile* isolates generated no visible DNA fragment band on the agarose electrophoresis gel for either the full or partial ghost binary toxin gene locus. These samples were verified for the non-toxigenic 68bp nucleotide sequence which occupies the region in place of the CDT locus (Carter *et al.*, 2007). Seven of the nine samples demonstrated a 700bp DNA band (Figure 4.8), with two isolates generating no PCR product. Subsequent

Sanger DNA sequencing of each PCR product revealed the 68bp fragment identical to that of the non-toxigenic CD37 strain (Figure 4.9). The two samples which demonstrated no DNA fragment are discussed in Chapter 4.2.5.

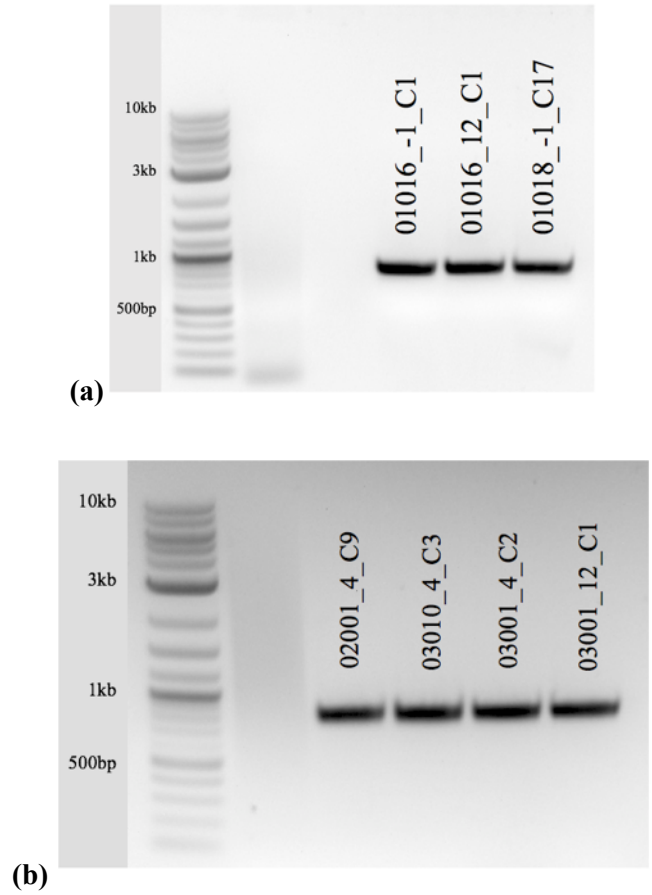


Figure 4.8 Non-Toxigenic CDT PCR for the RAPID *Clostridium difficile* Isolates

(a) and **(b)** 1% agarose electrophoresis gels to demonstrate the non-toxigenic CDT PCR products generated from the RAPID *C. difficile* CDT isolates. A 700bp band containing the 68bp non-toxigenic nucleotide sequence was observed for seven samples. Two samples generated no PCR product (data not shown). Key: -1; Diagnostic sample, 4; Week 4 check-up sample, 12; Week 12 check-up sample, C; Colony number picked. The negative gel is shown to highlight the lighter bands.

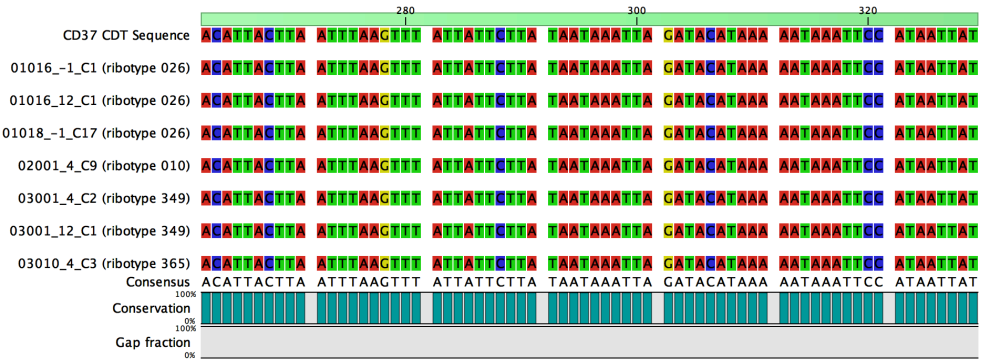


Figure 4.9 Non-Toxigenic CDT Sanger DNA Sequencing Alignment for the RAPID *Clostridium difficile* Isolates

The Sanger DNA sequencing alignment revealed each non-toxigenic CDT PCR product to contain the 68bp fragment identical to that of the non-toxigenic CD37 strain. Key: -1; Diagnostic sample, 4; Week 4 check-up sample, 12; Week 12 check-up sample, C; Colony number picked.

4.2.2.5. BINARY TOXIN GENE LOCUS SINGLE NUCLEOTIDE POLYMORPHISM ANALYSIS

The RAPID *C. difficile* isolates that contained either a full or partial ghost binary toxin gene locus were assessed for single nucleotide polymorphisms (SNPs) that caused an amino acid modification. Firstly, all isolates were Sanger DNA sequenced using specially designed walking primers (Chapter 2.10.4.) and the resulting nucleotide sequences were assembled into a complete gene locus contig. Alignments were created using either the R20291wildtype (WT) reference sequence; for the complete CDT gene locus samples (Appendix 1, 2 and 3), or the 630 WT reference sequence; for the ghost binary toxin gene locus (Appendix 4).

Basic variant detection was performed on all samples using a ploidy of 1, minimum coverage of 1, minimum count of 1 and minimum frequency of 100%. SNPs were identified using the R20291 WT reference sequence; for the complete and ghost CDT gene locus samples, as well as the 630 WT reference sequence; for the ghost binary toxin gene locus. SNPs which do not constitute an amino acid change were disregarded. Table 4.3 displays the SNPs and their corresponding modified amino acid for those RAPID isolates which contained a complete CDT gene locus. A total of 73 SNPs which lead to an amino acid change were identified, when compared to R20291 WT; 18 for *cdtR*, 23 for

cdtA and 32 for *cdtB*. The number of samples along with the calculated frequency percentage, and the identified ribotypes in which that SNP is observed are also indicated. The frequency of SNPs in this case ranged from 11% to 67% and were detected in ribotypes 023 and 078. The ribotyping was not completed on the third group of RAPID samples and so certain SNPs were not associated with a specific ribotype and are termed 'Unknown' in the table. The frequency of the SNPs as a percentage is also displayed graphically in Figure 4.10.

Table 4.3 RAPID *Clostridium difficile* Isolates Complete Binary Toxin Single Nucleotide Polymorphism Analysis

SNP	Amino Acid Change	Number of Samples (Out of 9)	Frequency (%)	Ribotypes
100A>G	Ile34Val	6	67%	023, 078
142A>G	Thr48Ala	4	44%	023
161A>G	Lys54Arg	6	67%	023, 078
178G>A	Val60Ile	4	44%	023
322G>T	Glu108*	2	22%	078
409G>A	Val137Ile	6	67%	023, 078
427C>T	Arg143Cys	4	44%	023
496T>A	Phe166Ile	1	11%	Unknown
508G>A	Ala170Thr	6	67%	023, 078
572G>A	Arg191Lys	6	67%	023, 078
574T>C	Ser192Pro	1	11%	Unknown
575C>A	Ser192Tyr	4	44%	023
601A>G	Ile201Val	6	67%	023, 078
607G>A	Val203Ile	4	44%	023
649C>A	Leu217Ile	2	22%	078
670A>G	Ile224Val	6	67%	023, 078
736A>G	Ile246Val	4	44%	023
743C>G	Thr248Arg	2	22%	078
19C>T	His7Tyr	4	44%	023
27G>T	Arg9Ser	6	67%	023, 078
50T>C	Leu17Ser	4	44%	023
76G>A	Gly26Ser	6	67%	023, 078
134G>A	Cys45Tyr	4	44%	023
179T>C	Leu60Pro	1	11%	023

194A>G	Lys65Arg	2	22%	078
299G>A	Ser100Asn	6	67%	023, 078
371A>G	Lys124Arg	6	67%	023, 078
532A>G	Asn178Asp	4	44%	023
564T>A	Asp188Glu	2	22%	078
598A>G	Lys200Glu	2	22%	078
673G>A	Val225Ile	4	44%	023
776G>A	Ser259Asn	4	44%	023
820G>A	Gly274Ser	1	11%	023
836A>G	Asn279Ser	4	44%	023
955C>T	Pro319Ser	4	44%	023
1085A>T	Asp362Val	1	11%	023
1097T>C	Leu366Pro	6	67%	023, 078
1138G>A	Ala380Thr	6	67%	023, 078
1325A>G	Asn442Ser	6	67%	023, 078
1350_1351delTAinsCG	Thr451Ala	2	22%	078
1351A>G	Thr451Ala	4	44%	023
7A>G	Ile3Val	6	67%	023, 078
46C>T	Leu16Phe	4	44%	023
74T>C	Val25Ala	2	22%	078
107_108delATinsGC	Asn36Ser	2	22%	078
115A>G	Asn39Asp	2	22%	078
120G>T	Lys40Asn	1	11%	Unknown
133G>A	Val45Ile	5	56%	023, 078
151C>A	Pro51Thr	2	22%	078
270_271delGGinsAA	Asp91Asn	2	22%	078
286G>A	Asp96Asn	2	22%	078
367G>A	Val123Ile	2	22%	078
386C>A	Thr129Asn	2	22%	078
528G>C	Thr136Lys	4	44%	023
445G>T	Val149Phe	2	22%	078
528G>C	Met176Ile	6	67%	023, 078
545A>C	Glu182Ala	2	22%	078
586G>A	Asp196Asn	6	67%	023, 078
626A>G	Lys209Arg	2	22%	078
1339C>A	Leu447Ile	4	44%	023
1605A>T	Leu535Phe	4	44%	023
1609G>A	Asp537Asn	2	22%	078
1795A>C	Lys599Gln	4	44%	023
1859C>T	Thr620Ile	4	44%	023
1892C>T	Thr631Ile	4	44%	023
1984G>A	Glu662Lys	2	22%	078
2108G>A	Gly703Glu	4	44%	023
2245G>A	Asp749Asn	2	22%	078
2286G>A	Met762Ile	6	67%	023, 078

2306T>C	Phe769Ser	6	67%	023, 078
2308G>T	Ala770Ser	4	44%	023
2341A>G	Thr781Ala	6	67%	023, 078
2365G>A	Ala789Thr	2	22%	078

Table 4.3 RAPID *Clostridium difficile* Isolates Complete Binary Toxin Single Nucleotide Polymorphism Analysis Legend - Key: *cdtR* SNPs, *cdtA* SNPs and *cdtB* SNPs

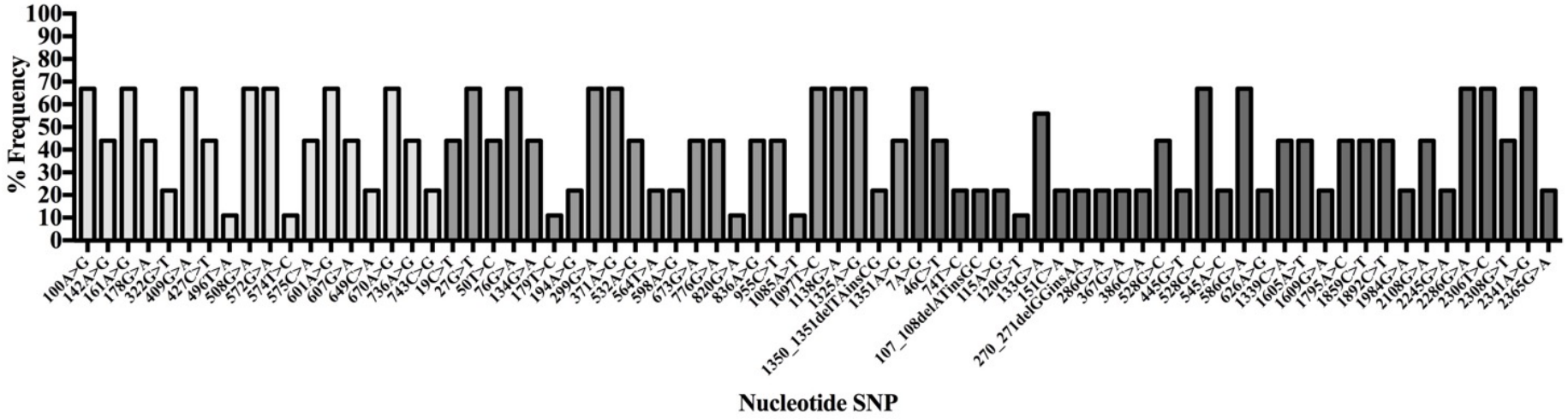


Figure 4.10 RAPID *Clostridium difficile* Isolates Complete Binary Toxin Single Nucleotide Polymorphism Frequency

Graph to show single nucleotide polmorphism (SNP) frequency as a percentage for the RAPID full CDT gene locus isolates when compared to the R20291 wildtype (WT) strain. Key: *cdtR* SNPs, *cdtA* SNPs and *cdtB* SNPs

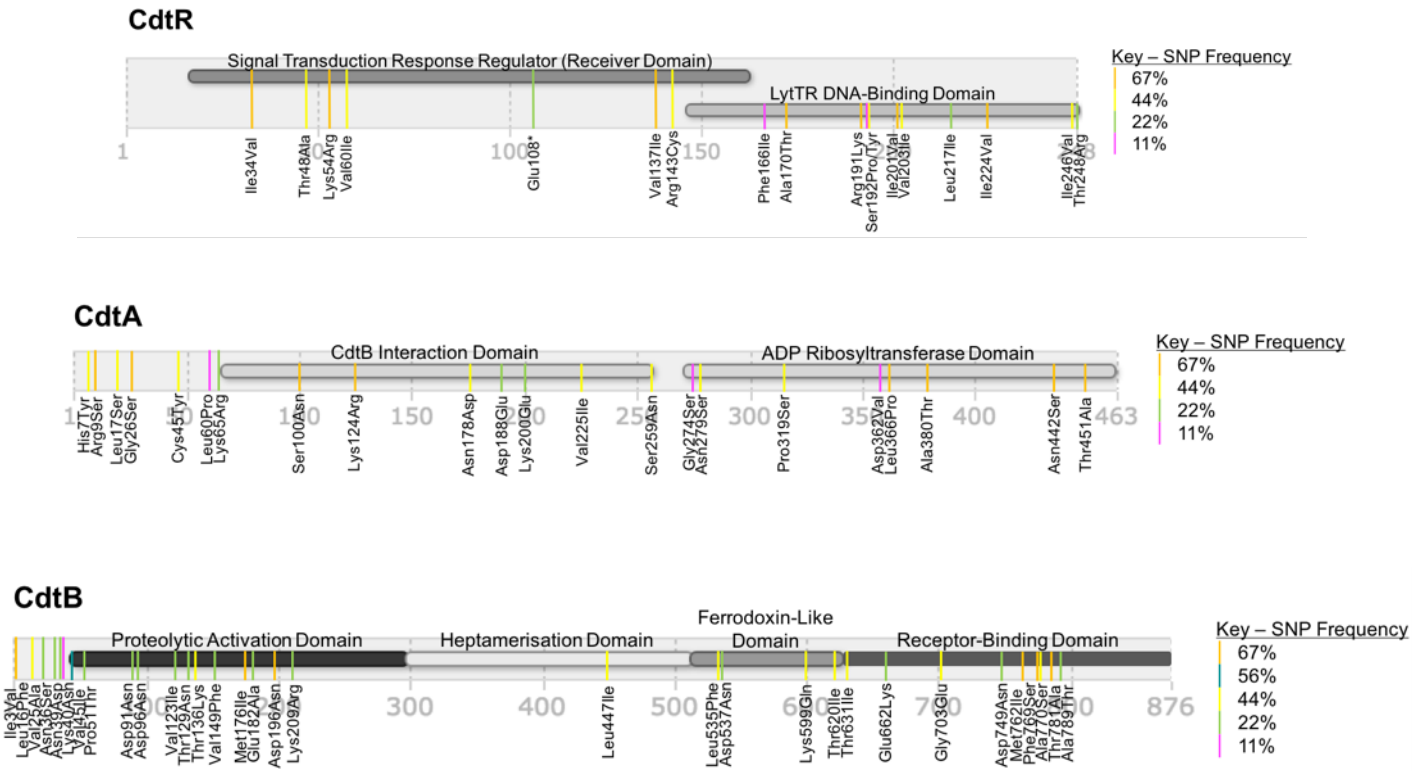


Figure 4.11 RAPID *Clostridium difficile* Isolates Complete Binary Toxin Amino Acid Substitution Positions

Figure to illustrate the amino acid substitution positions and associated single nucleotide polymorphism (SNP) frequency as a percentage for the RAPID full CDT gene locus isolates when compared to the R20291 wildtype (WT) strain.

The amino acid substitution for each corresponding SNP is illustrated in Figure 4.11, where the binary toxin genes are individually labelled with the appropriate protein domains. The frequency of the associated SNP is also illustrated as a percentage.

Table 4.4 RAPID *Clostridium difficile* Isolates Ghost *cdtR* vs. R20291 Wildtype Single Nucleotide Polymorphism Analysis

***cdtR* SNP**	**Amino Acid Change**	**Number of Samples (Out of 40)**	**Frequency (%)**	**Ribotypes**
42A>G	Ile14Met	40	100%	002, 005, 011, 014, 015, 020, 029, 070, 215
100A>G	Ile34Val	40	100%	002, 005, 011, 014, 015, 020, 029, 070, 215
112T>C	Tyr38His	40	100%	002, 005, 011, 014, 015, 020, 029, 070, 215
161A>G	Lys54Arg	40	100%	002, 005, 011, 014, 015, 020, 029, 070, 215
199A>G	Ser67Gly	3	8%	029
270A>G	Ile90Met	1	3%	015
409G>A	Val137Ile	40	100%	002, 005, 011, 014, 015, 020, 029, 070, 215
458C>T	Ser153Phe	40	100%	002, 005, 011, 014, 015, 020, 029, 070, 215
508G>A	Ala170Thr	40	100%	002, 005, 011, 014, 015, 020, 029, 070, 215
572G>A	Arg191Lys	40	100%	002, 005, 011, 014, 015, 020, 029, 070, 215
580T>C	Ser194Pro	40	100%	002, 005, 011, 014, 015, 020, 029, 070, 215

Table 4.4 displays the *cdtR* SNPs and their corresponding altered amino acid for those RAPID isolates which contained a partial ghost CDT gene locus. A total of 11 SNPs which lead to an amino acid change were identified, when compared to R20291 WT. The number of samples along with the calculated frequency percentage, and the identified ribotypes in which that SNP is observed are also specified. Nine of the eleven SNPs were detected in 100% of the ribotypes tested for including 002, 005, 011, 014, 015, 020, 029, 070 and 215. The tenth SNP was observed in 8% of ribotype 029 strains and the eleventh SNP was seen in 3% of ribotype 015 strains. The ribotyping was not completed on the third group of RAPID samples and so certain SNPs were not associated with a specific ribotype and are not included. The frequency of the SNPs as a percentage is also shown graphically in Figure 4.12.

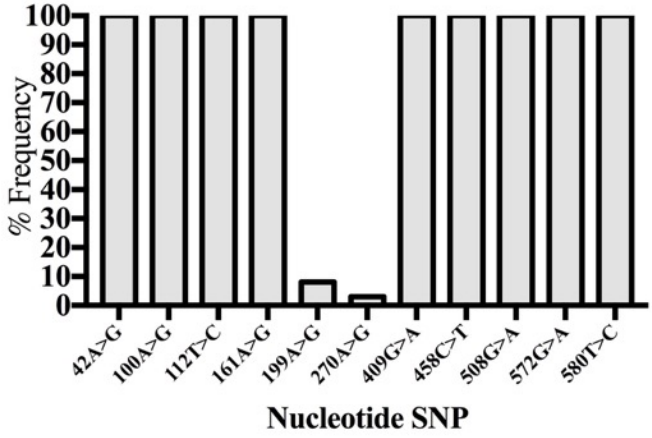


Figure 4.12 RAPID *Clostridium difficile* Isolates Ghost *cdtR* vs. R20291 Wildtype Single Nucleotide Polymorphism Frequency

Graph to show single nucleotide polymorphism (SNP) frequency as a percentage for the RAPID full CDT gene locus isolates when compared to the R20291 wildtype (WT) strain.

The amino acid substitution for each corresponding SNP when compared to the R20291 WT strain is illustrated in Figure 4.13, where CdtR is labelled with the appropriate protein domains. The frequency of the associated SNP is also demonstrated as a percentage.

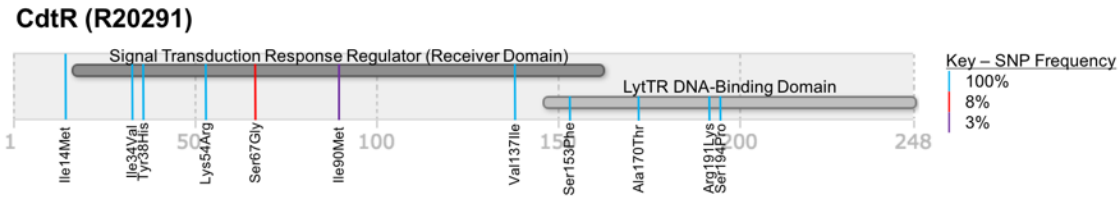


Figure 4.13 RAPID *Clostridium difficile* Isolates Ghost *cdtR* vs. R20291 Wildtype Amino Acid Substitution Positions

Figure to illustrate the amino acid substitution positions and associated single nucleotide polymorphism (SNP) frequency as a percentage for the RAPID ghost *cdtR* isolates when compared to the R20291 wildtype (WT) strain.

When the *cdtR* of the RAPID isolates, which contained a partial ghost CDT gene locus, were compared to 630 WT, a total of 2 SNPs which lead to an amino acid change were identified. Table 4.5 shows the *cdtR* SNPs and their corresponding amino acid change. The number of samples along with the calculated frequency percentage, and the identified ribotypes in which that SNP is observed are also detailed. The tenth and eleventh SNP seen when compared to the R20291 WT (8% of ribotype 029 strains and 3% of ribotype 015 strains, respectively) were also detected when compared to the 630 WT. No further SNPs were identified.

Table 4.5 RAPID *Clostridium difficile* Isolates Ghost *cdtR* vs. 630 Wildtype Single Nucleotide Polymorphism Analysis

***cdtR* SNP**	**Amino Acid Change**	**Number of Samples (Out of 40)**	**Frequency (%)**	**Ribotypes**
199A>G	Ser67Gly	3	8%	029
270A>G	Ile90Met	1	3%	015

The amino acid substitution for the two corresponding SNPs when compared to the 630 WT strain are illustrated in Figure 4.14, where CdtR is labelled with

the appropriate protein domains. The frequency of the associated SNP is also demonstrated as a percentage.

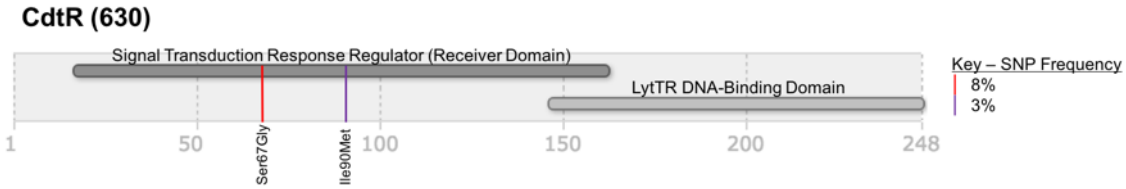


Figure 4.14 RAPID *Clostridium difficile* Isolates Ghost *cdtR* vs. 630 Wildtype Amino Acid Substitution Positions

Figure to illustrate the two amino acid substitution positions and associated single nucleotide polymorphism (SNP) frequency as a percentage for the RAPID ghost *cdtR* isolates when compared to the 630 wildtype (WT) strain.

4.2.3. SPORULATION RATES

4.2.3.1. CLOSTRON CONSTRUCTION

At the beginning of this study, the *C. difficile* 630Δ*erm*::CT26350 ClosTron mutant strain was generated by retargeting the group II intron using a specially designed algorithm (Chapter 2.7.3.). This putative spore envelope protein gene was initially identified using a reverse genetics approach; the methodical identification and knock-out of genes that are expected to play a role in *C. difficile* sporulation, built on sequence homology data of other spore-forming bacteria such as *B. subtilis*. Gene 26350 was disrupted by a sense orientation insert between positions 522bp and 523bp (as determined by the open reading frame [ORF]).

As previously described in Chapter 2.8.3.2., correct ClosTron insertion was established by junction PCR (Figure 4.15) and flanking PCR (data not shown). One erythromycin resistant clone from the five positive colonies observed for each screening PCR was confirmed by Sanger DNA sequencing.

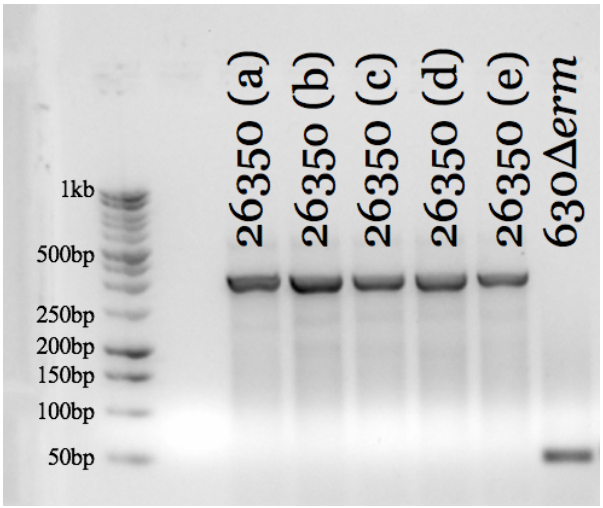


Figure 4.15 *Clostridium difficile* 630Δ*erm*::CT26350 ClosTron

A 1% agarose electrophoresis gel to illustrate the junction PCR used to confirm the erythromycin resistant *C. difficile* 630Δ*erm*::CT26350 ClosTron mutant strain. The ClosTron produced a DNA fragment of 286bp which was observed in all five colonies screened. The negative gel is shown to highlight the lighter bands.

4.2.3.2. CLOSTRON SPORULATION ANALYSIS

The confirmed *C. difficile* 630Δ*erm*::CT26350 ClosTron mutant strain was initially verified through a screening sporulation assay to assess phenotypic characteristics. To ensure that any observed phenotypes were not a result of a growth deficiency, ClosTron growth was compared to the 630Δ*erm* strain over a 24-hour period at OD_{600} (Figure 4.16). The phenotypic growth of 630Δ*erm*::CT26350 was observed to be indistinguishable from the 630Δ*erm* strain. A Paired T-Test demonstrated that there was no significant difference in growth between the two strains, and therefore the subsequent screening sporulation assay may be interpreted by excluding growth differences.

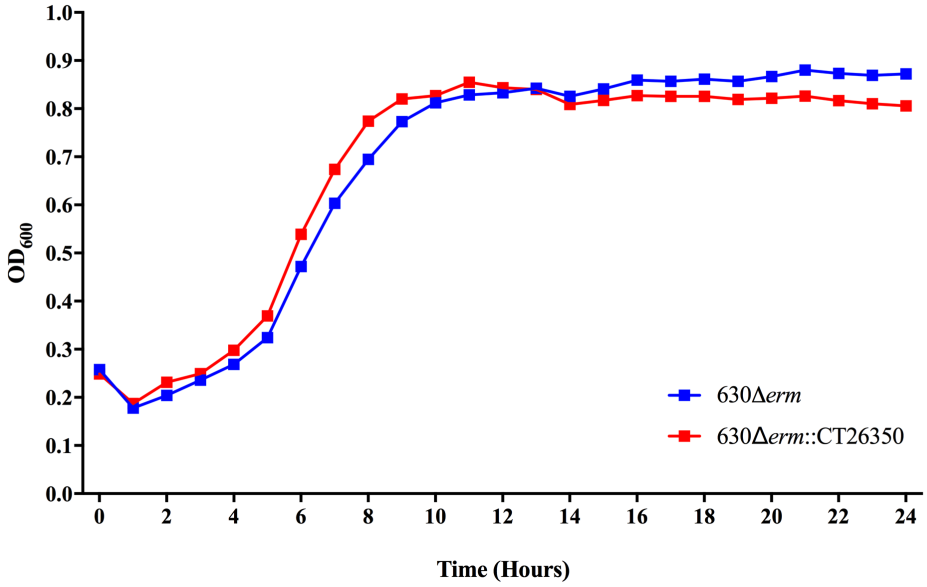


Figure 4.16 Growth Curve to Compare the 630Δ*erm*::CT26350 ClosTron and 630Δ*erm* Strain

Growth curve to show the *C. difficile* 630Δ*erm*::CT26350 ClosTron mutant strain growth compared to the 630Δ*erm* strain over a 24-hour period at OD_{600}. The phenotypic growth of 630Δ*erm*::CT26350 was observed to be indistinguishable from the 630Δ*erm* strain.

The *C. difficile* 630Δ*erm*::CT26350 ClosTron mutant strain was then compared to 630Δ*erm* and 630Δ*erm*Δ*spo0A* control strains in a screening sporulation assay, in which samples were only taken at the 120-hour time point and the heat-treated colony forming units per ml (cfu/ml) measured (Figure 4.17). 630Δ*erm* demonstrated a cfu/ml of $1x10^7$. In contrast, the 630Δ*erm*::CT26350 ClosTron mutant showed almost a 2-log decrease. The cfu/ml of the *spo0A* knock-out mutant, 630Δ*erm*Δ*spo0A*, was undetectable (above the detection limit of 50 cfu/ml). A significant difference in heat-treated cfu/ml at 120 hours was demonstrated between 630Δ*erm* and the 630Δ*erm*::CT26350 ClosTron ($p = 0.0247$), as well as 630Δ*erm* and 630Δ*erm*Δ*spo0A* ($p = 0.0216$). However, there was no significant difference between 630Δ*erm*Δ*spo0A* and the 630Δ*erm*::CT26350 ClosTron.

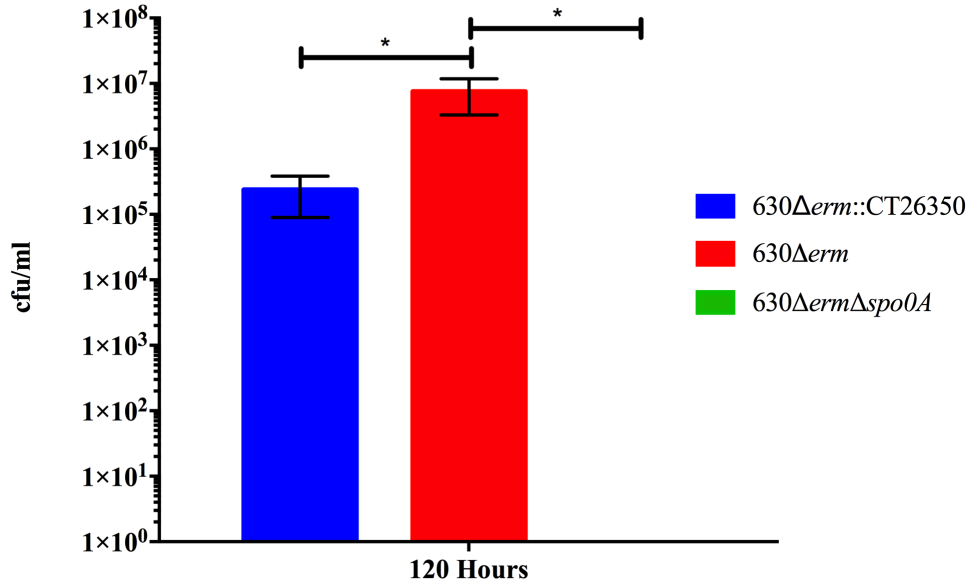


Figure 4.17 Screening Sporulation Assay to Compare the Heat-Treated 630Δ*erm*::CT26350 ClosTron, 630Δ*erm* Strain and 630Δ*erm*Δ*spo0A* Negative Control After 120 Hours

Bar chart to show the *C. difficile* 630Δ*erm*::CT26350 ClosTron mutant strain sporulation heat-treated colony forming units per ml (cfu/ml) compared to the 630Δ*erm* and 630Δ*erm*Δ*spo0A* control strains after 120 hours. Average of three biologically independent replicates with error bars displaying the mean with standard deviation (SD). 630Δ*erm* demonstrated a cfu/ml of $1x10^7$. In contrast, the 630Δ*erm*::CT26350 ClosTron mutant showed almost a 2-log decrease. The cfu/ml of the *spo0A* knock-out mutant, 630Δ*erm*Δ*spo0A*, was undetectable. A significant difference in cfu/ml between 630Δ*erm* and the 630Δ*erm*::CT26350 ClosTron ($p = 0.0247$), as well as 630Δ*erm* and 630Δ*erm*Δ*spo0A* ($p = 0.0216$) was demonstrated. Key: * $p = \leq 0.05$. The detection limit for colony counts was 50 cfu/ml.

4.2.3.3. RAPID ISOLATE SPORULATION ANALYSIS

The *C. difficile* RAPID isolate sporulation assays were conducted by a team of post-doctorate researchers and PhD students at the SBRC. I was allocated twelve samples, which were compared to the 630Δ*erm* and 630Δ*erm*Δ*spo0A* control strains. The 630Δ*erm*::CT26350 ClosTron mutant strain was also tested alongside the control strains and four RAPID isolates. Samples were taken at 0, 24, 48, 72, 96 and 120-hour time points, with the heat-treated colony forming units per ml (cfu/ml) measured at all time points (Figure 4.19, Figure 4.22 and Figure 4.25), and the non-heat-treated cfu/ml measured at 0 and 120-hour time points (Figure 4.18, Figure 4.21 and Figure 4.24). The 120-

hour heat-treated cfu/ml were also compared to 120-hour heat-treated spores/ml (Figure 4.20, Figure 4.23 and Figure 4.26).

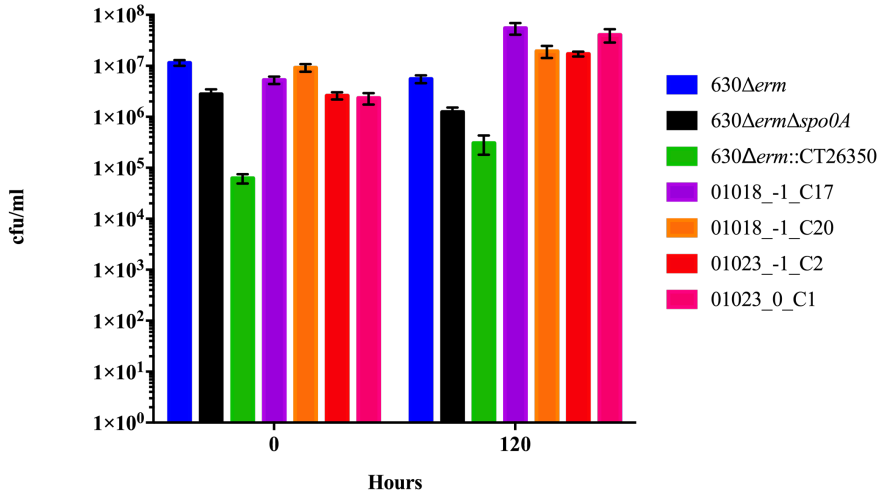


Figure 4.18 Sporulation Assay to Compare the 630Δ*erm* Strain, 630Δ*erm*Δ*spo0A* Strain, 630Δ*erm*::CT26350 ClosTron and 01018_-1_C17, 01018_-1_C20, 01023_-1_C2 and 01023_0_C1 RAPID Isolates - Non-Heat-Treated cfu/ml at 0 and 120 Hours

The 630Δ*erm*::CT26350 appeared to exhibit a lower cfu/ml when visually compared to the 01018_-1_C17, 01018_-1_C20, 01023_-1_C2 and 01023_0_C1 RAPID samples, and the two control strains at the non-heat-treated 0-hour time point. Average of three biologically independent replicates with error bars displaying the mean with standard deviation (SD). Key: -1; Diagnostic sample, 0; Sample after successful metronidazole or vancomycin treatment, C; Colony number picked. The detection limit for colony counts was 50 cfu/ml.

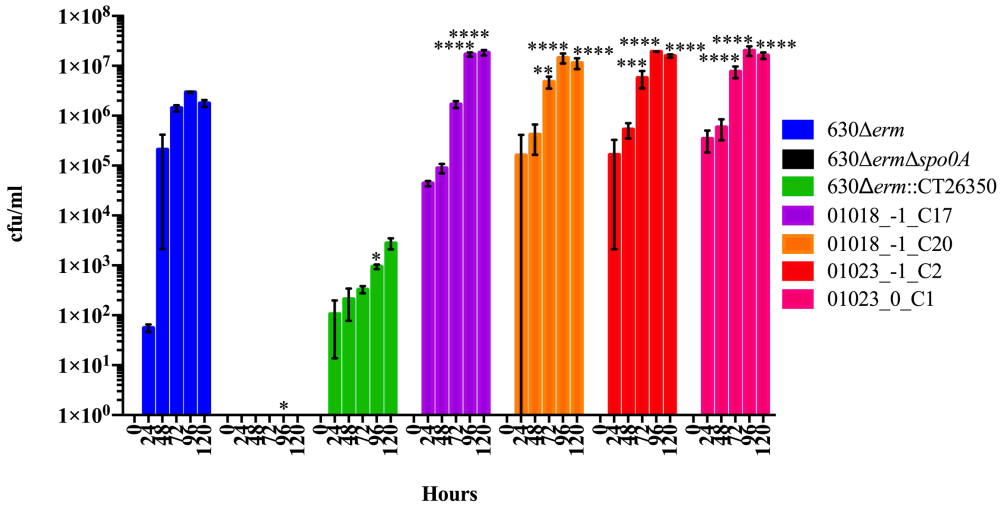


Figure 4.19 Sporulation Assay to Compare the 630Δ*erm* Strain, 630Δ*erm*Δ*spo0A* Strain, 630Δ*erm*::CT26350 ClosTron and 01018_-1_C17, 01018_-1_C20, 01023_-1_C2 and 01023_0_C1 RAPID Isolates - Heat-Treated cfu/ml at 24, 48, 72, 96 and 120 Hours

Four RAPID samples, the 630Δ*erm*::CT26350 ClosTron mutant strain and the 630Δ*erm*Δ*spo0A* negative control strain were compared to the 630Δ*erm* positive control strain for all heat-treated time points. Average of three biologically independent replicates with error bars displaying the mean with standard deviation (SD). The cfu/ml of the *spo0A* knock-out mutant, 630Δ*erm*Δ*spo0A*, was undetectable at all time points. The 630Δ*erm*::CT26350 ClosTron mutant demonstrated a visibly comparable cfu/ml to 630Δ*erm* at 0 and 24 hour time points, however, the cfu/ml count between 48 and 120 hours was notably decreased. The highest cfu/ml count for 630Δ*erm*::CT26350 was $1x10^{3.5}$ at 120 hours, which is a 2.5-log difference from that of the 630Δ*erm* cfu/ml count of $1x10^{6}$. In contrast, the 01018_-1_C17, 01018_-1_C20, 01023_-1_C2 and 01023_0_C1 RAPID isolates appeared more consistent with a typical sporulation pattern, although all samples appeared to reach slightly higher cfu/ml counts at the 96 and 120-hour time points, when compared to 630Δ*erm*. Key: -1; Diagnostic sample, 0; Sample after successful metronidazole or vancomycin treatment, C; Colony number picked, * $p = \leq 0.05$, ** $p = \leq 0.01$, *** $p = \leq 0.001$ and **** $p = \leq 0.0001$. The detection limit for colony counts was 50 cfu/ml.

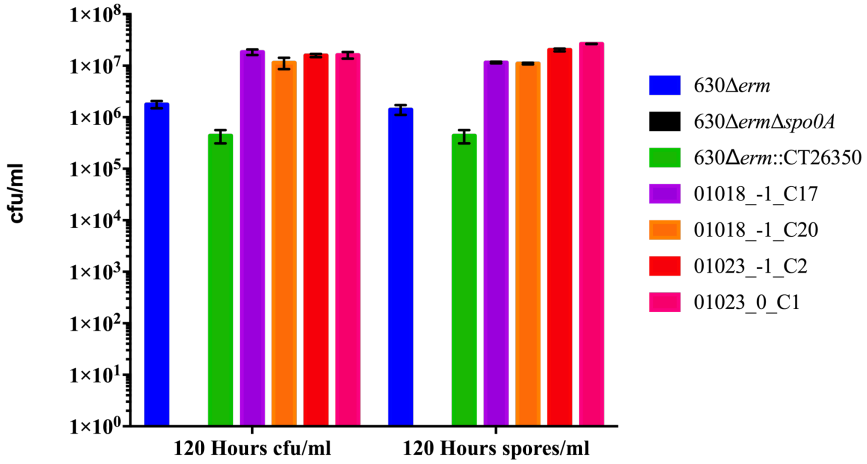


Figure 4.20 Sporulation Assay to Compare the 630Δ*erm* Strain, 630Δ*erm*Δ*spo0A* Strain, 630Δ*erm*::CT26350 ClosTron and 01018_-1_C17, 01018_-1_C20, 01023_-1_C2 and 01023_0_C1 RAPID Isolates - Heat-Treated cfu/ml at 120 Hours vs. Heat-Treated spores/ml at 120 Hours

The 120-hour heat-treated cfu/ml were compared to the 120-hour heat-treated spores/ml for each RAPID patient isolate. Average of three biologically independent replicates with error bars displaying the mean with standard deviation (SD). Visually, all samples appeared comparable with each other, however, a statistically significant difference was observed for 01018_-1_C17 ($p = <0.0001$), 01023_-1_C2 ($p = 0.0037$) and 01023_0_C1 ($p = <0.0001$). Key: -1; Diagnostic sample, 0; Sample after successful metronidazole or vancomycin treatment, C; Colony number picked. The detection limit for colony counts was 50 cfu/ml.

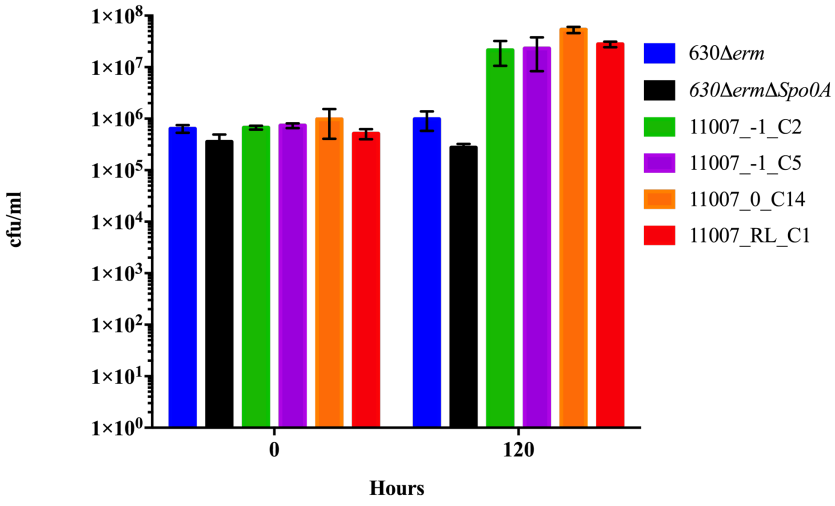


Figure 4.21 Sporulation Assay to Compare the 630Δ*erm* Strain, 630Δ*erm*Δ*spo0A* Strain and 11007_-1_C2, 11007_-1_C5, 11007_0_C14 and 11007_RL_C1 RAPID Isolates - Non-Heat-Treated cfu/ml at 0 and 120 Hours

Figure 4.21 Sporulation Assay to Compare the 630Δ*erm* Strain, 630Δ*erm*Δ*spo0A* Strain and 11007_-1_C2, 11007_-1_C5, 11007_0_C14 and 11007_RL_C1 RAPID Isolates - Non-Heat-Treated cfu/ml at 0 and 120 Hours Legend - The 11007_-1_C2, 11007_-1_C5, 11007_0_C14 and 11007_RL_C1 RAPID samples, and the two control strains appeared visually comparable to each other at the non-heat-treated 0-hour time point. Average of three biologically independent replicates with error bars displaying the mean with standard deviation (SD). Key: -1; Diagnostic sample, 0; Sample after successful metronidazole or vancomycin treatment, RL; Relapse sample, C; Colony number picked. The detection limit for colony counts was 50 cfu/ml.

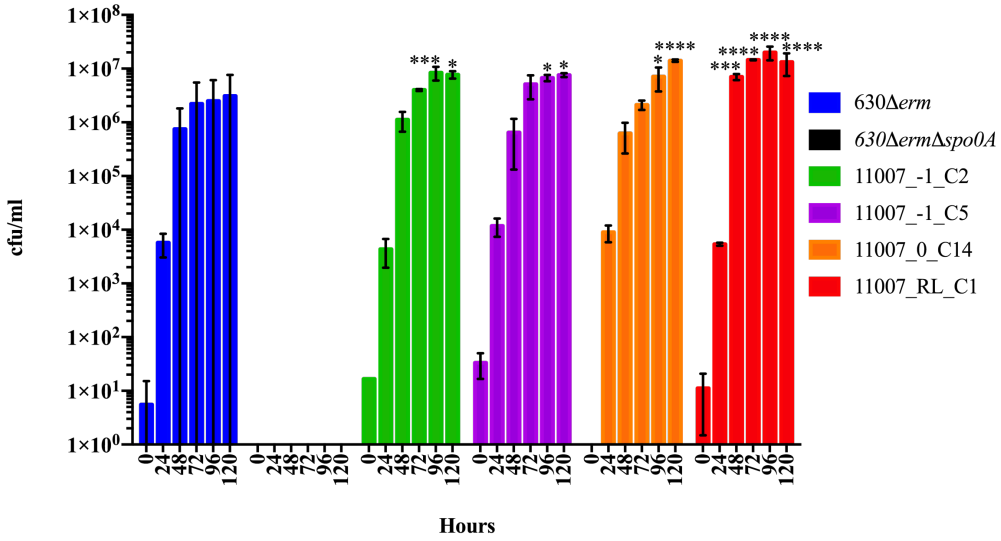


Figure 4.22 Sporulation Assay to Compare the 630Δ*erm* Strain, 630Δ*erm*Δ*spo0A* Strain and 11007_-1_C2, 11007_-1_C5, 11007_0_C14 and 11007_RL_C1 RAPID Isolates - Heat-Treated cfu/ml at 24, 48, 72, 96 and 120 Hours

Four RAPID samples and the 630Δ*erm*Δ*spo0A* negative control strain were compared to the 630Δ*erm* positive control strain for all heat-treated time points. Average of three biologically independent replicates with error bars displaying the mean with standard deviation (SD). The cfu/ml of the *spo0A* knock-out mutant, 630Δ*erm*Δ*spo0A*, was undetectable at all time points. In contrast, the 11007_-1_C2, 11007_-1_C5, 11007_0_C14 and 11007_RL_C1 RAPID isolates appeared more consistent with a typical sporulation pattern, although all samples appeared to reach slightly higher cfu/ml counts at the 96 and 120-hour time points, when compared to 630Δ*erm*. Key: -1; Diagnostic sample, 0; Sample after successful metronidazole or vancomycin treatment, RL; Relapse sample, C; Colony number picked, * $p = \leq 0.05$, *** $p = \leq 0.001$ and **** $p = \leq 0.0001$. The detection limit for colony counts was 50 cfu/ml.

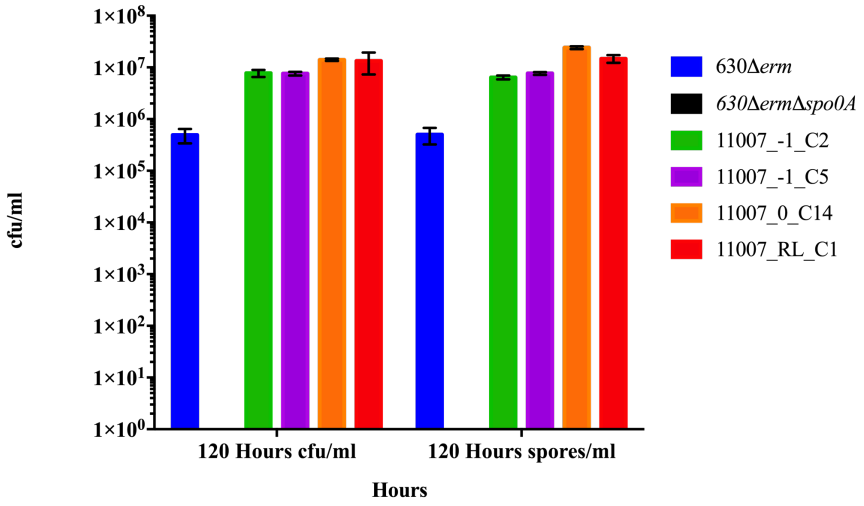


Figure 4.23 Sporulation Assay to Compare the 630Δ*erm* Strain, 630Δ*erm*Δ*spo0A* Strain and 11007_-1_C2, 11007_-1_C5, 11007_0_C14 and 11007_RL_C1 RAPID Isolates - Heat-Treated cfu/ml at 120 Hours vs. Heat-Treated spores/ml at 120 Hours

The 11007_-1_C2, 11007_-1_C5, 11007_0_C14 and 11007_RL_C1 RAPID samples, and the two control strains appeared visually comparable to each other at the non-heat-treated 0-hour time point. Average of three biologically independent replicates with error bars displaying the mean with standard deviation (SD). Key: -1; Diagnostic sample, 0; Sample after successful metronidazole or vancomycin treatment, RL; Relapse sample, C; Colony number picked. The detection limit for colony counts was 50 cfu/ml.

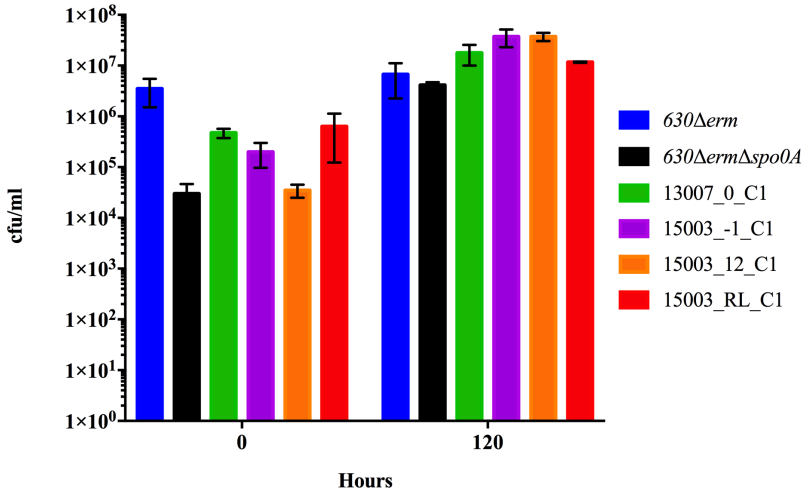


Figure 4.24 Sporulation Assay to Compare the 630Δ*erm* Strain, 630Δ*erm*Δ*spo0A* Strain and 13007_0_C1, 15003_-1_C1, 15003_12_C1 and 15003_RL_C1 RAPID Isolates - Non-Heat-Treated cfu/ml at 0 and 120 Hours

Figure 4.24 Sporulation Assay to Compare the 630Δ*erm* Strain, 630Δ*erm*Δ*spo0A* Strain and 13007_0_C1, 15003_-1_C1, 15003_12_C1 and 15003_RL_C1 RAPID Isolates - Non-Heat-Treated cfu/ml at 0 and 120 Hours Legend - The RAPID isolate 15003_12_C1 appeared to exhibit a lower cfu/ml when visually compared to the 13007_0_C1, 15003_-1_C1 and 15003_RL_C1 RAPID samples, and the two control strains at the non-heat-treated 0-hour time point. Average of three biologically independent replicates with error bars displaying the mean with standard deviation (SD). Key: -1; Diagnostic sample, 0; Sample after successful metronidazole or vancomycin treatment, 12; Week 12 check-up sample, RL; Relapse sample, C; Colony number picked. The detection limit for colony counts was 50 cfu/ml.

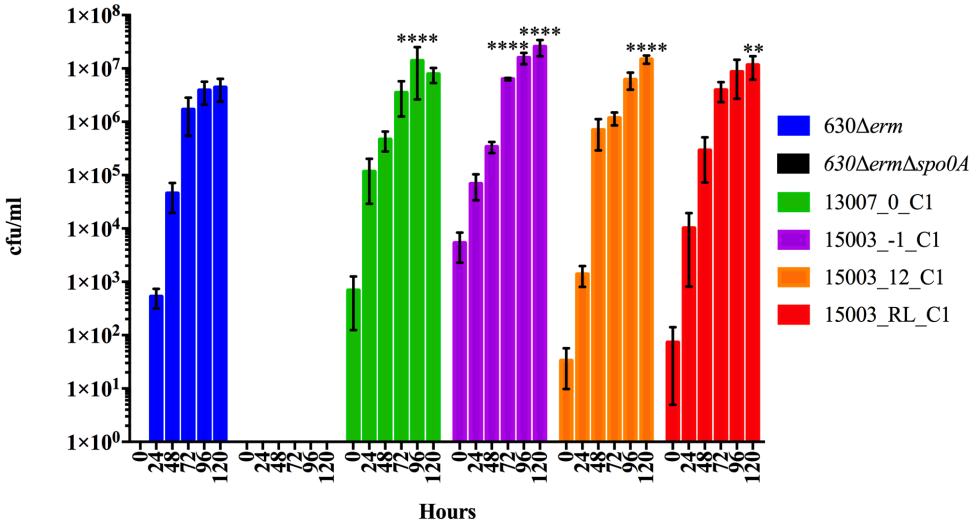


Figure 4.25 Sporulation Assay to Compare the 630Δ*erm* Strain, 630Δ*erm*Δ*spo0A* Strain and 13007_0_C1, 15003_-1_C1, 15003_12_C1 and 15003_RL_C1 RAPID Isolates - Heat-Treated cfu/ml at 24, 48, 72, 96 and 120 Hours

Four RAPID samples and the 630Δ*erm*Δ*spo0A* negative control strain were compared to the 630Δ*erm* positive control strain for all heat-treated time points. Average of three biologically independent replicates with error bars displaying the mean with standard deviation (SD). The cfu/ml of the *spo0A* knock-out mutant, 630Δ*erm*Δ*spo0A*, was undetectable at all time points. In contrast, the 13007_0_C1, 15003_-1_C1, 15003_12_C1 and 15003_RL_C1 RAPID isolates appeared more consistent with a typical sporulation pattern, although all samples appeared to reach slightly higher cfu/ml counts at the 96 or 120-hour time points, when compared to 630Δ*erm*. Key: -1; Diagnostic sample, 0; Sample after successful metronidazole or vancomycin treatment, 12; Week 12 check-up sample, RL; Relapse sample, C; Colony number picked, ** $p = \leq 0.01$ and **** $p = \leq 0.0001$. The detection limit for colony counts was 50 cfu/ml.

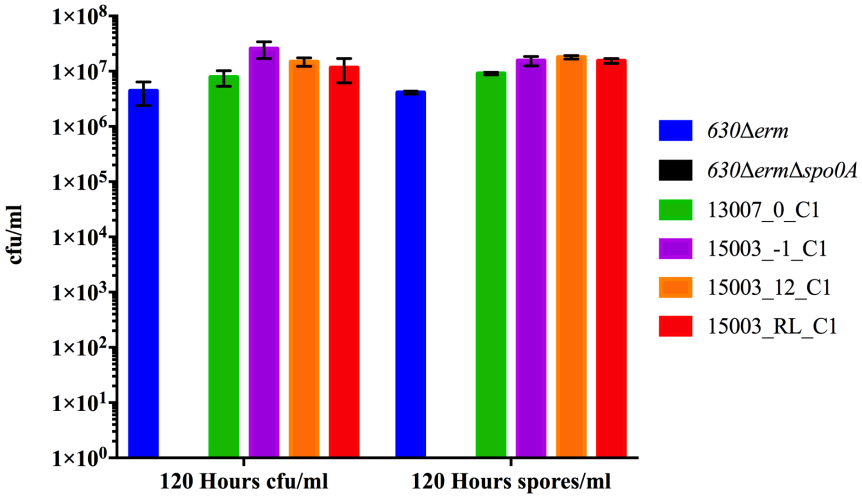


Figure 4.26 Sporulation Assay to Compare the 630Δ*erm* Strain, 630Δ*erm*Δ*spo0A* Strain and 13007_0_C1, 15003_-1_C1, 15003_12_C1 and 15003_RL_C1 RAPID Isolates - Heat-Treated cfu/ml at 120 Hours vs. Heat-Treated spores/ml at 120 Hours

The 120-hour heat-treated cfu/ml were compared to the 120-hour heat-treated spores/ml for each RAPID patient isolate. Average of three biologically independent replicates with error bars displaying the mean with standard deviation (SD). Visually, all samples appeared comparable with each other, however, a significant difference was observed for 15003_-1_C1 ($p = 0.0006$). Key: -1; Diagnostic sample, 0; Sample after successful metronidazole or vancomycin treatment, 12; Week 12 check-up sample, RL; Relapse sample, C; Colony number picked. The detection limit for colony counts was 50 cfu/ml.

Firstly, the non-heat-treated 0-hour time point was visually compared for each sample. The 630Δ*erm*::CT26350 ClosTron mutant and RAPID isolate 15003_12_C1 appeared to exhibit a lower cfu/ml when compared to the other samples. However, there was no statistically significant difference identified for the twelve RAPID samples, the 630Δ*erm*::CT26350 ClosTron mutant strain and the two control strains. It was essential to ensure there was no significant difference at the non-heat-treated 0-hour time point so that all samples were commencing at comparable growth.

All twelve RAPID samples, the 630Δ*erm*::CT26350 ClosTron mutant strain and the 630Δ*erm*Δ*spo0A* negative control strain were compared to the 630Δ*erm* positive control strain for all heat-treated time points. The cfu/ml of the *spo0A* knock-out mutant, 630Δ*erm*Δ*spo0A*, was undetectable at all time

points (above the detection limit of 50 cfu/ml). The 630Δ*erm*::CT26350 ClosTron mutant demonstrated a visibly comparable cfu/ml to 630Δ*erm* at 0 and 24 hour time points, however, the cfu/ml count between 48 and 120 hours was notably decreased. The highest cfu/ml count for 630Δ*erm*::CT26350 was $1x10^{3.5}$ at 120 hours, which is a 2.5-log difference from that of the 630Δ*erm* cfu/ml count of $1x10^{6}$. In contrast, the 01018_-1_C17, 01018_-1_C20, 01023_-1_C2, 01023_0_C1, 11007_-1_C2, 11007_-1_C5, 11007_0_C14, 11007_RL_C1, 13007_0_C1, 15003_-1_C1, 15003_12_C1 and 15003_RL_C1 RAPID isolates appeared more consistent with a typical sporulation pattern, although virtually all samples appeared to reach slightly higher cfu/ml counts at the 96 and 120-hour time points, when compared to 630Δ*erm*. The statistical P value and significance data is illustrated in Table 4.6.

Table 4.6 Statistical Analysis to Compare the RAPID Isolates with the 630Δ*erm* in the Sporulation Assay

Strain Comparison	P Value	Significant Difference
Heat-Treated cfu/ml at 0-Hour Measurement		
630Δ*erm* vs. 630Δ*erm*Δ*spo0A*	p = 0.9999	No
630Δ*erm* vs. 630Δ*erm*::CT26350 ClosTron	p = 0.9999	No
630Δ*erm* vs. 01018_-1_C17	p = 0.9999	No
630Δ*erm* vs. 01018_-1_C20	p = 0.9999	No
630Δ*erm* vs. 01023_-1_C2	p = 0.9999	No
630Δ*erm* vs. 01023_0_C1	p = 0.9999	No
630Δ*erm* vs. 11007_-1_C2	p = 0.9999	No
630Δ*erm* vs. 11007_-1_C5	p = 0.9999	No
630Δ*erm* vs. 11007_0_C14	p = 0.9999	No
630Δ*erm* vs. 11007_RL_C1	p = 0.9999	No
630Δ*erm* vs. 13007_0_C1	p = 0.9999	No
630Δ*erm* vs. 15003_-1_C1	p = 0.9999	No
630Δ*erm* vs. 15003_12_C1	p = 0.9999	No
630Δ*erm* vs. 15003_RL_C1	p = 0.9999	No
Heat-Treated cfu/ml at 24-Hour Measurement		
630Δ*erm* vs. 630Δ*erm*Δ*spo0A*	p = 0.9999	No
630Δ*erm* vs. 630Δ*erm*::CT26350 ClosTron	p = 0.9999	No
630Δ*erm* vs. 01018_-1_C17	p = 0.9999	No
630Δ*erm* vs. 01018_-1_C20	p = 0.9997	No
630Δ*erm* vs. 01023_-1_C2	p = 0.9997	No
630Δ*erm* vs. 01023_0_C1	p = 0.9979	No
630Δ*erm* vs. 11007_-1_C2	p = 0.9999	No
630Δ*erm* vs. 11007_-1_C5	p = 0.9999	No

630Δ*erm* vs. 11007_0_C14	p = 0.9999	No
630Δ*erm* vs. 11007_RL_C1	p = 0.9999	No
630Δ*erm* vs. 13007_0_C1	p = 0.9999	No
630Δ*erm* vs. 15003_-1_C1	p = 0.9999	No
630Δ*erm* vs. 15003_12_C1	p = 0.9999	No
630Δ*erm* vs. 15003_RL_C1	p = 0.9999	No
Heat-Treated cfu/ml at 48-Hour Measurement		
630Δ*erm* vs. 630Δ*erm*Δ*spo0A*	p = 0.9997	No
630Δ*erm* vs. 630Δ*erm*::CT26350 ClosTron	p = 0.9997	No
630Δ*erm* vs. 01018_-1_C17	p = 0.9998	No
630Δ*erm* vs. 01018_-1_C20	p = 0.9997	No
630Δ*erm* vs. 01023_-1_C2	p = 0.9982	No
630Δ*erm* vs. 01023_0_C1	p = 0.9974	No
630Δ*erm* vs. 11007_-1_C2	p = 0.9988	No
630Δ*erm* vs. 11007_-1_C5	p = 0.9999	No
630Δ*erm* vs. 11007_0_C14	p = 0.9999	No
630Δ*erm* vs. 11007_RL_C1	p = 0.0004	Yes
630Δ*erm* vs. 13007_0_C1	p = 0.9997	No
630Δ*erm* vs. 15003_-1_C1	p = 0.9998	No
630Δ*erm* vs. 15003_12_C1	p = 0.9973	No
630Δ*erm* vs. 15003_RL_C1	p = 0.9998	No
Heat-Treated cfu/ml at 72-Hour Measurement		
630Δ*erm* vs. 630Δ*erm*Δ*spo0A*	p = 0.4614	No
630Δ*erm* vs. 630Δ*erm*::CT26350 ClosTron	p = 0.5024	No
630Δ*erm* vs. 01018_-1_C17	p = 0.9996	No
630Δ*erm* vs. 01018_-1_C20	p = 0.0038	Yes
630Δ*erm* vs. 01023_-1_C2	p = 0.0001	Yes
630Δ*erm* vs. 01023_0_C1	p = 0.0001	Yes
630Δ*erm* vs. 11007_-1_C2	p = 0.6174	No
630Δ*erm* vs. 11007_-1_C5	p = 0.1885	No
630Δ*erm* vs. 11007_0_C14	p = 0.9999	No
630Δ*erm* vs. 11007_RL_C1	p = 0.0001	Yes
630Δ*erm* vs. 13007_0_C1	p = 0.8417	No
630Δ*erm* vs. 15003_-1_C1	p = 0.1024	No
630Δ*erm* vs. 15003_12_C1	p = 0.9408	No
630Δ*erm* vs. 15003_RL_C1	p = 0.7192	No
Heat-Treated cfu/ml at 96-Hour Measurement		
630Δ*erm* vs. 630Δ*erm*Δ*spo0A*	p = 0.0136	Yes
630Δ*erm* vs. 630Δ*erm*::CT26350 ClosTron	p = 0.0189	Yes
630Δ*erm* vs. 01018_-1_C17	p = 0.0001	Yes
630Δ*erm* vs. 01018_-1_C20	p = 0.0001	Yes
630Δ*erm* vs. 01023_-1_C2	p = 0.0001	Yes
630Δ*erm* vs. 01023_0_C1	p = 0.0001	Yes
630Δ*erm* vs. 11007_-1_C2	p = 0.0009	Yes
630Δ*erm* vs. 11007_-1_C5	p = 0.0242	Yes
630Δ*erm* vs. 11007_0_C14	p = 0.0117	Yes
630Δ*erm* vs. 11007_RL_C1	p = 0.0001	Yes
630Δ*erm* vs. 13007_0_C1	p = 0.0001	Yes
630Δ*erm* vs. 15003_-1_C1	p = 0.0001	Yes
630Δ*erm* vs. 15003_12_C1	p = 0.6910	No
630Δ*erm* vs. 15003_RL_C1	p = 0.1021	No
Heat-Treated cfu/ml at 120-Hour Measurement		
630Δ*erm* vs. 630Δ*erm*Δ*spo0A*	p = 0.2488	No
630Δ*erm* vs. 630Δ*erm*::CT26350 ClosTron	p = 0.3188	No

630Δ*erm* vs. 01018_-1_C17	p = 0.0001	Yes
630Δ*erm* vs. 01018_-1_C20	p = 0.0001	Yes
630Δ*erm* vs. 01023_-1_C2	p = 0.0001	Yes
630Δ*erm* vs. 01023_0_C1	p = 0.0001	Yes
630Δ*erm* vs. 11007_-1_C2	p = 0.0120	Yes
630Δ*erm* vs. 11007_-1_C5	p = 0.0147	Yes
630Δ*erm* vs. 11007_0_C14	p = 0.0001	Yes
630Δ*erm* vs. 11007_RL_C1	p = 0.0001	Yes
630Δ*erm* vs. 13007_0_C1	p = 0.3315	No
630Δ*erm* vs. 15003_-1_C1	p = 0.0001	Yes
630Δ*erm* vs. 15003_12_C1	p = 0.0001	Yes
630Δ*erm* vs. 15003_RL_C1	p = 0.0046	Yes

Those RAPID samples which were isolated from the same patient at different weeks were also compared. The statistical P value and significance data for these comparisons are illustrated in Table 4.7, Table 4.8 and Table 4.9.

Table 4.7 Statistical Analysis to Compare the 01023 RAPID Isolates in the Sporulation Assay

Strain Comparison	P Value	Significant Difference
Heat-Treated cfu/ml at 0-Hour Measurement		
01023_-1_C2 vs. 01023_0_C1	p = >0.9999	No
Heat-Treated cfu/ml at 24-Hour Measurement		
01023_-1_C2 vs. 01023_0_C1	p = >0.9999	No
Heat-Treated cfu/ml at 48-Hour Measurement		
01023_-1_C2 vs. 01023_0_C1	p = >0.9999	No
Heat-Treated cfu/ml at 72-Hour Measurement		
01023_-1_C2 vs. 01023_0_C1	p = 0.5401	No
Heat-Treated cfu/ml at 96-Hour Measurement		
01023_-1_C2 vs. 01023_0_C1	p = 0.9673	No
Heat-Treated cfu/ml at 120-Hour Measurement		
01023_-1_C2 vs. 01023_0_C1	p = 0.9999	No

Table 4.8 Statistical Analysis to Compare the 11007 RAPID Isolates in the Sporulation Assay

Strain Comparison	P Value	Significant Difference
Heat-Treated cfu/ml at 0-Hour Measurement		
11007_-1_C2 vs. 11007_0_C14	p = >0.9999	No
11007_-1_C2 vs. 11007_RL_C1	p = >0.9999	No
11007_0_C14 vs. 11007_RL_C1	p = >0.9999	No
Heat-Treated cfu/ml at 24-Hour Measurement		
11007_-1_C2 vs. 11007_0_C14	p = >0.9999	No
11007_-1_C2 vs. 11007_RL_C1	p = >0.9999	No
11007_0_C14 vs. 11007_RL_C1	p = >0.9999	No
Heat-Treated cfu/ml at 48-Hour Measurement		
11007_-1_C2 vs. 11007_0_C14	p = 0.9598	No
11007_-1_C2 vs. 11007_RL_C1	p = 0.0103	Yes
11007_0_C14 vs. 11007_RL_C1	p = 0.0056	Yes
Heat-Treated cfu/ml at 72-Hour Measurement		
11007_-1_C2 vs. 11007_0_C14	p = 0.5622	No
11007_-1_C2 vs. 11007_RL_C1	p = <0.0001	Yes
11007_0_C14 vs. 11007_RL_C1	p = <0.0001	Yes
Heat-Treated cfu/ml at 96-Hour Measurement		
11007_-1_C2 vs. 11007_0_C14	p = 0.7690	No
11007_-1_C2 vs. 11007_RL_C1	p = <0.0001	Yes
11007_0_C14 vs. 11007_RL_C1	p = <0.0001	Yes
Heat-Treated cfu/ml at 120-Hour Measurement		
11007_-1_C2 vs. 11007_0_C14	p = 0.0062	Yes
11007_-1_C2 vs. 11007_RL_C1	p = 0.0155	Yes
11007_0_C14 vs. 11007_RL_C1	p = 0.9123	No

Table 4.9 Statistical Analysis to Compare the 15003 RAPID Isolates in the Sporulation Assay

Strain Comparison	P Value	Significant Difference
Heat-Treated cfu/ml at 0-Hour Measurement		
15003_-1_C1 vs. 15003_12_C1	p = >0.9999	No
15003_-1_C1 vs. 15003_RL_C1	p = >0.9999	No
15003_12_C1 vs. 15003_RL_C1	p = >0.9999	No
Heat-Treated cfu/ml at 24-Hour Measurement		
15003_-1_C1 vs. 15003_12_C1	p = 0.9994	No
15003_-1_C1 vs. 15003_RL_C1	p = 0.9996	No
15003_12_C1 vs. 15003_RL_C1	p = >0.9999	No
Heat-Treated cfu/ml at 48-Hour Measurement		
15003_-1_C1 vs. 15003_12_C1	p = 0.9833	No
15003_-1_C1 vs. 15003_RL_C1	p = 0.9997	No
15003_12_C1 vs. 15003_RL_C1	p = 0.9784	No
Heat-Treated cfu/ml at 72-Hour Measurement		
15003_-1_C1 vs. 15003_12_C1	p = 0.2820	No
15003_-1_C1 vs. 15003_RL_C1	p = 0.4837	No
15003_12_C1 vs. 15003_RL_C1	p = 0.9169	No
Heat-Treated cfu/ml at 96-Hour Measurement		
15003_-1_C1 vs. 15003_12_C1	p = 0.0005	Yes
15003_-1_C1 vs. 15003_RL_C1	p = 0.0061	Yes
15003_12_C1 vs. 15003_RL_C1	p = 0.5060	No
Heat-Treated cfu/ml at 120-Hour Measurement		
15003_-1_C1 vs. 15003_12_C1	p = 0.0001	Yes
15003_-1_C1 vs. 15003_RL_C1	p = <0.0001	Yes
15003_12_C1 vs. 15003_RL_C1	p = 0.2746	No

Lastly, the 120-hour heat-treated cfu/ml were compared to the 120-hour heat-treated spores/ml for each RAPID patient isolate, the 630Δ*erm* and 630Δ*erm*Δ*spo0A* control strains, as well as the 630Δ*erm*::CT26350 ClosTron mutant strain. Visually, all samples appeared comparable with each other, however, 01018_-1_C17, 01023_-1_C2, 01023_0_C1, 11007_0_C14 and 15003_-1_C1 cfu/ml counts were statistically significantly different to the spores/ml detected at 120 hours (Table 4.10).

Table 4.10 Statistical Analysis to Compare the RAPID Isolate Heat-Treated cfu/ml with the Heat-Treated spores/ml at 120 hours in the Sporulation Assay

cfu/ml vs. spores/ml at 120 Hours	P Value	Significant Difference
630Δ*erm*	p = 0.9999	No
630Δ*erm*Δ*spo0A*	p = >0.9999	No
630Δ*erm*::CT26350 ClosTron	p = >0.9999	No
01018_-1_C17	p = <0.0001	Yes
01018_-1_C20	p = 0.9999	No
01023_-1_C2	p = 0.0037	Yes
01023_0_C1	p = <0.0001	Yes
11007_-1_C2	p = 0.9153	No
11007_-1_C5	p = >0.9999	No
11007_0_C14	p = <0.0001	Yes
11007_RL_C1	p = 0.8859	No
13007_0_C1	p = 0.9690	No
15003_-1_C1	p = 0.0006	Yes
15003_12_C1	p = 0.3951	No
15003_RL_C1	p = 0.2222	No

4.2.4. CLINICAL ISOLATE RIFAXIMIN AND FIDAXOMICIN ANTIBIOTIC RESISTANCE

4.2.4.1. RIFAXIMIN AND FIDAXOMICIN ANTIBIOTIC RESISTANCE PLATE ASSAY

Each *C. difficile* RAPID isolate was assessed for rifaximin and fidaxomicin antibiotic resistance. This was to determine whether patient exposure to rifaximin on the clinical trial led to resistance, and additionally, if any co-resistance was observed with fidaxomicin. Initially, a control assay was performed in which the plate antibiotic concentration was varied and tested against strains which were previously identified to be sensitive, intermediate or resistant to rifaximin or fidaxomicin (Kelly *et al.*, 2016; Stevenson *et al.*, 2016); Kuehne *et al*., under review) (Table 4.11 and Table 4.12).

Table 4.11 Rifaximin Control Plate Assay

Strain	4µg/ml	8µg/ml	16µg/ml	32µg/ml
RT002_A7 (Sensitive Strain)	×	×	×	×
RT002_F1 (Intermediate Strain)	✓	✓	✓ (Limited)	×

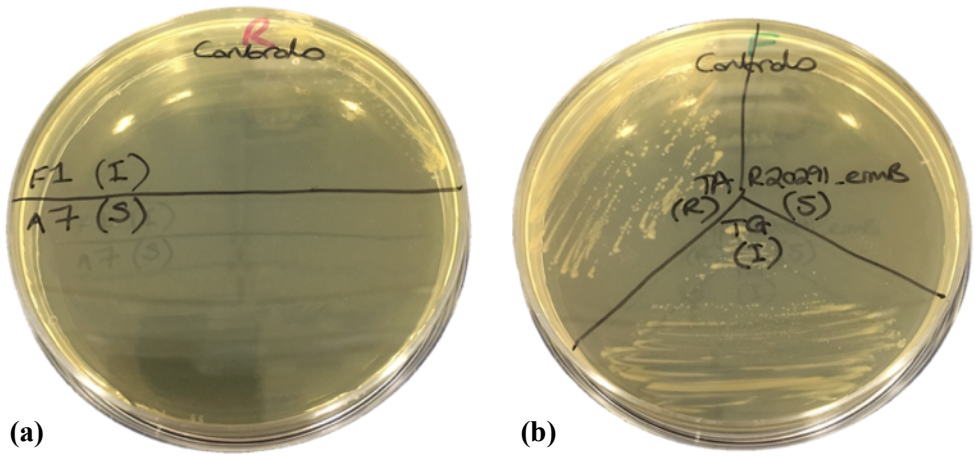
Key: × No Growth (Sensitive), ✓ Growth (Resistant)

Table 4.12 Fidaxomicin Control Plate Assay

Strain	1µg/ml	4µg/ml	8µg/ml	16µg/ml	32µg/ml
R20291_*ermB* (Sensitive Strain)	×	×	×	×	×
R20291_*ermB*_GT (Intermediate Strain)	✓	×	×	×	×
R20291_*ermB*_TG (Intermediate Strain)	✓	✓	×	×	×
R20291_*ermB*_TA (Resistant Strain)	✓	✓	✓	✓	✓

Key: × No Growth (Sensitive), ✓ Growth (Resistant)

Figure 4.27 illustrates the *C. difficile* growth observed for the control strains on an 8µg/ml rifaximin agar plate and a 1µg/ml fidaxomicin agar plate.



(a) **(b)**

Figure 4.27 Rifaximin and Fidaxomicin Control Plate Assay

The figure illustrates the *C. difficile* growth observed for **(a)** RT002_A7 (sensitive) and RT002_F1 (intermediate) control strains on an 8µg/ml rifaximin agar plate, and **(b)** R20291_*ermB* (sensitive), R20291_*ermB*_TG (intermediate) and R20291_*ermB*_TA (resistant) control strains on a 1µg/ml fidaxomicin agar plate.

Primarily, all relapse patient RAPID samples were tested for rifaximin resistance (including intermediate resistance) on an 8µg/ml rifaximin agar plate and for fidaxomicin resistance (including intermediate resistance) on a 1µg/ml fidaxomicin agar plate. There were no fidaxomicin resistant relapse patient RAPID isolates identified (Table 4.13), and so non-relapse patient RAPID samples were not investigated. Seven relapse patient RAPID isolates were shown to be rifaximin resistant (Table 4.14); 01016_12_C1 (Figure 4.28), 12001_RL_C14 (Figure 4.30), 15003_-1_C1, 15003_12_C1 and 15003_RL_C1 (Figure 4.31), 17002_RL2_C1 and 17002_RL3_C1 (Figure 4.32). The non-relapse patient RAPID samples were subsequently tested and one isolate exhibited resistance to rifaximin (Table 4.14); 03008_4_C2 (Figure 4.29). The 12006_12_C1 and 13007_4_C3 RAPID isolate rifaximin resistance data are discussed in Chapter 4.2.5.

Table 4.13 Relapse Patient RAPID Isolates Fidaxomicin Plate Assay

RAPID Isolates	Ribotype	Treatment	1µg/ml Plate Growth
11001_4_C1	029	Placebo	×
11001_RL_C3	029		×
11007_-1_C2	014	Placebo	×
11007_-1_C5	029		×
11007_0_C14	014		×
11007_RL_C1	014		×
12001_0_C5	070	Rifaximin	×
12001_RL_C14	070		×
12004_0_C2	020	Placebo	×
12004_RL_C14	020		×
15003_-1_C1	Not Ribotyped	Placebo	×
15003_12_C1	Not Ribotyped		×
15003_RL_C1	Not Ribotyped		×
17002_-1_C1	Not Ribotyped	Rifaximin	×
17002_0_C1	Not Ribotyped		×
17002_RL1_C1	Not Ribotyped		×
17002_RL2_C1	Not Ribotyped		×
17002_RL3_C1	Not Ribotyped		×
18001_0_C6	020	Rifaximin	×
18001_RL_C5	020		×

Key: × No Growth (Sensitive), ✓ Growth (Resistant), -1; Diagnostic sample, 0; Sample after successful metronidazole or vancomycin treatment, 4; Week 4 check-up sample, 12; Week 12 check-up sample, RL; Relapse sample, C; Colony number picked.

Table 4.14 Relapse and Non-Relapse Patient RAPID Isolates Rifaximin Plate Assay

RAPID Isolates	Ribotype	Treatment	8µg/ml Plate Growth
01006_-1_C10	014	Rifaximin	×
01006_0_C1	014		×
01006_12_C11	014		×
01011_-1_C20	215	Rifaximin	×
01013_-1_C17	002	Rifaximin	×
01013_12_C19	014		×
01015_-1_C1	023	Placebo	×
01015_4_C1	023		×
01016_-1_C1	026	Rifaximin	×
01016_12_C1	026		✓
01017_-1_C3	005	Rifaximin	×
01017_4_C4	005		×
01018_-1_C17	026	Placebo	×
01018_-1_C20	015		×
01023_-1_C2	011	Placebo	×
01023_0_C1	011		×
01026_-1_C3	002	Placebo	×
01027_-1_C1	078	Rifaximin	×
01028_-1_C12	023	Placebo	×
01028_0_C3	023		×
01029_-1_C19	014	Placebo	×
01036_-1_C4	002	Placebo	×
02001_4_C9	010	Placebo	×
03001_4_C2	349	Placebo	×
03001_12_C1	349		×
03008_0_C2	002	Rifaximin	×
03008_4_C2	002		✓
03010_4_C3	365	Placebo	×
11001_4_C1	029	Placebo	×
11001_RL_C3	029		×
11007_-1_C2	014	Placebo	×
11007_-1_C5	029		×

11007_0_C14	014		×
11007_RL_C1	014		×
12001_0_C5	070	Rifaximin	×
12001_RL_C14	070		✓
12004_0_C2	020	Placebo	×
12004_RL_C14	020		×
12006_12_C1	Not Applicable	Placebo	✓
13002_-1_C13	015	Rifaximin	×
13007_0_C1	Not Ribotyped	Rifaximin	×
13007_4_C3	Not Applicable		✓
15003_-1_C1	Not Ribotyped	Placebo	✓
15003_12_C1	Not Ribotyped		✓
15003_RL_C1	Not Ribotyped		✓
17001_-1_C1	Not Ribotyped	Placebo	×
17001_0_C1	Not Ribotyped		×
17001_4_C1	Not Ribotyped		×
17002_-1_C1	Not Ribotyped	Rifaximin	×
17002_0_C1	Not Ribotyped		×
17002_RL1_C1	Not Ribotyped		×
17002_RL2_C1	Not Ribotyped		✓
17002_RL3_C1	Not Ribotyped		✓
18001_-1_C4	020	Rifaximin	×
18001_0_C6	020		×
18001_RL_C5	020		×
21008_0_C1	Not Ribotyped	Placebo	×
21008_4_C1	Not Ribotyped		×

Table 4.14 Relapse and Non-Relapse Patient RAPID Isolates Rifaximin Plate Assay Legend - Key: × No Growth (Sensitive), ✓ Growth (Resistant), -1; Diagnostic sample, 0; Sample after successful metronidazole or vancomycin treatment, 4; Week 4 check-up sample, 12; Week 12 check-up sample, RL; Relapse sample, C; Colony number picked.

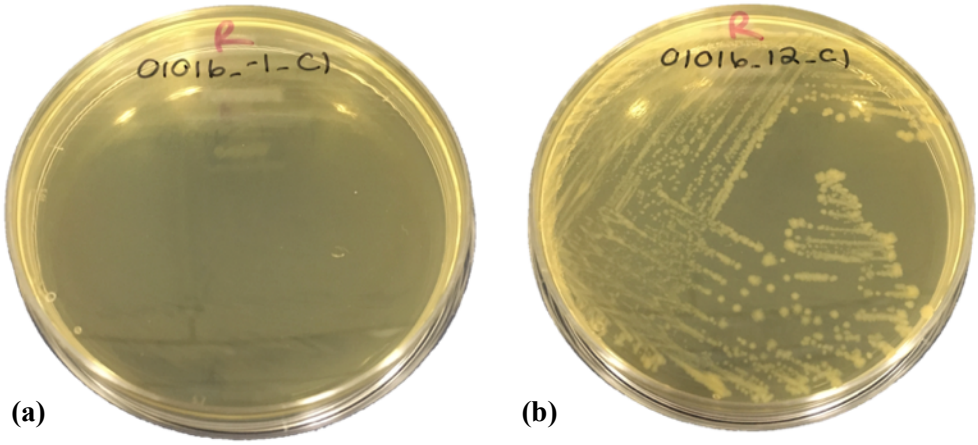


Figure 4.28 Non-Relapse Patient 01016 RAPID Isolate Rifaximin Plate Assay

The figure illustrates the *C. difficile* growth observed for relapse patient **(a)** 01016_-1_C1 and **(b)** 01016_12_C1 RAPID isolates on 8µg/ml rifaximin agar plates. No growth was demonstrated on the 01016_-1_C1 plate. Growth was illustrated on the 01016_12_C1 plate.

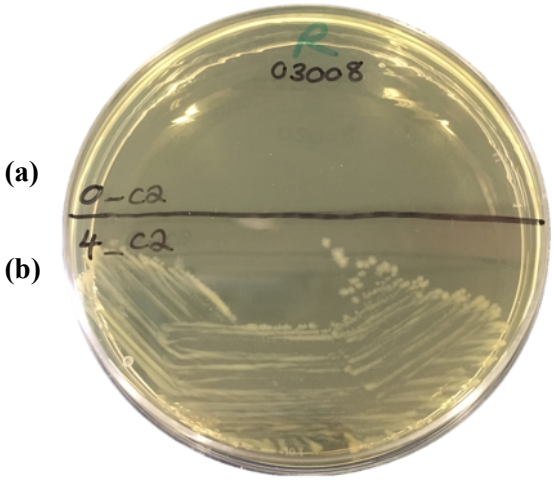


Figure 4.29 Non-Relapse Patient 03008 RAPID Isolate Rifaximin Plate Assay

The figure illustrates the *C. difficile* growth observed for non-relapse patient **(a)** 03008_0_C2 and **(b)** 03008_4_C2 RAPID isolates on an 8µg/ml rifaximin agar plate. No growth was demonstrated on the 03008_0_C2 plate. Growth was illustrated on the 03008_4_C2 plate.

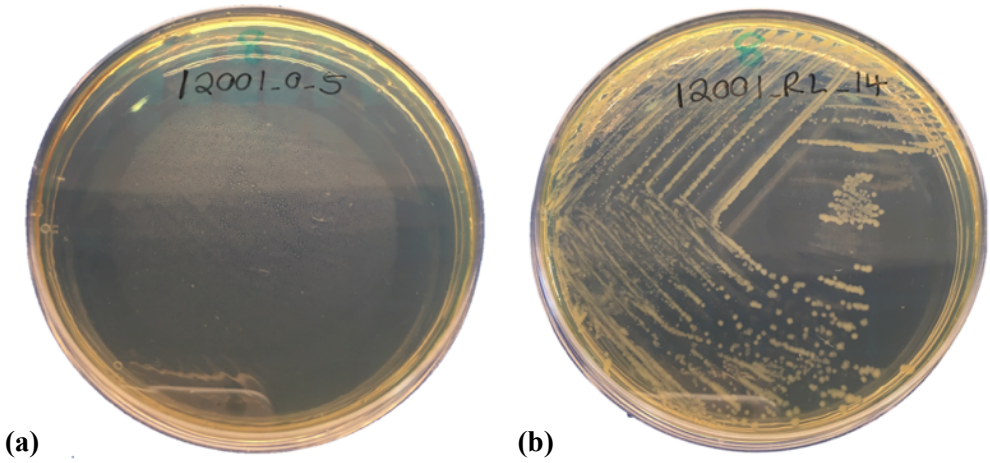


Figure 4.30 Relapse Patient 12001 RAPID Isolate Rifaximin Plate Assay

The figure illustrates the *C. difficile* growth observed for relapse patient **(a)** 12001_0_C5 and **(b)** 12001_RL_14 RAPID isolates on 8µg/ml rifaximin agar plates. No growth was demonstrated on the 12001_0_C5 plate. Growth was illustrated on the 12001_RL_14 plate.

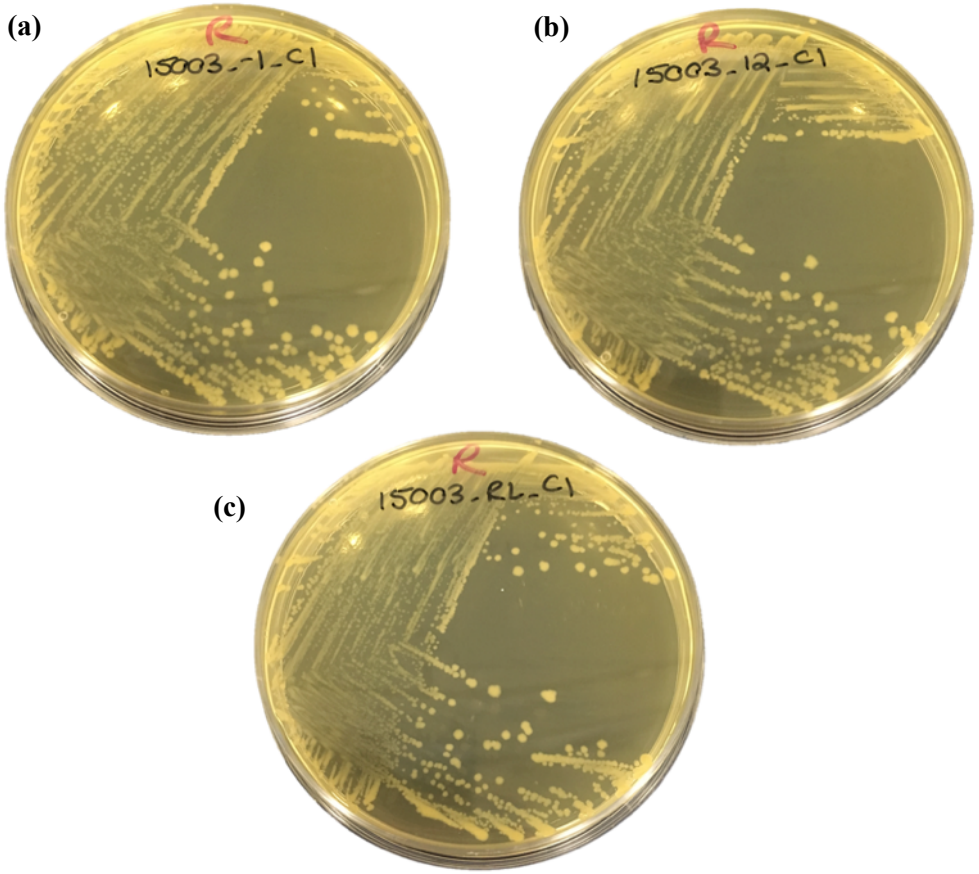


Figure 4.31 Relapse Patient 15003 RAPID Isolate Rifaximin Plate Assay

Figure 4.31 Relapse Patient 15003 RAPID Isolate Rifaximin Plate Assay Legend The figure illustrates the *C. difficile* growth observed for relapse patient **(a)** 15003_-1_C1, **(b)** 15003_12_C1 and **(c)** 15003_RL_C1 RAPID isolates on 8µg/ml rifaximin agar plates. Growth was illustrated on the 15003_-1_C1, 15003_12_C1 and 15003_RL_C1 plates.

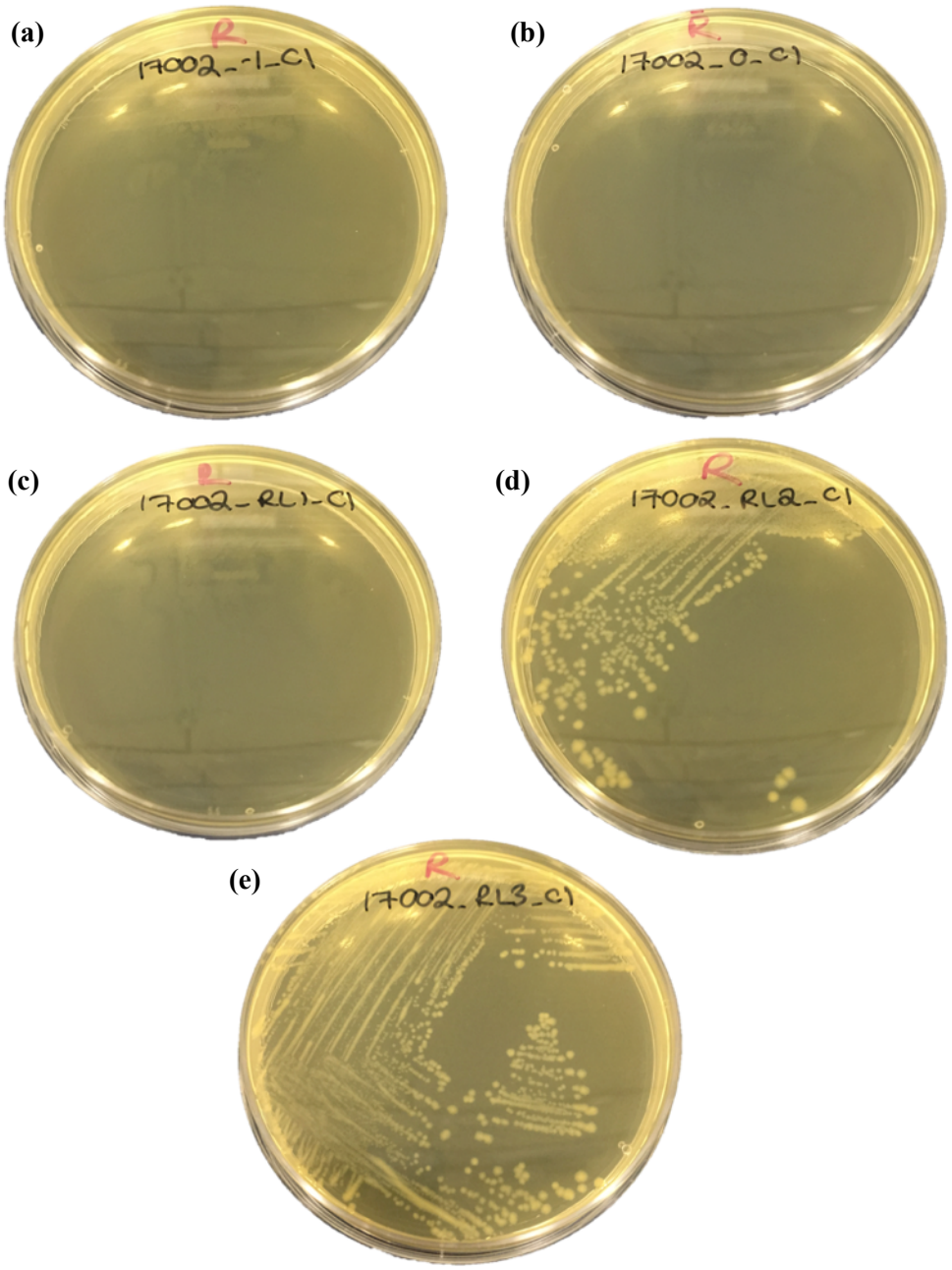


Figure 4.32 Relapse Patient 17002 RAPID Isolate Rifaximin Plate Assay

Figure 4.32 Relapse Patient 17002 RAPID Isolate Rifaximin Plate Assay Legend The figure illustrates the *C. difficile* growth observed for relapse patient **(a)** 17002_-1_C1, **(b)** 17002_0_C1, **(c)** 17002_RL1_C1, **(d)** 17002_RL2_C1 and **(e)** 17002_RL3_C1 RAPID isolates on 8µg/ml rifaximin agar plates. No growth was demonstrated on the 17002_-1_C1, 17002_0_C1 and 17002_RL1_C1 plates. Growth was illustrated on the 17002_RL2_C1 and 17002_RL3_C1 plates.

4.2.4.2. RIFAXIMIN ANTIBIOTIC RESISTANCE MINIMUM INHIBATORY CONCENTRATION

The 01016_12_C1, 03008_4_C2, 12001_RL_C14, 15003_-1_C1, 15003_12_C1, 15003_RL_C1, 17002_RL2_C1 and 17002_RL3_C1 RAPID isolates, which were resistant to rifaximin on the plate assay, were confirmed with a rifaximin broth dilution assay to determine the MIC. The rifaximin concentrations tested ranged from 512µg/ml to 0.5µg/ml, and the *C. difficile* isolate absorbance at 600nm (A_{600}) values minus the blank baseline BHIS broth A_{600} values were plotted (Figure 4.33).

Visually, the RAPID isolate growth in the BHIS broth containing rifaximin concentrations ranging from 0.5µg/ml to 16µg/ml was consistent with exposure to no antibiotic. At 32µg/ml, growth decreased steadily until 128µg/ml, where minimal growth was observed for this rifaximin concentration, as well as for 256µg/ml and 512µg/ml. A statistically significant difference was observed between 512µg/ml and 32µg/ml for 01016_12_C1 ($p = 0.0001$), 03008_4_C2 ($p = 0.0001$), 12001_RL_C14 ($p = 0.0001$), 15003_-1_C1 ($p = 0.0001$) and 17002_RL2_C1 ($p = 0.0012$), and so the MIC for these RAPID isolates was determined to be 32µg/ml. A significant difference was observed between 512µg/ml and 64µg/ml for 15003_12_C1 ($p = 0.0001$), 15003_RL_C1 ($p = 0.0009$) and 17002_RL3_C1 ($p = 0.0032$), and so the MIC for these RAPID isolates was determined to be 64µg/ml.

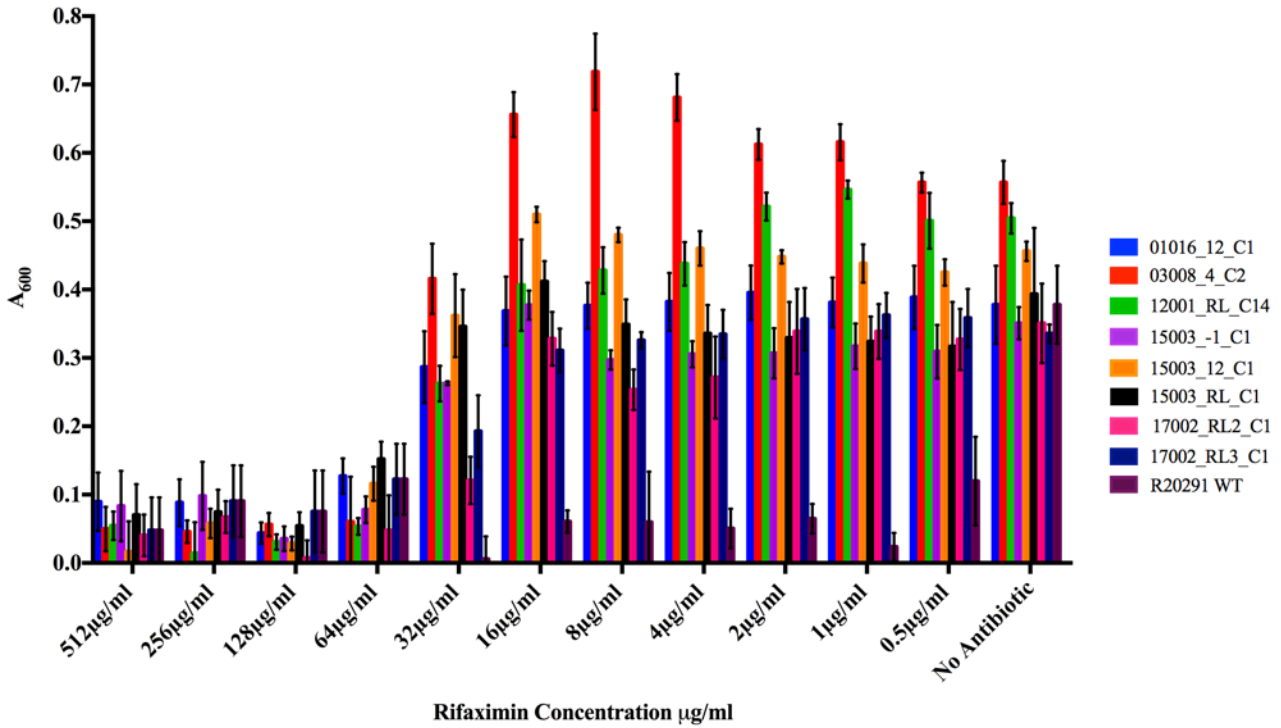


Figure 4.33 Rifaximin Dilution Broth Assay to Determine the Minimum Inhibitory Concentration

Bar chart to show the rifaximin dilution broth assay to determine the minimum inhibitory concentration (MIC). Average of three biologically independent replicates with error bars displaying the mean with standard deviation (SD). Visually, the RAPID isolate growth in the BHIS broth containing rifaximin concentrations ranging from 0.5µg/ml to 16µg/ml is consistent with exposure to no antibiotic. At 32µg/ml, growth decreases steadily until 128µg/ml, where minimal growth is observed for this rifaximin concentration, as well as for 256µg/ml and 512µg/ml. Key: -1; Diagnostic sample, 4; Week 4 check-up sample, 12; Week 12 check-up sample, RL; Relapse sample, C; Colony number picked, WT; Wild type.

4.2.4.3. COMPARISON OF THE RIFAXIMIN RESISTANCE DATA WITH THE RAPID TRIAL PARTICIPANT DATA

Figure 4.34 shows a context diagram to illustrate the summarised RAPID clinical trial data in relation to the rifaximin resistance results from the plate assay. Eight isolates of patients who were given 'follow on' rifaximin, did not demonstrate antibiotic resistance, with only one of the patients (18001) relapsing. Furthermore, two strains (01016 and 03008) of the four, which were found to be resistance, did not cause patient relapse. With regard to the placebo group, only one isolate (15003) was resistant to rifaximin, with the patient subsequently relapsing. In the rifaximin sensitive placebo group, three patients (11001, 11007 and 12004) relapsed. The 12006_12_C1 and 13007_4_C3 RAPID isolate rifaximin resistance data were excluded in this analysis and discussed in Chapter 4.2.5.

When the figures were analysed, it was determined that a total of 24.13% of the patients on the RAPID clinical trial relapsed within 12 weeks. Of that percentage, 25.00% of the 'follow on' rifaximin group relapsed compared to 23.53% of the placebo group.

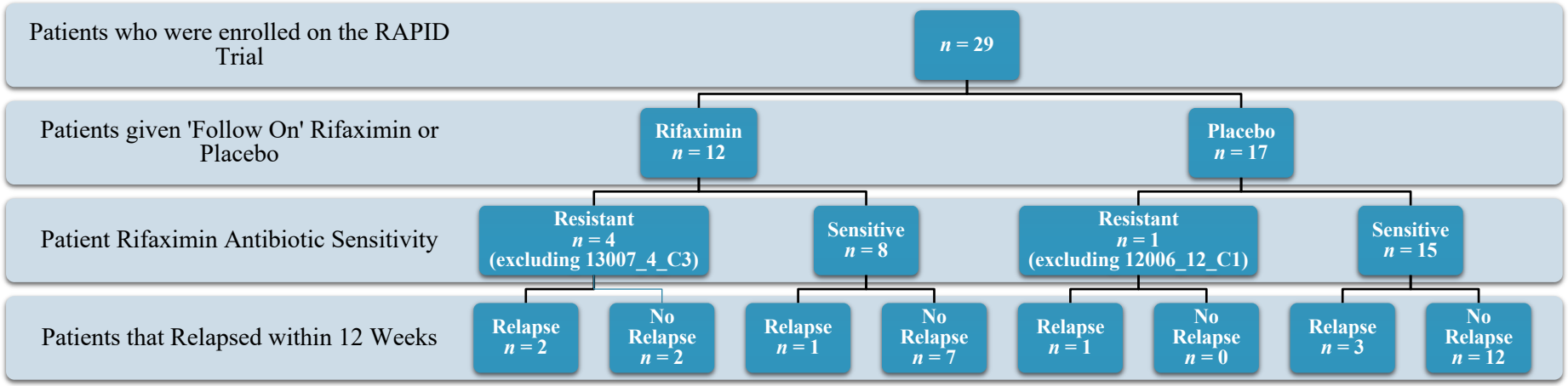


Figure 4.34 RAPID Clinical Trial Participant Data and Rifaximin Resistance Context Diagram

The figure shows a context diagram to illustrate the summarised RAPID clinical trial data in relation to the rifaximin resistance results from the plate assay. Eight isolates of patients who were given 'follow on' rifaximin, did not demonstrate antibiotic resistance, with only one of the patients (18001) relapsing. Furthermore, two strains (01016 and 03008) of the four, which were found to be resistance, did not cause patient relapse. With regard to the placebo group, only one isolate (15003) was resistant to rifaximin, with the patient subsequently relapsing. In the rifaximin sensitive placebo group, three patients (11001, 11007 and 12004) relapsed. The 12006_12_C1 and 13007_4_C3 RAPID isolate rifaximin resistance data were excluded in this analysis and discussed in Chapter 4.2.5. Key: *n*; Number, 4; Week 4 check-up sample, 12; Week 12 check-up sample, C; Colony number picked.

4.2.4.4. RPOB HOTSPOT SINGLE NUCLEOTIDE POLYMORPHISM ORGANISM COMPARISON

Mutations in the RpoB hotspot region leading to rifaximin resistance have been reported previously (O'Connor *et al.*, 2008; Curry *et al.*, 2009; Huhulescu *et al.*, 2011; Miller *et al.*, 2011b; Pecavar *et al.*, 2012; Pons *et al.*, 2012; Kothary *et al.*, 2013; Molodtsov *et al.*, 2017) in various organisms including *Escherichia coli*, *Mycobacterium tuberculosis* and *Staphylococcus aureus*, as well as *C. difficile*. Table 4.15 demonstrates the locations of amino acid changes within the conserved RpoB hotspot region of rifaximin resistant *C. difficile*, *E. coli*, *M. tuberculosis* and *S. aureus*, when compared to *Thermus aquaticus* and *T. thermophilus*. This is to illustrate that a number of the more common RpoB hotspot mutations are located in identical positions within different bacterial species. Of particular note, all four microorganisms reported SNPs in the following five amino acids, three of which have been shown to directly interact with rifaximin in a *T. aquaticus* model (red boxed amino acids) (O'Connor *et al.*, 2008): leucine at position 487 for *C. difficile* (or position 511 for *E. coli*, 430 for *M. tuberculosis* and 466 for *S. aureus*); serine at position 488 for *C. difficile* (or position 512 for *E. coli*, 431 for *M. tuberculosis* and 467 for *S. aureus*); aspartic acid at position 492 for *C. difficile* (or position 516 for *E. coli*, 435 for *M. tuberculosis* and 471 for *S. aureus*); serine at position 498 for *C. difficile* (or position 522 for *E. coli*, 441 for *M. tuberculosis* and 477 for *S. aureus*); and histidine at position 502 for *C. difficile* (or position 526 for *E. coli*, 445 for *M. tuberculosis* and 481 for *S. aureus*).

Table 4.15 RpoB Hotspot Single Nucleotide Polymorphism Organism Comparison

Thermus aquaticus and *T. thermophilus*	384	E	F	F	S	R	S	Q	L	S	Q	F	K	D	E	T	N	P	L	S	S	L	R	H	K	R	R	I	S	A	L	G	P	G	G	L	418
Clostridium difficile	480	E	F	F	G	S	S	Q	L	S	Q	F	M	D	Q	T	N	P	L	S	E	L	T	H	K	R	R	L	S	A	L	G	P	G	G	L	514
									F	T/P	K			N/V						T				N/R/Y/L			K										
Escherichia coli	504	E	F	F	G	S	S	Q	L	S	Q	F	M	D	Q	N	N	P	L	S	E	I	T	H	K	R	R	I	S	A	L	G	P	G	G	L	538
									P	F/Y	H/P/L/C/D			N/V/Y/G		D				F/Y			R	N/L/Y			S/L/C/H		F/Y	E	P/H/R	S/A					
Mycobacterium tuberculosis	423	E	F	F	G	T	S	Q	L	S	Q	F	M	D	Q	N	N	P	L	S	G	L	T	H	K	R	R	L	S	A	L	G	P	G	G	L	457
		A	L/S		D/S	A/S/P/H	Q/T	K/H	P/R/V	T/I/G/R	P/L/N/K/E/H/R	L/V	I	Y/V/N/H/E/G/A/F	H/L	D/Y/T/H/I	K		M/P	L/Q/W	A/W	S/W	P/I	N/L/Y/F/D/T/S/G/C/R/P/Q	N/Q	P/H			F/Y/C/W/L/Q		P		S/H			P	
Staphylococcus aureus	459	E	F	F	G	S	S	Q	L	S	Q	F	M	D	Q	A	N	P	L	A	E	L	T	H	K	R	R	L	S	A	L	G	P	G	G	L	493
							P		S	R/K/L				V/E/G/Y			K			V/D				N/R/Y/D			H/S/C		L							

Table to demonstrate the locations of amino acid changes within the conserved RpoB hotspot region of rifaximin resistant *C. difficile*, *E. coli*, *M. tuberculosis* and *S. aureus*, when compared to *T. aquaticus* and *T.* thermophilus. This is to illustrate that a number of the more common RpoB hotspot mutations are located in identical positions within different bacterial species. Key: Dark Grey = Original amino acid, Light Grey = All reported amino acid substitutions, Red Boxed Amino Acids = Residues equivalent to *T. aquaticus* RpoB amino acids that directly interact with rifaximin. Data gathered from the following published studies: (O'Connor *et al.*, 2008; Curry *et al.*, 2009; Huhulescu *et al.*, 2011; Miller *et al.*, 2011b; Pecavar *et al.*, 2012; Pons *et al.*, 2012; Kothary *et al.*, 2013; Stevenson *et al.*, 2016; Molodtsov *et al.*, 2017). The beginning and end of the RpoB hotspot region for each organism are highlighted in blue.

4.2.4.5. RPOB SINGLE NUCLEOTIDE POLYMORPHISM ANALYSIS

A PCR was performed on the rifaximin resistant 01016_12_C1, 03008_4_C2, 12001_RL_C14, 15003_-1_C1, 15003_12_C1, 15003_RL_C1, 17002_RL2_C1 and 17002_RL3_C1 RAPID isolates. An identical PCR was carried out simultaneously on the additional rifaximin sensitive isolates for the same patient to use as a negative control strain (Chapter 2.10.5.). The PCR amplified an 835bp DNA fragment, which included the RpoB hotspot region (data not shown), as the majority of previously reported SNPs have been isolated in this area.

Each DNA band was extracted and sent for Sanger DNA sequencing. Alignments were produced utilising the *C. difficile* 630 RpoB hotspot sequence (used to design the RpoB PCR primers) and the sequence of the rifaximin sensitive isolate(s) from the same patient as reference strains. The RAPID isolate nucleotide sequences were analysed for SNPs by comparing to the sequence of the isolate(s) from the same patient which was sensitive to rifaximin. Subsequently, the protein sequence was examined to identify those SNPs which had led to an amino acid change.

The 01016_12_C1 rifaximin resistant isolate when compared to the 01016_-1_C1 rifaximin sensitive isolate and *C. difficile* 630 strain RpoB sequence, demonstrated only one SNP from a G to an A at position 1514. This SNP created an amino acid change from an arginine (R) to a lysine (K) at position 505 (Figure 4.35).

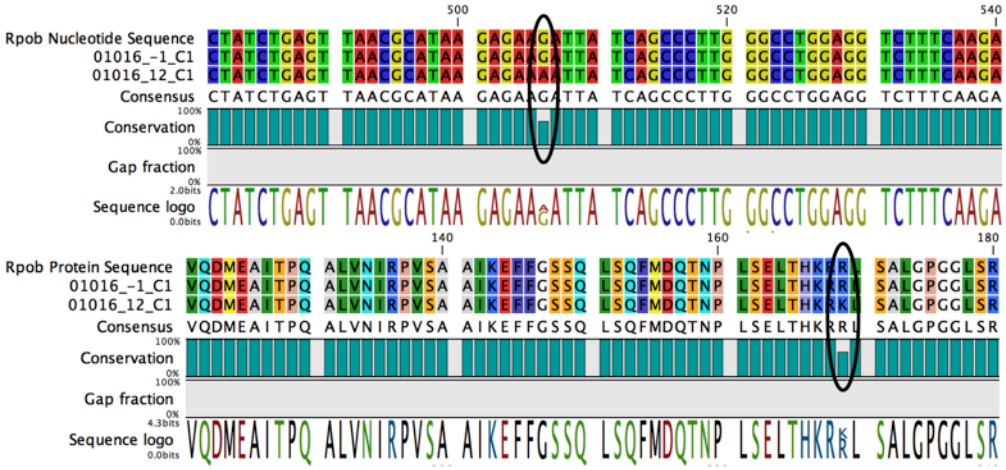


Figure 4.35 RAPID Isolate 01016_12_C1 RpoB Hotspot Single Nucleotide Polymorphism Analysis

The alignment demonstrates SNP analysis for the 01016_12_C1 rifaximin resistant isolate when compared to the 01016_-1_C1 rifaximin sensitive isolate and *C. difficile* 630 strain RpoB sequence. Only one SNP from a G to an A at position 1514 was determined. This SNP created an amino acid change from an arginine (R) to a lysine (K) at position 505.

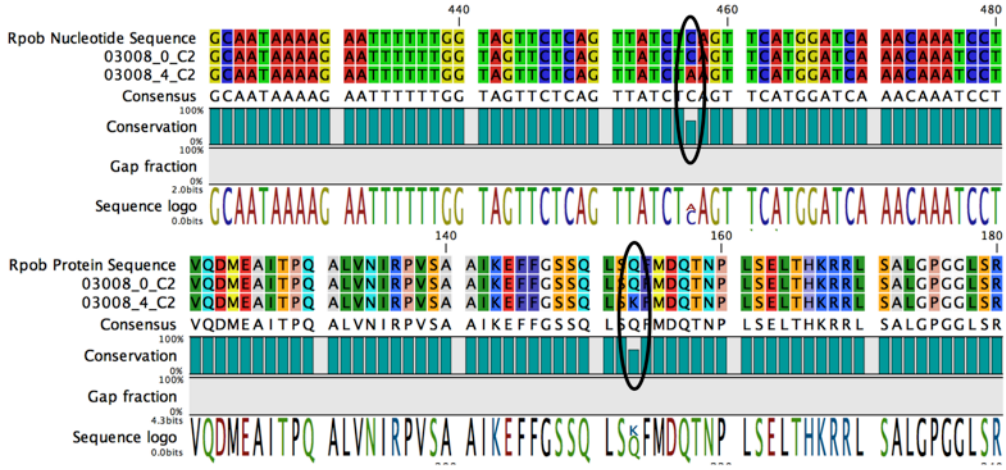


Figure 4.36 RAPID Isolate 03008_4_C2 RpoB Hotspot Single Nucleotide Polymorphism Analysis

The alignment demonstrates SNP analysis for the 03008_4_C2 rifaximin resistant isolate when compared to the 03008_0_C2 rifaximin sensitive isolate and *C. difficile* 630 strain RpoB sequence. Only one SNP from a C to an A at position 1465 was determined. This SNP created an amino acid change from a glutamine (Q) to a lysine (K) at position 489.

The 03008_4_C2 rifaximin resistant isolate when compared to the 03008_0_C2 rifaximin sensitive isolate and *C. difficile* 630 strain RpoB sequence, demonstrated only one SNP from a C to an A at position 1465. This SNP created an amino acid change from a glutamine (Q) to a lysine (K) at position 489 (Figure 4.36).

The 12001_RL_C14 rifaximin resistant isolate when compared to the 12001_0_C5 rifaximin sensitive isolate and *C. difficile* 630 strain RpoB sequence, demonstrated only one SNP from a G to an A at position 1514. This SNP created an amino acid change from an arginine (R) to a lysine (K) at position 505 (Figure 4.37). This SNP is identical to the alteration observed in the 01016_12_C1 rifaximin resistant isolate.

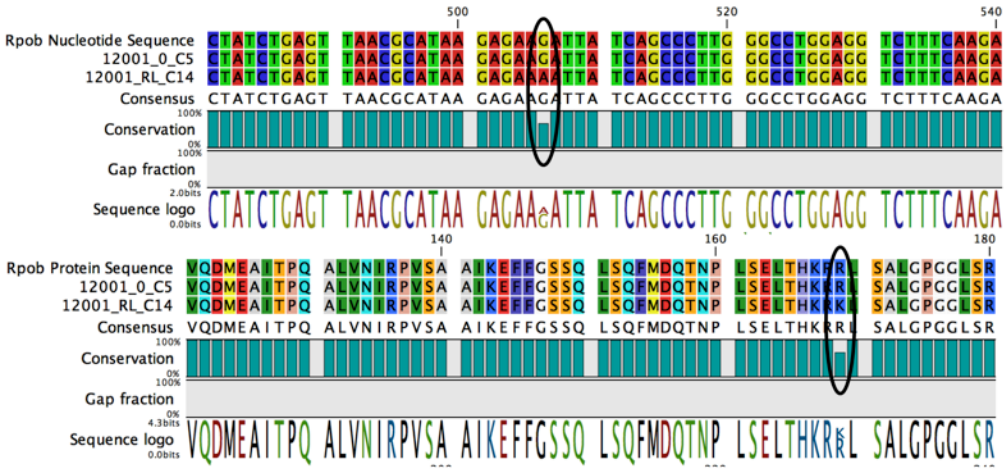


Figure 4.37 RAPID Isolate 12001_RL_C14 RpoB Hotspot Single Nucleotide Polymorphism Analysis

The alignment demonstrates SNP analysis for the 12001_RL_C14 rifaximin resistant isolate when compared to the 12001_0_C5 rifaximin sensitive isolate and *C. difficile* 630 strain RpoB sequence. Only one SNP from a G to an A at position 1514 was determined. This SNP created an amino acid change from an arginine (R) to a lysine (K) at position 505.

The 15003_-1_C1, 15003_12_C1 and 15003_RL_C1 rifaximin resistant isolates when compared to the *C. difficile* 630 strain RpoB sequence, demonstrated only one SNP from a G to an A at position 1514. This SNP

created an amino acid change from an arginine (R) to a lysine (K) at position 505 (Figure 4.38). This SNP is therefore identical to the modification observed in the 01016_12_C1 and 12001_RL_C14 rifaximin resistant isolates.

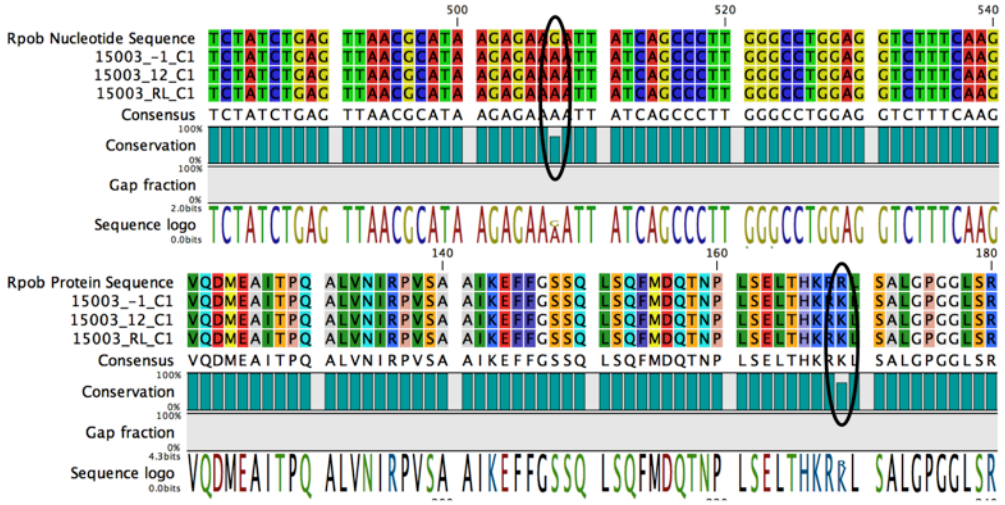


Figure 4.38 RAPID Isolates 15003_-1_C1, 15003_12_C1 and 15003_RL_C1 RpoB Hotspot Single Nucleotide Polymorphism Analysis

The alignment demonstrates SNP analysis for the 15003_-1_C1, 15003_12_C1 and 15003_RL_C1 rifaximin resistant isolates when compared to the *C. difficile* 630 strain RpoB sequence. Only one SNP from a G to an A at position 1514 was determined. This SNP created an amino acid change from an arginine (R) to a lysine (K) at position 505.

The 17002_RL3_C1 rifaximin resistant isolate when compared to the 17002_-1_C1, 17002_0_C1 and 17002_RL1_C1 rifaximin sensitive isolates and *C. difficile* 630 strain RpoB sequence, demonstrated only one SNP from a C to a T at position 1520. This SNP created an amino acid change from a serine (S) to a leucine (L) at position 507 (Figure 4.39).

RAPID isolate 17002_RL2_C1 had accumulated 11 SNPs in the RpoB hotspot region, however, none of these led to an amino acid change. Therefore, a PCR was carried out to amplify the entire RpoB gene. This produced a DNA fragment of 3,717bp (data not shown), which was Sanger DNA sequenced.

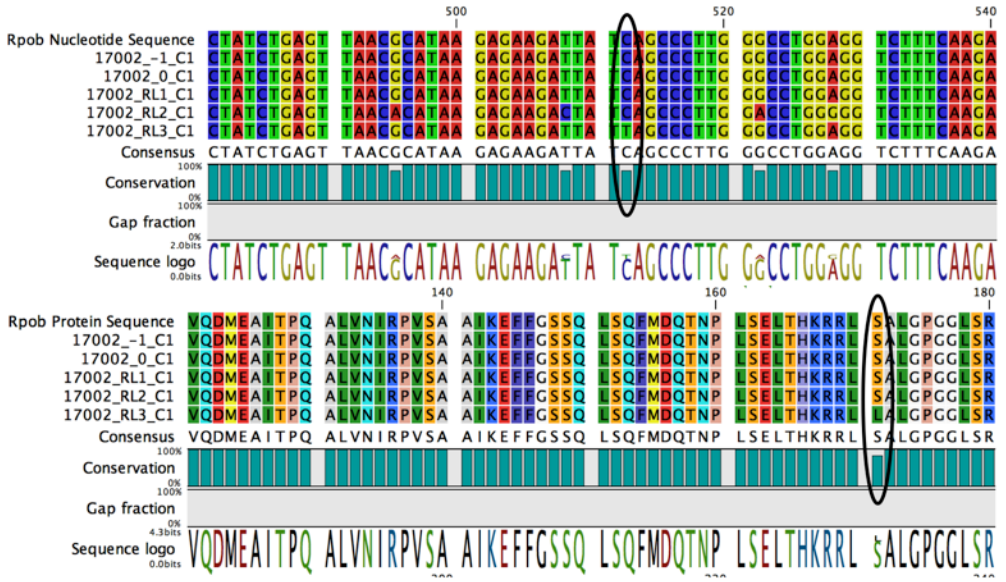


Figure 4.39 RAPID Isolates 17002_RL2_C1 and 17002_RL3_C1 RpoB Hotspot Single Nucleotide Polymorphism Analysis

The alignment demonstrates SNP analysis for the 17002_RL3_C1 rifaximin resistant isolate when compared to the 17002_-1_C1, 17002_0_C1 and 17002_RL1_C1 rifaximin sensitive isolates and *C. difficile* 630 strain RpoB sequence. Only one SNP from a C to a T at position 1520 was determined. This SNP created an amino acid change from a serine (S) to a leucine (L) at position 507. RAPID isolate 17002_RL2_C1 had accumulated 11 SNPs in the RpoB hotspot region, however, none of these led to an amino acid change.

Analysis of the complete RpoB gene sequence for RAPID isolate 17002_RL2_C1 identified a total of 78 SNPs. Of those 78, nine nucleotide polymorphisms led to an amino acid change. The A to a G SNP at position 2250 produced an amino acid change from Isoleucine (I) to methionine (M) at position 750. The C to G and A to G SNPs at positions 3109 and 3111, respectively, generated an amino acid modification from glutamine (Q) to glutamate (E) at position 1037. The TG to CA double nucleotide polymorphism (DNP) at position 3614 and 3615 created an amino acid change from valine (V) to alanine (A) at position 1205. The GC to AA DNP at position 3619 and 3620 generated an amino acid modification from alanine to asparagine (N) at position 1207. The A to G SNP observed at position 3622 produced an amino acid change from threonine (T) to alanine at position 1208 (Figure 4.40).

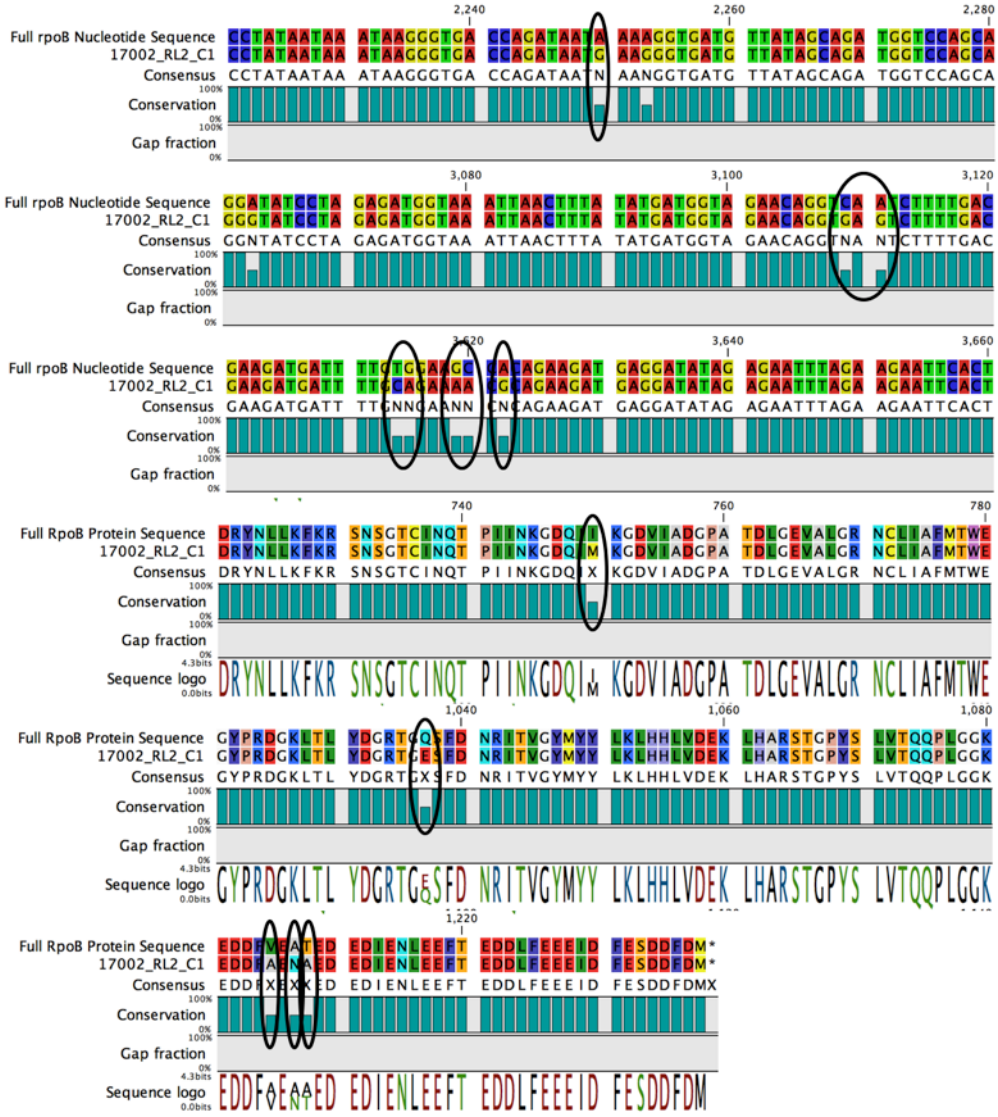


Figure 4.40 RAPID Isolate 17002_RL2_C1 Complete RpoB Single Nucleotide Polymorphism Analysis

The alignment demonstrates SNP analysis of the complete RpoB gene sequence for RAPID isolate 17002_RL2_C1. A total of 78 SNPs were identified. Of those 78, nine nucleotide polymorphisms led to an amino acid change. The A to a G SNP at position 2250 produced an amino acid change from Isoleucine (I) to methionine (M) at position 750. The C to G and A to G SNPs at positions 3109 and 3111, respectively, generated an amino acid modification from glutamine (Q) to glutamate (E) at position 1037. The TG to CA double nucleotide polymorphism (DNP) at position 3614 and 3615 created an amino acid change from valine (V) to alanine (A) at position 1205. The GC to AA DNP at position 3619 and 3620 generated an amino acid modification from alanine to asparagine (N) at position 1207. The A to G SNP observed at position 3622 produced an amino acid change from threonine (T) to alanine at position 1208.

The RAPID isolate *rpoB* SNP gene analysis and RpoB amino acid change details are summarised, along with the ribotyping information and unblinded patient 'follow on' treatment data, in Table 4.16.

Table 4.16 RAPID Isolate RpoB Single Nucleotide Polymorphism and Amino Acid Change Summary Table

RAPID Isolate	Ribotype	Treatment	SNP	Amino Acid Change
01016_12_C1	026	Rifaximin	G1514A	R505K
12001_RL_C14	070	Rifaximin		
15003_-1_C1	Not Ribotyped	Placebo		
15003_12_C1	Not Ribotyped	Placebo		
15003_RL_C1	Not Ribotyped	Placebo		
03008_4_C2	002	Rifaximin	C1465A	Q489K
17002_RL2_C1	Not Ribotyped	Rifaximin	A2250G C3109G A3111G TG3614-3615CA GC3619-3620AA A3622G	I750M Q1037E Q1037E V1205A A1207N T1208A
17002_RL3_C1	Not Ribotyped	Rifaximin	C1520T	S507L

Key: -1; Diagnostic sample, 4; Week 4 check-up sample, 12; Week 12 check-up sample, RL; Relapse sample, C; Colony number picked. DNA Bases – Adenine (A), Cytosine (C), Guanine (G) and Thymine (T). Amino Acids – Arginine (R), Lysine (K), Glutamine (Q), Isoleucine (I), Methionine (M), Glutamic Acid (E), Valine (V), Alanine (A), Asparagine (N) and Threonine (T).

4.2.5. *ROBINSONIELLA PEORIENSIS*

The 12006_12_C1 and 13007_4_C3 RAPID isolates were characterised alongside the third group of RAPID samples. Both isolates grew well on Braziers agar and BHIS agar supplemented with *C. difficile* selective supplement. Upon *C. difficile* binary toxin screening, no DNA bands at the correct size were observed for a full CDT gene locus, ghost CDT gene locus or the non-toxigenic CDT region (Figure 4.41).

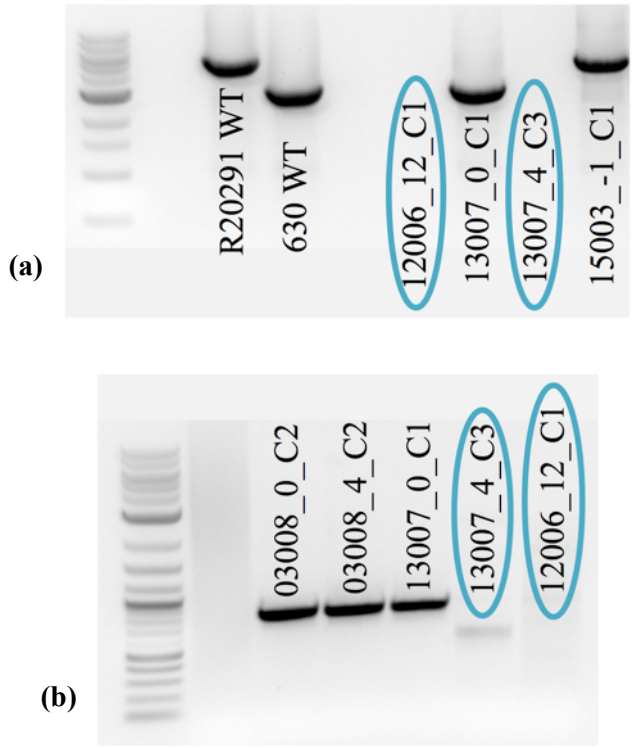


Figure 4.41 RAPID Isolates 12006_12_C1 and 13007_4_C3 CDT PCR Screening

A 1% agarose electrophoresis gel to demonstrate the CDT PCR screening for 12006_12_C1 and 13007_4_C3 RAPID isolates. No DNA bands at the correct size were observed for **(a)** the full or ghost CDT gene locus, and **(b)** the non-toxigenic CDT region. The negative gel is shown to highlight the lighter bands.

With regards to sporulation, the 13007_4_C3 RAPID isolate was tested together with the 13007_0_C1 RAPID isolate from the same patient (Figure 4.42). Firstly, the non-heat-treated 0-hour time point results were visually compared for each sample. There was no observed difference in cfu/ml at this time point. A Two-Way ANOVA statistical test followed by a Tukey multiple comparison test, in which each strain triplicate mean was compared with the triplicate mean of every other strain, was performed to confirm visual observations. There was no significant difference identified for the two RAPID samples and the 630Δ*erm* and 630Δ*erm*Δ*spo0A* control strains, which demonstrated that all samples were commencing at comparable growth (data not shown).

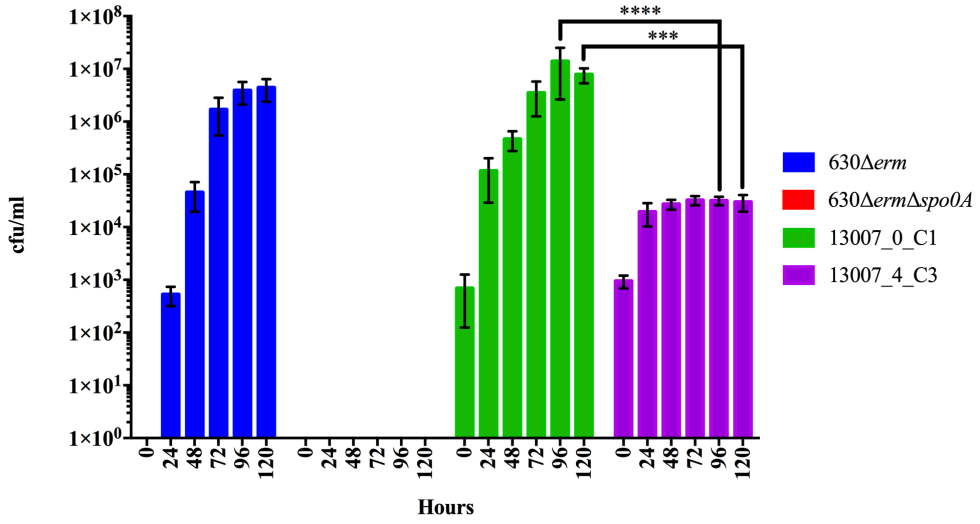


Figure 4.42 Sporulation Assay to Compare the 630Δ*erm* Strain, 630Δ*erm*Δ*spo0A* Strain, and 13007_0_C1 and 13007_4_C3 RAPID Isolates

Bar chart to demonstrate the sporulation assay to compare the 630Δ*erm* strain, 630Δ*erm*Δ*spo0A* strain, and 13007_0_C1 and 13007_4_C3 RAPID isolates. Average of three biologically independent replicates with error bars displaying the mean with standard deviation (SD). 13007_4_C3 produced an unusual sporulation profile where the cfu/ml between the 24-hour time point and the 120-hour time point plateaued compared to 13007_0_C1 and the 630Δ*erm* strain. A significant difference was detected between 13007_0_C1 and 13007_4_C3 at 96 hours (p = <0.0001) and 120 hours (p = 0.0009). Key: *** p = ≤ 0.001 and **** p = ≤ 0.0001.

13007_4_C3 produced an unusual sporulation profile, which was observed at the time. The cfu/ml between the 24-hour time point and the 120-hour time point plateaued compared to 13007_0_C1 and the 630Δ*erm* strain. A Two-Way ANOVA statistical test followed by a Tukey multiple comparison test detected a significant difference between 13007_0_C1 and 13007_4_C3 at 96 hours (p = <0.0001) and 120 hours (p = 0.0009). A sporulation assay was not completed on RAPID isolate 12006_12_C1 as no earlier patient samples were received for comparison.

In the rifaximin plate assay, both 12006_12_C1 and 13007_4_C3 RAPID isolates demonstrated growth on the 8µg/ml rifaximin agar plates. The 12006_12_C1 displayed limited growth, indicating intermediate resistance

(Figure 4.43). However, no DNA bands were observed at the correct size when the RpoB hotspot region was PCR amplified (data not shown).

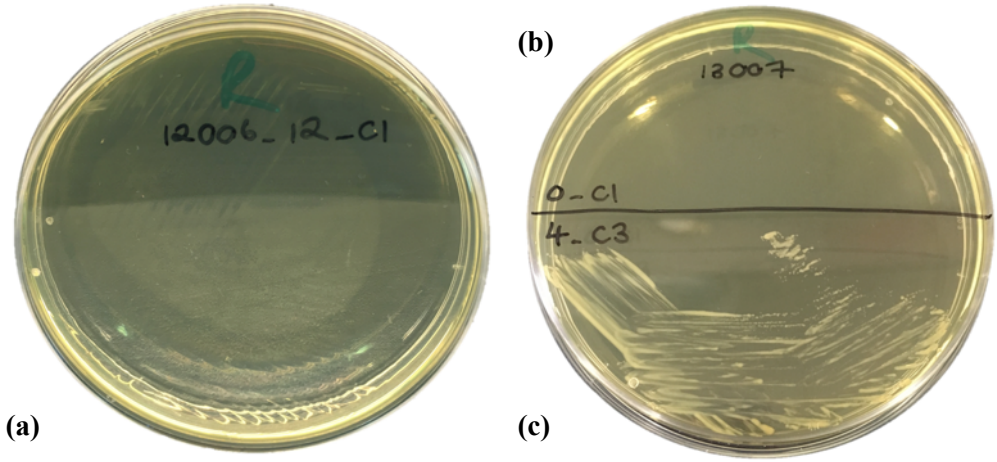


Figure 4.43 12006_12_C1 and 13007 RAPID Isolates Rifaximin Plate Assay

The figure illustrates the *C. difficile* growth observed for **(a)** 12006_12_C1 **(b)** 13007_0_C1 and **(c)** 13007_4_C3 RAPID isolates on 8µg/ml rifaximin agar plates. No growth was demonstrated on the 13007_0_C1 plate. Growth was illustrated on the 12006_12_C1 and 13007_4_C3 plates. The 12006_12_C1 displayed limited growth, indicating intermediate resistance.

A 16s rRNA PCR was performed on both the 12006_12_C1 and 13007_4_C3 RAPID isolates to verify if they were indeed *C. difficile* (Figure 4.44). The DNA fragments were extracted and sent for Sanger DNA sequencing.

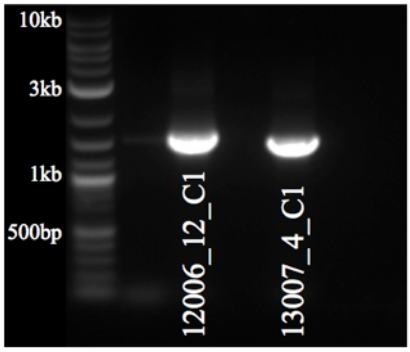


Figure 4.44 12006_12_C1 and 13007_4_C1 16s rRNA PCR

A 1% agarose electrophoresis gel to demonstrate the 16s rRNA PCR performed on both the 12006_12_C1 and 13007_4_C3 RAPID isolates for organism verification. A DNA fragment of approximately 2,000bp was observed.

The BLAST database search with the subsequent sequencing results was conducted. RAPID isolate 12006_12_C1 was 94% identical (with a 90% query cover) to *Robinsoniella peoriensis* strain HGUE-09/9434 (GenBank accession number GU322806.1). RAPID isolate 13007_4_C3 was 98% identical (with a 97% query cover) to *R. peoriensis* strain PPC44 (GenBank accession number AF445283.2).

4.3. DISCUSSION

4.3.1. *CLOSTRIDIUM DIFFICILE* BINARY TOXIN

4.3.1.1. CDT PCR

Firstly, RAPID patient samples that contained a *C. difficile* strain with a full or ghost CDT gene locus, or were non-toxigenic for CDT, were confirmed. In 56 RAPID *C. difficile* isolates investigated (excluding the two isolates which were confirmed to be *R. peoriensis*), nine contained the full binary toxin gene locus. This equates to 16.1%, which is marginally lower when compared to the 17% to 23% reported by previous studies (Geric *et al.*, 2006; Eckert *et al.*, 2015). The ribotypes confirmed to contain the full CDT gene locus included

023 and 078, which have been previously established in the literature (Bacci *et al.*, 2011; Gerding *et al.*, 2014). Unfortunately, four of the nine samples were in the third group of RAPID isolates, and so were not ribotyped due to time limitations.

Forty of the 56 samples contained the ghost CDT gene locus (71.4%). The non-functional ghost binary toxin has not been as well characterised, however, a 2009 national survey (Eckert *et al.*, 2013) conducted in France determined 74.1% of the clinical samples contained a pseudogene of the binary toxin, which is comparable to the outcome in this research. The ribotypes confirmed to contain the ghost CDT gene locus included 002, 005, 011, 014 (previously established (Knight *et al.*, 2017)), 015, 020, 029, 070 and 215. Unfortunately, ten of the forty samples were in the third group of RAPID isolates, and so were not ribotyped due to time limitations.

A non-toxigenic CDT 68bp sequence was detected in seven of the 56 isolates (12.5%). The ribotypes confirmed to contain the non-toxigenic CDT region included 010 (previously established (Janezic *et al.*, 2014)), 026, 349 and 365.

4.3.1.2. FULL CDT SNP ANALYSIS

The full binary toxin SNP analysis, when compared to strain R20291, revealed several interesting results. The *cdtR* in 01027_-1_C1 contained a G to a T SNP at position 322, which led to a premature stop codon. This would cause translation of a truncated CdtR protein of only 107 amino acids, instead of the 249 normally seen (Bouvet and Popoff, 2008; Metcalf and Weese, 2011). The TAA stop codon is found in most ribotype 078 strains (Peng *et al.*, 2017b). This SNP was also observed in RAPID isolate 17002_RL2_C1 and so this sample was also expected to be a ribotype 078, however, this was not established (Figure 4.45). As illustrated in Figure 4.11, the CdtR consists of two domains; the signal transduction response regulator (N-terminal) and the LytTR DNA-binding domain (C-terminal). Phosphorylation of the N-terminal by a sensor histidine kinase induces a conformational change in the C-terminal (Metcalf and Weese, 2011). The Glu108* amino acid modification, which causes the truncated CdtR protein resides in the N-terminal, suggesting that

CdtR may be non-functional in this case. Nonetheless, a previous study (Lyon *et al.*, 2016) reported that ribotype 078 strains produce CDT, even with the conserved *cdtR* SNP. The researchers observed that the level of CDT activity was comparable to other ribotypes without the truncated CdtR. However, the expression of a functional CdtR led to increased binary toxin production.

Unfortunately, due to time constraints, the RAPID isolates containing the complete binary toxin gene locus were not assessed for CDT production. This would have been particularly informative for the 01027_-1_C1 and 17002_RL2_C1 RAPID isolates, due to the truncated CdtR. CDT production of each full CDT RAPID isolate would have been investigated with the TaqMan Protein Assay described in Chapter 3.2.7.3., however, the assay was never completely optimised.

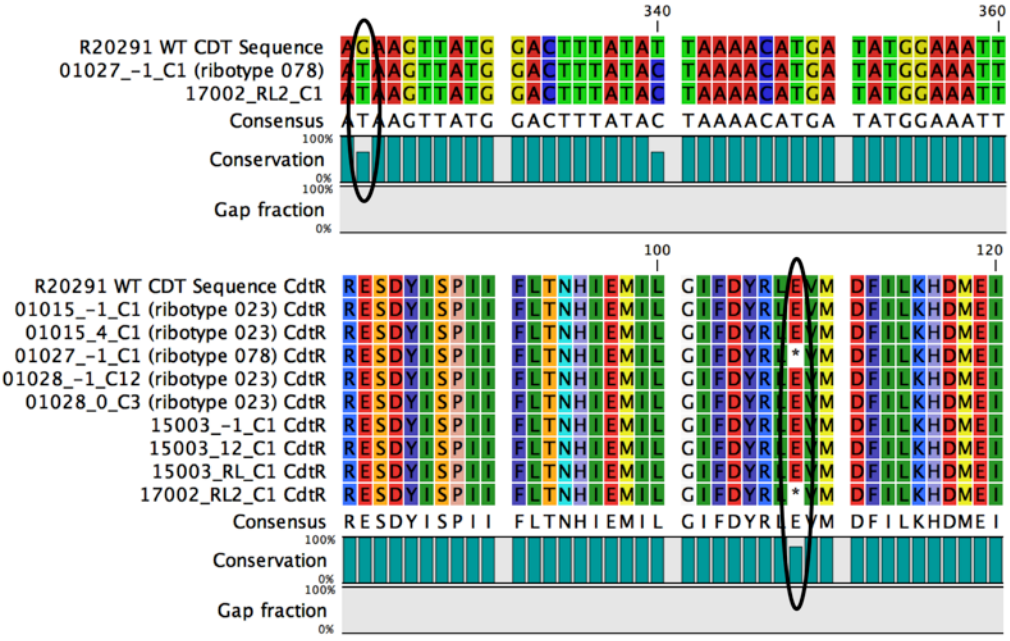


Figure 4.45 The Truncated CdtR Protein in Ribotype 078

The alignment demonstrates the truncated CdtR protein in RAPID isolates 01027_-1_C1 and 17002_RL2_C1. The *cdtR* contained a G to a T SNP at position 322, which led to a premature stop codon. This would cause translation of a truncated CdtR protein of only 107 amino acids, instead of the 248 normally seen (Metcalf and Weese, 2011). The TAA stop codon is found in most ribotype 078 strains (Peng *et al.*, 2017b), however, the ribotype was not confirmed for the RAPID isolate 17002_RL2_C1.

The 49kDA mature CdtA protein also has a two-domain structure; a CdtB interaction or binding domain (N-terminal, residues 1-215) and the ADP-ribosyltransferase activity domain (C-terminal, residues 216-420) (Sundriyal *et al.*, 2009; Roth *et al.*, 2016) (Figure 4.11). The first 43 amino acids represent a signal sequence and are proteolytically cleaved (Perelle *et al.*, 1997). As with other ADP-ribosyltransferases, CdtA contains a crucial arginine residue. In this case, it is the arginine at position 302 (Gerding *et al.*, 2014). Mutation of this arginine to lysine blocked ADP-ribosyltransferase activity in one study (Gulke *et al.*, 2001). Furthermore, ADP-ribosyltransferases contain conserved STS and EXE motifs. In CdtA, the STS motif begins with a serine at position 345, and the EXE motif starts at position 385 with a glutamate. The second glutamate in the EXE motif at position 387 has been identified as the catalytic residue (Gerding *et al.*, 2014). In the same study (Gulke *et al.*, 2001), the Ser345 was changed to an alanine and the Glu385 and Glu387 residues were altered to glutamine. These mutations all abolished ADP-ribosyltransferase activity. Other important amino acids that form part of the catalytic cleft of CdtA and bind nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADPH) include Arg303, Arg359, Gln307 and Asn342 (Sundriyal *et al.*, 2009). The nine RAPID isolates confirmed to contain the full binary toxin gene locus did not contain SNPs in any of the crucial and important amino acid residues, which suggest that these are necessary for CDT production *in vivo*.

The CdtB component of CDT is 99kDA (Sundriyal *et al.*, 2009) and separated into four distinct domains; a proteolytic activation domain (N-terminal, 257 residues), a heptamerisation domain (until residue 480), a ferrodoxin-like domain and a receptor-binding domain (C-terminal, residues 592-876). There is also a 42 amino acid signal sequence present (Gerding *et al.*, 2014) (Figure 4.11). CdtB is activated by protease cleavage to create a 20 and a 75kDa peptide, with the larger peptide binding to the host cell lipolysis-stimulated lipoprotein receptor (LSR). The essential amino acids are less well characterised for CdtB, however, it is thought that cleavage occurs between the lysine at position 209 and the leucine at position 210 (Gerding *et al.*,

2014). RAPID isolates 01027_-1_C1 (ribotype 078) and 17002_RL2_C1 (expected to be ribotype 078, but unconfirmed) both contain a SNP at Lys209 to an arginine. Further investigation into whether this SNP hinders production of CDT and if this is a conserved SNP in other ribotype 078 strains would certainly be interesting. A recent study (Hemmasi *et al.*, 2015), also demonstrated from a series of N-terminal and C-terminal CdtB truncations, that only amino acids 757-866 are necessary for the binding of CdtB to the LSR. Several SNPs were identified in this region, including Met762Ile (67%), Phe769Ser (67%), Ala770Ser (44%), Thr781Ala (67%) and Ala789Thr (22%), however, it is unclear whether these mutations would impact on CDT production *in vivo*. It could be speculated that only truncations and not individual SNPs in this region would impact on the production of binary toxin.

Furthermore, there appeared to be no correlation of these findings to patient outcome. Of the five patients (01015, 01027, 01028, 15003 and 17002) infected with *C. difficile* isolates containing the full binary toxin gene locus, only two relapsed (15003 and 17002). The hypothesis that the presence of the full binary toxin gene locus in patient isolates is associated with recurrent episodes, specifically relapse, cannot be accepted in this case.

4.3.1.3. GHOST CDT SNP ANALYSIS

The ghost binary toxin SNP analysis of *cdtR* illustrated an interesting result. Far less SNPs were demonstrated for the ghost *cdtR*, when compared to the partial CDT 630 strain, than with the full gene locus *cdtR*, when compared to the complete CDT R20291 strain (11 against 18 SNPs). When the ghost *cdtR* was compared to R202091 *cdtR*, the gene was even more well conserved, with a total of 2 SNPs. Strains that contain the ghost binary toxin gene locus do not produce CDT, and so the gene conservation was unexpected. The purpose of this remains unknown, however, it was speculated that the ghost *cdtR* could be regulating other genes in the genome. Recently published research (Lyon *et al.*, 2016) demonstrated that *cdtR* in two ribotype 027 strains also regulated *C. difficile* toxin A and B production. Conversely, this was shown not to be the case in an 078 strain and the 630 (ribotype 012) strain. The authors did conclude, however, that variation in virulence factor regulatory mechanisms is

evident. This is particularly important as the two ghost CDT gene locus strains the researchers tested contained either a truncated non-functional CdtR (ribotype 078) or a CdtR protein with 9 SNPs, when compared to strain R20291 (strain 630). Therefore, further investigation into the role of the ghost *cdtR*, with a selection of different ribotypes, is necessary. Moreover, examination of CDT horizontal gene transfer (as discussed in Chapter 3.3.4.) in a strain containing the ghost binary toxin, such as *C. difficile* 630, may give insight into the reason behind the high frequency of strains carrying this non-functional gene locus.

4.3.2. SPORULATION

4.3.2.1. 630Δ*erm*::CT26350 CLOSTRON

The CD630_26350 gene in *C. difficile* strain 630 has been identified as a putative spore envelope protein (GenBank accession number CAJ69521.1). It is thought to be part of the YIEGIA protein family (IPR025918), which includes the *Bacillus subtilis* YphB sporulation protein. *B. subtilis yphB* gene expression is regulated by an early forespore-specific sigma factor *sigF* (σF), and is not essential in sporulation or germination (Decatur and Losick, 1996). The sporulation data for the ClosTron mutant strain indicated that the 630 protein may also not be essential as sporulation did still occur. However, the assay additionally demonstrated that the sporulation cfu/ml was reduced and significantly different at the 96-hour time point when compared to the 630Δ*erm* WT strain. This suggests the CD630_26350 gene may play an important role in achieving an optimal sporulation rate and so the hypothesis that this putative spore envelope protein gene would negatively affect sporulation rate was confirmed. Furthermore, it could be speculated *in vivo* that this may hinder sporulation sufficiently in the host that the risk of patient relapse, as well as exogenous spread in the environment, would be reduced.

4.3.2.2. RAPID ISOLATE SPORULATION

All twelve RAPID samples tested reached a significantly higher cfu/ml count at the later time points compared to the 630Δ*erm* WT control strain. Two studies (Wilcox and Fawley, 2000; Fawley *et al.*, 2007) researching the

inhibitory effect of cleaning agents on the sporulation rate of *C. difficile* demonstrated that exposure to a low level of cleaning agent (particularly non-chlorine based products) compared to no cleaning agent increased sporulation capacity. The authors determined that certain hospital environmental stresses, such as cleaning and the choice of cleaning agent used, may promote sporulation and be associated with *C. difficile* persistence. One could speculate that this conclusion could equally explain the results observed for the RAPID patient samples. However, there is no evidence to suggest whether this effect is long-lasting and would promote increased sporulation *in vivo* or only occurs when the strains are directly exposed to the cleaning agents *in vitro*.

It was hypothesised that there may be individual patients that have strains with unique sporulation profiles, which could be correlated with relapse. When comparing the RAPID isolates at different time points for the same patient, the 11007 relapse (11007_RL_C1) sample sporulated higher at a faster rate, when compared to the diagnostic (11007_-1_C2) and week 0 (11007_0_C14) samples. However, the 15003 week 12 (15003_12_C1) and relapse (15003_RL_C1) samples sporulated at a lower rate at the later time points, when compared to the diagnostic (15003_-1_C1) isolate. It is difficult to reach any sound conclusions on the effect of sporulation on patient outcome. The sporulation profiles observed for 11007 and 15003 may have impacted on patient clinical outcome, as both did relapse, however, this conclusion would be merely speculation, due to the small cohort examined. Analysis of the collective sporulation data for all RAPID isolates is currently being completed and may reveal more significant evidence for an impact on clinical outcome.

4.3.3. RIFAXIMIN AND FIDAXOMICIN RESISTANCE

The antibiotic resistance mechanisms of *C. difficile* are typical of other Gram-positive bacteria, including mutation, selection and acquisition of mobile genetic elements (MGEs) that encode antibiotic resistance genes (Huang *et al.*, 2009). Rifaximin and fidaxomicin cause antibiotic resistance by modifying their target and creating SNPs in the *rpoB* gene.

It was hypothesised that neither fidaxomicin antibiotic resistance nor cross-resistance with rifaximin would be observed in the RAPID clinical isolates, which was confirmed upon completion of the antibiotic resistance plate assay. This outcome was unsurprising as it has been previously reported (Venugopal and Johnson, 2012; Babakhani *et al.*, 2014) that no cross-resistance has been observed between rifaximin and fidaxomicin, although they are both RNAP inhibitors. Complete fidaxomicin resistance has also never been reported *in vivo* from a patient derived strain, however, a strain isolated from a patient in a phase III trial had an elevated fidaxomicin MIC of 16μg/ml when they relapsed (Goldstein *et al.*, 2011). The Glu1073Lys, Gln1074Lys, and Val1143Phe SNPs in RpoB that confer resistance to fidaxomicin have been previously demonstrated in an *in vitro* study (Babakhani *et al.*, 2014). In the same research, the Asp237Tyr SNP in the *rpoC* gene has also been shown to provide fidaxomicin resistance.

Rifaximin resistance was observed in six RAPID isolates from three different relapse patients (12001_RL_C14, 15003_-1_C1, 15003_12_C1, 15003_RL_C1, 17002_RL2_C1 and 17002_RL3_C1) and two non-relapse patient RAPID isolates (01016_12_C1 and 03008_4_C2). The unblinded trial data revealed that all but one of the patients (15003) had been given the 4-week course of 'follow on' rifaximin, after metronidazole or vancomycin therapy. The diagnostic (15003_-1_C1), week 12 (15003_12_C1) and relapse samples (15003_RL_C1) from the patient who had received the placebo all contained a *C. difficile* strain resistant to rifaximin. This suggests that the patient was infected with a previously rifaximin resistant strain and that resistance did not develop whilst on the clinical trial, which has been previously documented (Curry *et al.*, 2009).

The three SNPs identified in the RpoB hotspot region were Gln489Lys, Arg505Lys and Ser507Leu (Figure 4.46).

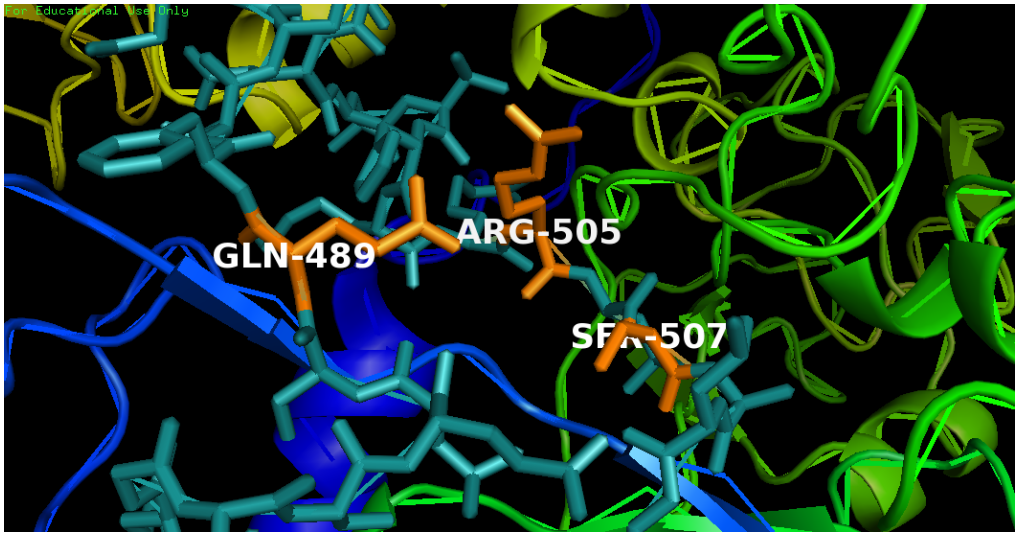


Figure 4.46 Identified RpoB Hotspot Single Nucleotide Polymorphisms

The figure illustrates the three residues (orange) in the RpoB hotspot region (teal) where SNPs were identified; Gln489Lys, Arg505Lys and Ser507Leu.

The glutamine to lysine SNP at position 489 in *C. difficile* has only previously been described once in a case study (Stevenson *et al.*, 2016) involving a patient also enrolled in the RAPID clinical trial. However, this SNP has been observed in *M. tuberculosis* rifaximin resistant strains (O'Sullivan *et al.*, 2005; Molodtsov *et al.*, 2017). The equivalent glutamine amino acid in *T. aquaticus* (position 393) has been shown to directly interact with rifaximin (Campbell *et al.*, 2001; O'Connor *et al.*, 2008). Glutamine is a polar, hydrophilic, neutral amino acid, and prefers to be on the protein surface in an aqueous environment. The residue is normally found in the protein active or binding sites as the polar side chain interacts well with other polar or charged atoms. Lysine is a polar, hydrophilic positively charged amino acid, and a substitution with this residue can affect protein activity, particularly if the uncharged glutamine is in the protein active site (Betts and Russell, 2003) (Figure 4.47).

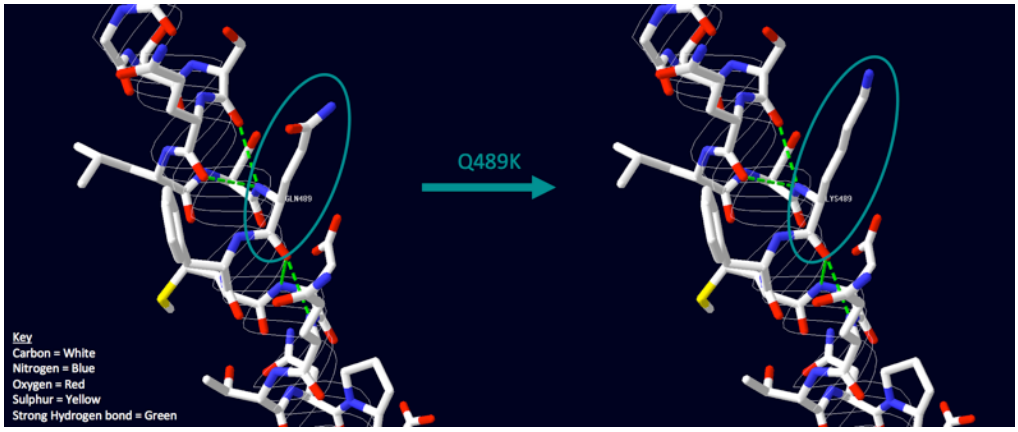


Figure 4.47 Gln489Lys Single Nucleotide Polymorphism

The figure illustrates the Gln489Lys SNP. Glutamine is a polar, hydrophilic, neutral amino acid, and prefers to be on the protein surface in an aqueous environment. The residue is normally found in the protein active or binding sites as the polar side chain interacts well with other polar or charged atoms. Lysine is a polar, hydrophilic positively charged amino acid, and a substitution with this residue can affect protein activity, particularly if the uncharged glutamine is in the protein active site (Betts and Russell, 2003).

One study (Dang *et al.*, 2016) determined that the arginine to lysine SNP at position 505 does not impose an *in vitro* fitness cost. It is therefore the most dominant mutation observed in *C. difficile* clinical isolates, and has been published in numerous papers (O'Connor *et al.*, 2008; Curry *et al.*, 2009; Miller *et al.*, 2011b; Pecavar *et al.*, 2012). The equivalent arginine amino acid in *T. aquaticus* (position 409) has been shown to directly interact with rifaximin (Campbell *et al.*, 2001; O'Connor *et al.*, 2008). Both arginine and lysine are positively charged, polar, hydrophilic amino acids. Arginine plays an important part in protein structure and contains a guanidinium group on the side-chain which pairs with negatively charged amino acids to create multiple protein stabilising hydrogen bonds. It is also commonly found in protein active or binding sites. Lysine can similarly form hydrogen bonds, however, as it contains only a single amino acid group, the number of bonds are limited, and so protein stability can be compromised when substituted (Betts and Russell, 2003) (Figure 4.48).

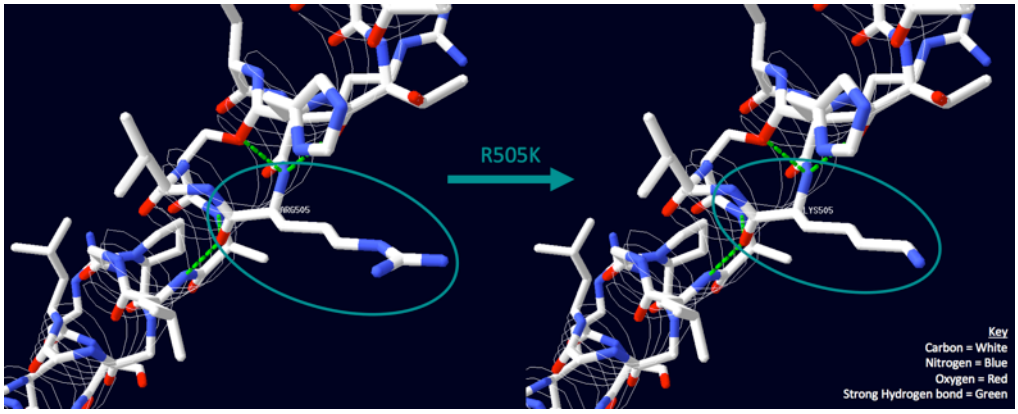


Figure 4.48 Arg505Lys Single Nucleotide Polymorphism

The figure illustrates the Arg505Lys SNP. Both arginine and lysine are positively charged, polar, hydrophilic amino acids. Arginine plays an important part in protein structure and contains a guanidinium group on the side-chain which pairs with negatively charged amino acids to create multiple protein stabilising hydrogen bonds. It is also commonly found in protein active or binding sites. Lysine can similarly form hydrogen bonds, however, as it contains only a single amino acid group, the number of bonds are limited, and so protein stability can be compromised when substituted (Betts and Russell, 2003).

The serine to leucine SNP at position 507 has been described previously in *S. aureus* (O'Connor *et al.*, 2008) and *M. tuberculosis* (O'Sullivan *et al.*, 2005; Molodtsov *et al.*, 2017), however, this is the first reported case in a *C. difficile* clinical isolate. Serine is a polar, hydrophilic, neutral amino acid and is usually observed within tight turns on the protein surface, where the side-chain hydroxyl oxygen can form a hydrogen bond with the protein backbone. It is also frequently found in protein active sites. Leucine is a non-polar, hydrophobic amino acid, and so substitution is disfavoured as the side chain is very non-reactive and rarely involved in protein function (Betts and Russell, 2003) (Figure 4.49).

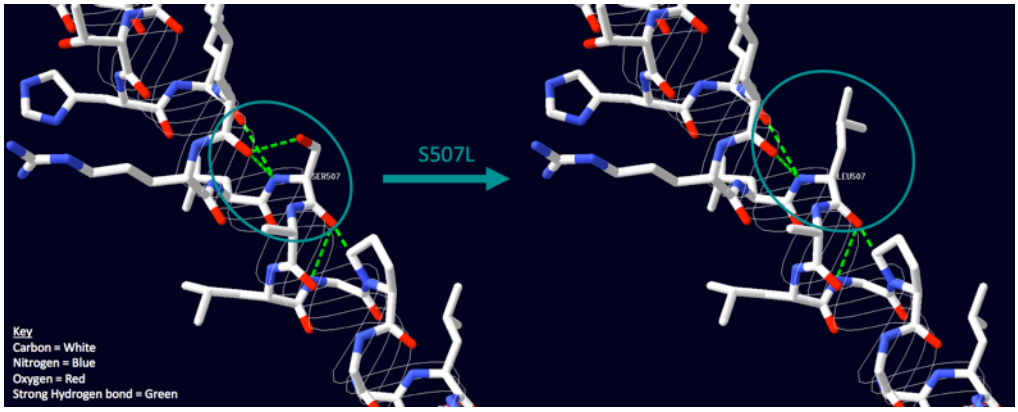


Figure 4.49 Ser507Leu Single Nucleotide Polymorphism

The figure illustrates the Ser507Leu SNP. Serine is a polar, hydrophilic, neutral amino acid and is usually observed within tight turns on the protein surface, where the side-chain hydroxyl oxygen can form a hydrogen bond with the protein backbone. It is also frequently found in protein active sites. Leucine is a non-polar, hydrophobic amino acid, and so substitution is disfavoured as the side chain is very non-reactive and rarely involved in protein function (Betts and Russell, 2003).

RAPID isolate 17002_RL2_C1 contained five separate SNPs, which were all located outside of the RpoB hotspot region; Ile750Met, Gln1037Glu, Val1205Ala, Ala1207Asn and Thr1208Ala. BLAST search results of the complete RpoB gene indicated that Gln1037Glu, Val1205Ala, Ala1207Asn and Thr1208Ala substitutions were very common and that those residues were not well conserved. Therefore, the rifaximin resistance demonstrated in the strain is thought to be due to the isoleucine to methionine SNP at position 750. Both isoleucine and methionine are non-polar, hydrophobic, non-charged amino acids. Isoleucine is Cβ carbon branched and contains two non-hydrogen substitutes. The side chain is also very non-reactive, however, it can be involved in binding of hydrophobic ligands. Methionine has a very similar function, although additionally it contains a sulphur atom which can bind to atoms such as metals. It is also not one of the three amino acids (which include valine, threonine and isoleucine) that are Cβ carbon branched and so backbone conformation is less restricted. Therefore, a methionine substitution may result in loss of protein structural integrity (Betts and Russell, 2003) (Figure 4.50).

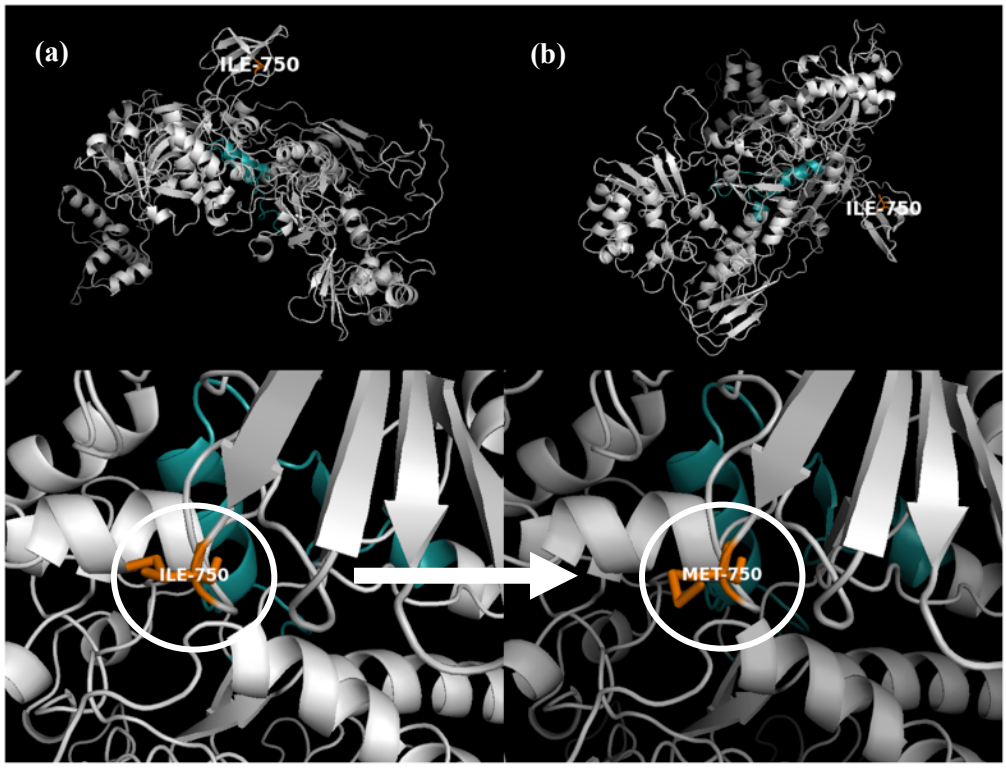


Figure 4.50 Ile750Met Single Nucleotide Polymorphism

The figure illustrates the Ile750Met SNP. **(a)** and **(b)** demonstrate the isoleucine at position 750 (orange) in relation to the RpoB hotspot (teal). Both isoleucine and methionine are non-polar, hydrophobic, non-charged amino acids. Isoleucine is Cβ carbon branched and contains two non-hydrogen substitutes. The side chain is also very non-reactive, however, it can be involved in binding of hydrophobic ligands. Methionine has a very similar function, although additionally it contains a sulphur atom which can bind to atoms such as metals (Betts and Russell, 2003).

Rifaximin resistance has been observed in clinical isolates (as discussed previously in Chapter 4.1.2.3), and so it was hypothesised that emergence of resistance in the RAPID strains would be observed in at least one patient. This study had shown this to be the case in five patients, and demonstrated *rpoB* SNPs which have been previously reported in *C. difficile* (the glutamine to lysine SNP at position 489 (Stevenson *et al.*, 2016) and the arginine to lysine SNP at position 505 (O'Connor *et al.*, 2008; Curry *et al.*, 2009; Miller *et al.*, 2011; Pecavar *et al.*, 2012)).

As resistance was observed, it was important to correlate the findings with patient outcome. However, it is difficult to ascertain whether the resistance to rifaximin caused patient recurrent episodes. Eight patients who were given 'follow on' rifaximin, did not demonstrate antibiotic resistance. Furthermore, two patients (01016 and 03008) of the four, who were found to have resistance, did not relapse and in the rifaximin sensitive placebo group, three patients (11001, 11007 and 12004) relapsed. Additionally, the difference in percentage of patients that relapsed in the rifaximin group compared to the placebo group were minimal. One could speculate that given the amount of evidence in the literature (Marchese *et al.*, 2000; Hecht *et al.*, 2007; Johnson *et al.*, 2007; O'Connor *et al.*, 2008; Curry *et al.*, 2009; Johnson *et al.*, 2009; Carman *et al.*, 2012; Obuch-Woszczatyński *et al.*, 2013; Freeman *et al.*, 2015b; Kullin *et al.*, 2017), it *is* likely that exposure to rifaximin increases the risk of developing resistance in certain individuals, however, patient 18001, who was given 'follow on' rifaximin, did not demonstrate rifaximin resistance, but they did relapse within the 12 weeks. (Johnson *et al.*, 2009) noted previously that *C. difficile* isolates from patients that failed treatment did not necessarily demonstrate rifaximin resistance. A larger cohort of rifaximin resistant patients would be necessary to verify the correlation between resistance and patient outcome.

Furthermore, due to time constraints, five of the eight RAPID isolates (15003_-1_C1, 15003_12_C1, 15003_RL_C1, 17002_RL2_C1 and 17002_RL3_C1), which were rifaximin resistant, were not ribotyped, and so it is difficult to draw any conclusions about patient outcome regarding ribotype. With regard to patient 17002, it cannot be confirmed that the initial diagnostic strain was the same strain that the patient relapsed with, due to a lack of ribotyping data, and so it could be argued that the patient may have experienced a recurrent episode with a previously rifaximin resistant strain.

4.3.4. *ROBINSONIELLA PEORIENSIS*

This section of the research was not originally part of the aims and objectives of this study, however, it is included in the work as a point of interest. Both 12006_12_C1 and 13007_4_C3 were identified as *R. peoriensis* from the

BLAST results. *R. peoriensis* was originally identified in 2009 from a swine-manure storage pit and a patient clinical source (Cotta *et al.*, 2009). The human specimen was isolated from a deep heel wound of a 79-year old female and deposited in the University of Göteborg (Sweden) culture collection. The bacterium was characterised as an anaerobic, spore-forming (sub-terminal), non-motile, Gram-positive short bacillus, which resides within the clostridial rRNA cluster XIVa subgroup (Figure 4.51).

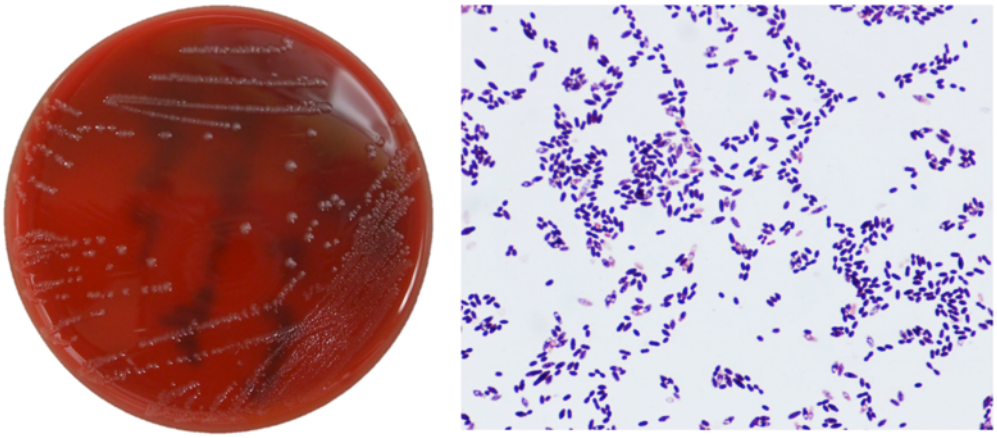


Figure 4.51 Robinsoniella peoriensis Culture and Gram Stain Appearance (Gomez et al., 2011)

The blood agar plate displays non-haemolytic smooth *R. peoriensis* colonies after 48 hours' incubation. The Gram stain illustrates Gram-positive coccobacilli with evident sub-terminal spores.

The occurrence of *R. peoriensis* in animal stool, other than swine manure, has been reported, including a wood turtle (Braasch *et al.*, 2015) and an endangered Yangtze finless porpoise (McLaughlin *et al.*, 2013). Very few human cases have been described since identification of the organism. *R. peoriensis* has been confirmed as the causative organism in four bacteraemia cases (Shen *et al.*, 2010; Gomez *et al.*, 2011; Jeon *et al.*, 2012; Lim *et al.*, 2017), two automobile accident surgical wounds (Gomez *et al.*, 2011), two orthopaedic prosthetic device infections (Cassir *et al.*, 2012; Rieber *et al.*, 2016), an abdominal fluid infection (Gomez *et al.*, 2011), a muscular

haematoma (Lopez *et al.*, 2010) and an aortic homograft infection (Mertes *et al.*, 2017).

R. peoriensis has also been identified in the stool of premature neonates (Ferraris *et al.*, 2012), and in the ascending colon of an 11th century A.D. pre-Colombian Andean mummy (Santiago-Rodriguez *et al.*, 2015), suggesting that this organism could be part of the commensal gastrointestinal flora. Furthermore, it has been shown to dominate in a mouse germ-free gut, even in the presence of other bacterial communities isolated from a zebrafish and termite gut (Seedorf *et al.*, 2014).

Last year, a draft genome sequence of *R. peoriensis* strain 6600698 was published (Roberts *et al.*, 2016). This was of particular interest as the strain was isolated from an 82-year old male patient that had elevated faecal lactoferrin levels, indicating intestinal inflammation. The isolate was initially classified as a non-toxigenic *C. difficile* due to patient clinical symptoms, growth on *C. difficile* selective agar, a positive *C. difficile* glutamate dehydrogenase (GDH) test, but a negative enzyme immunoassay for the detection of *C. difficile* toxin A and B. Whole genome sequencing was completed when *C. difficile* ribotyping produced an unusual banding pattern, which do not match existing standards. Specific 16s rRNA PCR primers have now been recently developed to aid with the rapid identification of *R. peoriensis* (Whitehead *et al.*, 2017). The *in vivo* importance of *R. peoriensis* in the RAPID stool samples is uncertain. Unfortunately, patient 12006 had no additional stool samples submitted to the clinical trial for comparison to the week 12 isolate, however, it may be assumed that the patient was confirmed to be infected with *C. difficile* using the initial diagnostic sample. Patient 13007 also had a week 0 stool sample taken. Although this was one of the RAPID isolates which were not ribotyped, it did present with a typical *C. difficile* sporulation profile, when compared to the week 4 *R. peoriensis* isolate. Whether *R. peoriensis* has merely colonised the gut, alongside the CDI, or is causing diarrhoea in these patients is not known. It is certainly an organism of interest and the *in vivo* significance should be further investigated.

CHAPTER 5. GENERAL DISCUSSION

5.1. KEY OUTCOMES OF THIS RESEARCH

5.1.1. THE *CLOSTRIDIUM DIFFICILE* R20291 CDT GENE LOCUS IS TRANSCRIPTIONALLY LINKED

Transcriptional linkage of the binary toxin gene locus was assessed in *C. difficile* R20291 and examined in Chapter 3. The characterisation of CDT transcription was initially completed to aid with CDT complementation insert design. The reverse transcriptase (RT) PCR results demonstrated that the *C. difficile* R20291 CDT gene locus is transcriptionally linked, with one mRNA transcript produced. RNA expression was qualitatively detected through the amplification of complementary DNA (cDNA) from the introns between *cdtR* and *cdtA*, and *cdtA* and *cdtB*. It also revealed that transcription occurs at both early and late exponential phase, as well as in stationary phase.

Interestingly, the *in silico* promoter sequence analysis revealed putative promoters upstream of the *cdtR* and *cdtA* genes. This suggested that transcription of the CDT gene locus is regulated by *Escherichia coli* sigma70 (σ^{70})-like sigma factors (Metcalf and Weese, 2011). Although the study of CDT transcriptional linkage indicated that one mRNA transcript is generated, it has been described in a previously published paper (Lyon *et al.*, 2016) that CdtA and CdtB proteins are still produced at decreased levels in a *cdtR* mutant strain, therefore, indicating that *cdtR* is not essential for CDT production *in vivo*. This inconsistency could be due to the secondary promoter identified upstream of the *cdtA* gene that is continuing to allow *cdtA* and *cdtB* transcription, without the presence of *cdtR*.

5.1.2. FIRST REPORTED *CLOSTRIDIUM DIFFICILE* R20291 CDT IN-FRAME DELETION AND COMPLEMENTED STRAINS CREATED AND CHARACTERISED

The work discussed in Chapter 3, detailed the creation of CDT binary toxin allelic exchange knock-out strains in *Clostridium difficile* R20291, PaLoc allelic exchange knock-out strains in *C. difficile* R20291 and the CDT knock-out strains, as well as, CDT complemented strains. The following four binary toxin allelic exchange in-frame deletion mutant strains are the first reported;

R20291Δ*cdtA*Δ*cdtB*, R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*, R20291ΔPaLocΔ*cdtA*Δ*cdtB* and R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB*. Furthermore, the following two CDT complemented strains have never been described previously; R20291Δ*cdtR***cdtR* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB*. Accompanied by R20291Δ*cdtR*, R20291ΔPaLoc and R20291ΔPaLocΔ*cdtR*, all strains were characterised in terms of growth, cytotoxicity and biofilm formation.

Growth was significantly increased between the R20291 WT strain and the R20291ΔPaLocΔ*cdtR*, R20291ΔPaLocΔ*cdtA*Δ*cdtB* and R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* strains (although R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* visibly appeared indistinguishable from the WT control). This is thought to be associated with the lack of toxin production as studies (Carlson *et al.*, 2013; Tschudin-Sutter *et al.*, 2016) have shown that ribotype 027 isolates, which exhibit high toxin production, also have a low growth rate. With regards to the CDT knock-out strains, both R20291Δ*cdtA*Δ*cdtB* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB* displayed significantly increased growth, when compared to the R20291 WT strain. This result may again be explained by the finding that certain strains with increased toxin production exhibit a low growth rate (Carlson *et al.*, 2013; Tschudin-Sutter *et al.*, 2016), as well as the presence of the secondary promoter upstream of the *cdtA* gene, identified in the *in silico* promoter sequence analysis, which would allow the *cdtA* and *cdtB* genes to be transcribed without the presence of the *cdtR* gene. The R20291Δ*cdtR***cdtR* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* complemented strains both displayed a significantly increased growth rate when compared to the R20291 WT strain (although R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* visibly appeared indistinguishable from the WT control). However, only the growth of R20291Δ*cdtR***cdtR* was significantly increased when compared to the original CDT knock-out strain. In both cases, the *cdtR* gene appears to be up-regulated at the *pyrE* gene locus, overexpressing the phenotype.

A visible and statistically significant difference was observed between the R20291 WT strain and the R20291ΔPaLoc strain for cytotoxicity. The substantial decrease in toxin A and B cytotoxic effect when the PaLoc is

deleted was anticipated. The remaining cytotoxicity observed was considered to be due to the binary toxin. No cytotoxicity was observed for all three CDT ΔPaLoc knock-out strains, when compared to the R20291 WT strain. The evidence that no cytotoxic effect was demonstrated for the R20291ΔPaLocΔ*cdtR* knock-out strain opposes the data in the (Lyon *et al.*, 2016) study. However, in that research it was Western blot analysis that illustrated *cdtR* mutants were still able to produce CdtA and CdtB. Therefore, it appears that although the *cdtA* and *cdtB* genes are transcribed and translated, the Vero cell cytotoxic effect is inconsequential (Bilverstone *et al.*, 2017). All the CDT knock-out strains displayed a visible and statistically significant cytotoxicity difference to the R20291 WT strain. Interestingly, the Vero cell cytotoxic effect was significantly decreased in these strains, despite the presence of the PaLoc. A previous study (Lyon *et al.*, 2016), recently demonstrated that toxin A and B cytotoxic activity was lower in supernatant from a *cdtR* mutant when compared to the WT strain. Other *in vivo* research (Kuehne *et al.*, 2014) has demonstrated that the presence of CDT in a toxin B mutant which continues to produce toxin A displayed increased virulence in a hamster model, with the animals dying at a significantly faster rate, when compared to that of a toxin A positive, toxin B and CDT negative mutant strain. It appears that CDT may play a synergistic role with the production of toxin A and B. The two R20291Δ*cdtR***cdtR* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* complemented strains both displayed cytotoxic activity comparable to the R20291 WT strain. However, only the R20291Δ*cdtR***cdtR* strain exhibited no significant difference. There was also a significant difference when the *cdtR* complemented strain was compared to the original knock-out mutant strain. This outcome again demonstrated that the *cdtR* gene appears to be up-regulated at the *pyrE* gene locus.

Finally, investigation into the effect of binary toxin deletion on the formation of biofilms revealed that there was no clear elimination of biofilm formation in the CDT knock-out mutant strains, with correlates with the findings found (Dapa *et al.*, 2013) in with *cdtA* and *cdtB* ClosTron mutants.

5.1.3. THE FULL BINARY TOXIN *cdtR* IN RIBOTYPE 078 STRAINS POSSESS A PREMATURE STOP CODON

Rifaximin for Preventing Relapse of *Clostridium difficile* Associated Diarrhoea (RAPID) patient samples that contained a *C. difficile* strain with a full CDT gene locus were confirmed and discussed in Chapter 4. In 56 RAPID *C. difficile* isolates investigated (excluding the two isolates which were confirmed to be *R. peroriensis*), 16.1% contained the full binary toxin gene locus (ribotype 023 and 078, which have been previously established in the literature (Bacci *et al.*, 2011; Gerding *et al.*, 2014)).

Single nucleotide polymorphism (SNP) analysis of the full binary toxin gene locus determined that the *cdtR* of 01027_-1_C1 (ribotype 078) and 17002_RL2_C1 (expected to be ribotype 078, but unconfirmed) contained a G to a T SNP at position 322, which led to a premature stop codon. This would cause translation of a truncated CdtR protein of only 107 amino acids, instead of the 249 usually observed (Bouvet and Popoff, 2008; Metcalf and Weese, 2011). The TAA stop codon is found in most ribotype 078 strains (Peng *et al.*, 2017b).

All nine full CDT gene locus RAPID isolates did not contain SNPs in any of the crucial and important amino acid residues (Arg302, Arg303, Arg359, Gln307, Asn342, Ser345, Glu385 and Glu387 (Sundriyal *et al.*, 2009; Gerding *et al.*, 2014)) for the *cdtA* gene.

With regards to *cdtB*, RAPID isolates 01027_-1_C1 (ribotype 078) and 17002_RL2_C1 (ribotype not confirmed) both contained a SNP at Lys209 to an arginine. It is thought that CdtB cleavage occurs between the lysine at position 209 and the leucine at position 210 (Gerding *et al.*, 2014). A recent study (Hemmasi *et al.*, 2015), also demonstrated that only amino acids 757-866 are necessary for the binding of CdtB to the host cell lipolysis-stimulated lipoprotein receptor (LSR). Several SNPs were identified in this region, including Met762Ile (67% of full CDT gene locus samples), Phe769Ser (67%), Ala770Ser (44%), Thr781Ala (67%) and Ala789Thr (22%). It is unclear, however, whether any of these mutations would impact on CDT production. Further work would primarily include assessing the production of

binary toxin in the full CDT gene locus RAPID isolates. Additionally, point mutations could be created in key amino acid residues, with the CDT production evaluated in these mutant strains. This investigation has been carried out previously (Gulke *et al.*, 2001), however, only a small number of mutations in the *cdtA* gene were examined.

5.1.4. *cdtR* IS WELL CONSERVED IN CLINICAL STRAINS CONTAINING THE GHOST CDT GENE LOCUS

RAPID patient samples that contained a *C. difficile* strain with a ghost CDT gene locus were verified and examined in Chapter 4. In 56 RAPID *C. difficile* isolates investigated (excluding the two isolates which were confirmed to be *R. peroriensis*), 71.4% contained the ghost CDT gene locus (ribotype 002, 005, 011, 014 [previously established (Knight *et al.*, 2017)], 015, 020, 029, 070 and 215).

Far less SNPs were demonstrated for the ghost *cdtR*, when compared to the partial CDT 630 strain, than with the full gene locus *cdtR*, when compared to the complete CDT R20291 strain (11 versus 18 SNPs). When the ghost *cdtR* was compared to R20291 *cdtR*, the gene was even more well conserved, with a total of 2 SNPs. The purpose of this remains unknown, however, it was speculated that the ghost *cdtR* could be regulating other genes in the genome. Recently published research (Lyon *et al.*, 2016) demonstrated that *cdtR* in two ribotype 027 strains also regulated *C. difficile* toxin A and B production. Conversely, this was shown not to be the case in an 078 strain and the 630 (ribotype 012) strain. Further investigation into the role of the ghost *cdtR*, with a variety of different ribotypes, is necessary.

5.1.5. A NUMBER OF *rpoB* SNPs CAUSE RIFAXIMIN ANTIBIOTIC RESISTANCE IN CLINICAL ISOLATES

Rifaximin resistance was observed in six RAPID isolates from three different relapse patients (12001_RL_C14, 15003_-1_C1, 15003_12_C1, 15003_RL_C1, 17002_RL2_C1 and 17002_RL3_C1) and two non-relapse patient RAPID isolates (01016_12_C1 and 03008_4_C2). The unblinded trial data revealed that all but one of the patients (15003) had been given the 4-

week course of ‘follow on’ rifaximin after metronidazole or vancomycin therapy. For the patient who received the placebo, all three samples (15003_-1_C1, 15003_12_C1 and 15003_RL_C1) contained a *C. difficile* strain previously resistant to rifaximin before clinical trial commencement.

The SNPs conferring resistance to rifaximin were detailed in Chapter 4. The three SNPs identified in the RpoB hotspot region were Gln489Lys, Arg505Lys and Ser507Leu. The glutamine to lysine SNP at position 489 in *C. difficile* has only previously been described once in a case study (Stevenson *et al.*, 2016) involving a patient also enrolled in the RAPID clinical trial. This particular case was complicated, as the patient relapsed twice and presented with coinfection of ribotype 002 and 014. Rifaximin resistance was detected in the ribotype 002 strain in the first relapse sample, taken after the week 0 sample. At the time of publication, it was not known whether patient 01008 was on rifaximin or placebo therapy, however, the unblinded trial data revealed the patient had received ‘follow on’ rifaximin. The Gln489Lys SNP has been observed in *Mycobacterium tuberculosis* rifaximin resistant strains (O'Sullivan *et al.*, 2005; Molodtsov *et al.*, 2017). The equivalent glutamine amino acid in *Thermus aquaticus* (position 393) has been shown to directly interact with rifaximin (Campbell *et al.*, 2001; O'Connor *et al.*, 2008).

One study (Dang *et al.*, 2016) determined that the arginine to lysine SNP at position 505 does not impose an *in vitro* fitness cost. It is therefore the most dominant mutation observed in *C. difficile* clinical isolates, and has been published in numerous papers (O'Connor *et al.*, 2008; Curry *et al.*, 2009; Miller *et al.*, 2011b; Pecavar *et al.*, 2012). The equivalent arginine amino acid in *T. aquaticus* (position 409) has been shown to directly interact with rifaximin (Campbell *et al.*, 2001; O'Connor *et al.*, 2008).

The serine to leucine SNP at position 507 has been described previously in *Staphylococcus aureus* (O'Connor *et al.*, 2008) and *M. tuberculosis* (O'Sullivan *et al.*, 2005; Molodtsov *et al.*, 2017), however, this is the first reported case in a *C. difficile* clinical isolate.

RAPID isolate 17002_RL2_C1 contained five separate SNPs, which were all located outside of the RpoB hotspot region; Ile750Met, Gln1037Glu,

Val1205Ala, Ala1207Asn and Thr1208Ala. BLAST search results of the complete *rpoB* gene indicated that Gln1037Glu, Val1205Ala, Ala1207Asn and Thr1208Ala substitutions were very common, and present in sensitive strains. Consequently, the rifaximin resistance demonstrated in the strain is thought to be due to the isoleucine to a methionine SNP at position 750.

As resistance was observed, it was important to correlate the findings with patient outcome. However, it is difficult to ascertain whether the resistance to rifaximin caused patient recurrent episodes. Eight patients who were given 'follow on' rifaximin, did not demonstrate antibiotic resistance. Furthermore, two patients (01016 and 03008) of the four, who were found to have resistance, did not relapse and in the rifaximin sensitive placebo group, three patients (11001, 11007 and 12004) relapsed. Additionally, the difference in percentage of patients that relapsed in the rifaximin group compared to the placebo group were minimal. One could speculate that given the amount of evidence in the literature (Marchese *et al.*, 2000; Hecht *et al.*, 2007; Johnson *et al.*, 2007; O'Connor *et al.*, 2008; Curry *et al.*, 2009; Johnson *et al.*, 2009; Carman *et al.*, 2012; Obuch-Woszczatyński *et al.*, 2013; Freeman *et al.*, 2015b; Kullin *et al.*, 2017), it *is* likely that exposure to rifaximin increases the risk of developing resistance in certain individuals, however, there may be other strain dependent and host risk factors, which increase the risk. It is necessary to research this interaction further, with a larger cohort of rifaximin resistant patients, before recommending that rifaximin is appropriate for the prevention of *C. difficile* relapse.

5.1.6. THE RATE OF SPORULATION IN CLINICAL STRAINS IS INCREASED AT LATER TIME POINTS

To determine whether the RAPID patient isolates exhibited any differences in sporulation, twelve RAPID isolates (01018_-1_C17, 01018_-1_C20, 01023_-1_C2, 01023_0_C1, 11007_-1_C2, 11007_-1_C5, 11007_0_C14, 11007_RL_C1, 13007_0_C1, 15003_-1_C1, 15003_12_C1 and 15003_RL_C1) were analysed and the results described in Chapter 4. All strains tested reached a significantly higher cfu/ml count at the later time points compared to the 630Δ*erm* WT control strain. Two studies (Wilcox and

Fawley, 2000; Fawley *et al.*, 2007) researching the inhibitory effect of cleaning agents on the sporulation rate of *C. difficile* demonstrated that exposure to a low level of cleaning agent compared to no cleaning agent increased sporulation capacity. The authors determined that hospital cleaning and the choice of cleaning agent used, may promote sporulation and be associated with *C. difficile* persistence. One could speculate that this conclusion could similarly explain the results observed for the RAPID patient samples. However, there is no evidence to suggest whether this effect is long-lasting and increases sporulation *in vivo* or only occurs when the strains are directly exposed to the cleaning agents *in vitro*.

5.1.7. CD630_26350 MAY PLAY AN IMPORTANT ROLE IN SPORULATION

In Chapter 4, a ClosTron mutant was generated to knock out the *C. difficile* 630 gene 26350, which encodes for a putative spore envelope protein. It is thought to be part of the YIEGIA protein family, which includes the *Bacillus subtilis* YphB sporulation protein. *B. subtilis yphB* gene expression is regulated by an early forespore-specific sigma factor *sigF* (σF), and has been previously shown not to be essential in sporulation or germination (Decatur and Losick, 1996). Sporulation did still occur in the ClosTron mutant, however, the assay additionally demonstrated that the cfu/ml was reduced and significantly different at the 96-hour time point when compared to the 630Δ*erm* WT strain. This suggests that gene 26350 is not crucial for sporulation but may play an important role in achieving an optimal sporulation rate. Furthermore, it could be speculated *in vivo* that this mutation may hinder sporulation sufficiently in the host that the risk of patient relapse would be reduced.

5.1.8. *ROBINSONIELLA PERORIENSIS* CAN BE CONFUSED FOR *CLOSTRIDIUM DIFFICILE* IN CLINICAL SAMPLES USING CONVENTIONAL MICROBIOLOGICAL TECHNIQUES

Both 12006_12_C1 and 13007_4_C3 RAPID isolates were confirmed to be *Robinsoniella peroriensis* after 16s rRNA analysis was performed in Chapter

4. Initially, they both grew well on Braziers agar and BHIS agar supplemented with *C. difficile* selective supplement. However, when characterising the strains, neither performed as expected and so it was necessary to confirm the organism.

R. peroriensis is characterised as an anaerobic, spore-forming (sub-terminal), non-motile, Gram-positive short bacillus, which resides within the clostridial rRNA cluster XIVa subgroup (Cotta *et al.*, 2009). The bacterium has been confirmed as the causative organism in four bacteraemia cases (Shen *et al.*, 2010; Gomez *et al.*, 2011; Jeon *et al.*, 2012; Lim *et al.*, 2017), two automobile accident surgical wounds (Gomez *et al.*, 2011), two orthopaedic prosthetic device infections (Cassir *et al.*, 2012; Rieber *et al.*, 2016), an abdominal fluid infection (Gomez *et al.*, 2011), a muscular haematoma (Lopez *et al.*, 2010) and an aortic homograft infection (Mertes *et al.*, 2017). It has also been isolated from the stool of premature neonates (Ferraris *et al.*, 2012) suggesting that this organism could be part of the commensal gastrointestinal flora, but equally become an opportunistic pathogen.

The significance of *R. peroriensis* in the RAPID stool samples is uncertain. It is not known whether the bacterium has merely colonised the gut or is causing diarrhoea in these patients. A recently published draft genome sequence of *R. peroriensis* strain 6600698 (Roberts *et al.*, 2016), which was isolated from an 82-year old male patient with elevated faecal lactoferrin levels, was initially classified as a non-toxigenic *C. difficile* strain. The patient clinical symptoms, growth on *C. difficile* selective agar, a positive *C. difficile* glutamate dehydrogenase (GDH) test, but a negative enzyme immunoassay for the detection of *C. difficile* toxin A and B all suggested the diagnosis. However, whole genome sequencing was completed when *C. difficile* ribotyping produced an unusual banding pattern, confirming the isolate was in fact *R. peroriensis*. This organism could therefore have a significant impact on the correct diagnosis of *C. difficile* when using standard diagnostic techniques.

5.2. LIMITATIONS OF THIS WORK AND PROPOSED FUTURE RESEARCH

5.2.1. TRANSCRIPTIONAL LINKAGE OF THE CDT GENE LOCUS

The RT-PCR results, analysed in Chapter 3, demonstrated that the *C. difficile* R20291 CDT gene locus is transcriptionally linked, with one mRNA transcript produced, however, it is important to note that this method is not quantitative and so the size of the mRNA transcript cannot be accurately predicted. RT-qPCR or a Northern blot are both quantitative gene expression methods, which could be employed in future transcriptional studies investigating the amount of CDT produced.

5.2.2. GENERATION OF THE CDT IN-FRAME DELETION AND COMPLEMENTED STRAINS

As discussed in Chapter 3, R20291Δ*cdtR*, R20291Δ*cdtA*Δ*cdtB*, R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*, R20291ΔPaLoc, R20291ΔPaLocΔ*cdtR*, R20291ΔPaLocΔ*cdtA*Δ*cdtB*, R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* knock-out strains, and the R20291Δ*cdtR***cdtR* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* complemented strains were produced. The in-frame deletion mutant generation was challenging at certain points. This was particularly the case with the SOEing PCR, where problems with non-specific binding and low target yields were encountered. A high level of optimisation was required, which was very time consuming. To counteract this problem in the future, the LHA and RHA amplification can be completed, as previously demonstrated, with each subsequent fragment (including the linearised vector) assembled using Gibson Assembly or NEBuilder HiFi DNA Assembly (NEB). Gibson Assembly was used successfully in this study to produce the pMTL-YN18_cdtA_cdtB allelic exchange plasmid and the pMTL-YN2C_PR_cdtR_cdtA complementation plasmid. The NEBuilder HiFi DNA Assembly has a comparable format to Gibson Assembly but has been shown to offer improved efficiency and accuracy, particularly with low DNA inputs and larger fragments.

C. difficile R20291 is also known to have a low conjugation frequency compared to the 630 strain (Kirk and Fagan, 2016). Initial conjugation experiments were conducted using a previously optimised protocol (Heap *et al.*, 2009), which did yield transconjugant colonies. However, to increase conjugation efficiency, the alterations suggested in a protocol published more recently (Kirk and Fagan, 2016) were accommodated. This increased subsequent conjugation frequency from 10 to 20-fold.

Future generation of in-frame deletion mutant strains may be accomplished using a novel tool in molecular biology. While conducting this work, a precise gene editing tool was developed based on the type II prokaryotic CRISPR (clustered regularly interspaced short palindromic repeats)/Cas adaptive immune system from *Streptococcus pyogenes*. This technique allows for targeted and efficient site-specific DNA cleavage using the RNA-guided Cas9 nuclease (Cong *et al.*, 2013). The CRISPR/Cas9 tool to generate markerless gene deletion in the chromosome has been achieved in a number of *Clostridium* species, including *Clostridium beijerinckii* (Wang *et al.*, 2015; Li *et al.*, 2016; Wang *et al.*, 2016), *Clostridium cellulolyticum* (Xu *et al.*, 2015), *Clostridium acetobutylicum* (Li *et al.*, 2016), *Clostridium pasteurianum* (Pyne *et al.*, 2016) and *Clostridium ljungdahlii* (Huang *et al.*, 2016). However, as of yet, CRISPR/Cas 9 gene deletion in *C. difficile* has not been demonstrated.

5.2.3. CDT MUTANT STRAIN CHARACTERISATION

The generated R20291Δ*cdtR*, R20291Δ*cdtA*Δ*cdtB*, R20291Δ*cdtR*Δ*cdtA*Δ*cdtB*, R20291ΔPaLoc, R20291ΔPaLocΔ*cdtR*, R20291ΔPaLocΔ*cdtA*Δ*cdtB*, R20291ΔPaLocΔ*cdtR*Δ*cdtA*Δ*cdtB* knock-out strains, and the R20291Δ*cdtR***cdtR* and R20291Δ*cdtR*Δ*cdtA*Δ*cdtB***cdtR***cdtA***cdtB* complemented strains were assessed for growth, cytotoxicity and biofilm formation, as discussed in Chapter 3.

To further enhance knowledge on the binary toxin mechanism of action, a cytotoxicity assay using the human colon adenocarcinoma cell line HT29 (Martínez-Maqueda *et al.*, 2015), could be performed. They are considered a superior *in vitro* model when compared to the Vero cell line, due to their

similarities with small intestine enterocytes. An ADP-ribosyltransferase assay (Stubbs *et al.*, 2000; Barbut *et al.*, 2005), which assesses the production of actin-specific ADP-ribosylating activity, could additionally be used to detect the presence of binary toxin. In this case, it was not performed as the assay requires a radioactive material; nicotinamide adenine dinucleotide (NAD) ^{32}P, labelled on the adenylate phosphate. It is necessary to have specific training and handling precautions for phorphorus-32.

The biofilm assay, to assess formation of biofilms in the CDT knock-out mutants, demonstrated replicate error bars which were relatively large. This is thought to be due to the fragile biofilms lack of robustness at the phosphate buffered saline (PBS) washing steps (Chapter 2.9.5). In this work, the 24-well plate biofilm assay employed was adapted from (Dapa *et al.*, 2013). Other published papers (O'Toole, 2011; Coffey *et al.*, 2014) have described using a 96-well plate, which may lead to thicker biofilm formation, as there is less surface area. This could prevent the fragile biofilms from breaking apart at the PBS washing steps, reducing the observed error bars among replicates.

Lastly, much research has been carried out on the role of CDT and colonisation (as discussed in Chapter 3.1.2.2.) and so investigating adherence with the CDT in-frame deletion mutant strains using Caco-2 cells would complement this research.

5.2.4. *IN VIVO* ANIMAL INFECTION MODEL EXPERIMENTS

The CDT in-frame deletion mutant strains from Chapter 3 could be used to determine if binary toxin-producing strains are more virulent and play a synergistic role with the production of toxin A and B, in a hamster infection model. A similar experiment was carried out utilising previously generated CDT ClosTron mutants (Kuehne *et al.*, 2014), which demonstrated that the presence of CDT in a toxin B mutant which continues to produce toxin A displayed increased virulence when compared to that of a toxin A positive, toxin B and CDT negative mutant strain. Adherence investigations may also be performed *in vivo* with human microbiota-associated mice (Baban *et al.*, 2013).

5.2.5. CDT AND THE IMMUNE RESPONSE

A recent study (Cowardin *et al.*, 2016) established that binary toxin enhances *C. difficile* virulence by suppressing the protective host eosinophil response via the Toll-like receptor 2 (TLR2)-dependent pathway. Another paper (Jafari *et al.*, 2013) suggested that the CDT-producing R20291 strain elicits an increased interleukin (IL)-12 cytokine response, which culminates in greater type 1 T helper (Th1) cell immunity, when compared to strain 630. Furthermore, in preliminary data (Kuehne *et al.*, unpublished), a CDT and toxin A and B synergistic effect was observed on C57BL6 mouse dendritic cells, indicating that the binary toxin may also have a role in IL-23 activation. The CDT in-frame deletion mutant strains from Chapter 3 could be employed in prospective binary toxin immune response investigations.

5.2.6. RAPID CLINICAL STUDY

5.2.6.1. RAPID TRIAL RECRUITMENT AND PARTICIPANT DATA

The main limitations when conducting research on the RAPID trial (the results of which were analysed in Chapter 4) were those which were not under the control of the investigator. Initially, a total of 151 patients were enrolled onto the study from twenty different hospital sites. However, due to patient and hospital site nonfulfillment, only 58 stool samples from 29 patients collected from nine hospital sites were analysed. There was a distinct lack of compliance from satellite hospital sites, as well as a number of patients withdrawing consent throughout the study.

Unfortunately, severity of disease (Table 5.1) data for each patient were not obtained in the RAPID clinical study. Although this was never intended to be collected, this would have been particularly useful when analysing the RAPID isolates and relating findings to patient outcome, specifically relapse.

Table 5.1 Severity of *Clostridium difficile* Infection, as Defined by Public Health England (Public Health England and Wilcox, 2013)

Disease Severity	Definition
Mild	Not associated with an increased white cell count (WCC). It is typically associated with less than three episodes of loose stools (5-7 on the Bristol Stool Chart) per day.
Moderate	Associated with an increased WCC (but less than 15 x 10^9/L) and typically associated with 3–5 loose stools per day.
Severe	Associated with a WCC greater than 15 x 10^9/L, or an acutely increased serum creatinine concentration (greater than 50% increase above baseline), or a temperature higher than 38.5°C, or evidence of severe colitis (abdominal or radiological signs). The number of stools may be a less reliable indicator of severity.
Life-Threatening	Signs and symptoms include hypotension, partial or complete ileus, toxic megacolon, or computerised tomography (CT) evidence of severe disease.

However, it was intended to document which patients had received metronidazole or vancomycin as their standard therapy. This information could have been used to provide a greater understanding of the initial CDI episode, as patients with mild to moderate CDI are usually treated with metronidazole (500mg, three times a day for 10-14 days), whereas vancomycin (125mg, four times a day for 10-14 days) is reserved for more severe infections (Cohen *et al.*, 2010; Public Health England and Wilcox, 2013; Debast *et al.*, 2014; Gerding *et al.*, 2016). Regrettably, for the majority of patients, principle treatment was never documented, as several hospital sites did not collect this data. It is also important to note that the primary treatment data alone is not reliable enough to correlate to disease severity.

5.2.6.2. RAPID STOOL SAMPLE CULTURE

C. difficile was isolated and cultured from the RAPID stool samples using the heat-shock method and Braziers agar supplemented with egg yolk emulsion and *C. difficile* selective supplement, as described in Chapter 2.4.7.2. This method was based on the findings of (Hink *et al.*, 2013a), who determined that the heat-shock method yielded the best recovery of *C. difficile* when compared to ethanol-shock and direct plating out. Although the CDRN uses the UK Standards for Microbiology Investigations for the Processing of Faeces for *C.*

difficile (Public Health England, 2018) alcohol-shock method (Borriello and Honour, 1981), both sample preparations select for *C. difficile* spores and eliminate non-sporing bacterial growth.

The plating out of the supernatant after centrifugation of the sample in the RAPID stool sample culture method did not follow the method described by (Hink *et al.*, 2013b), which plated out the pellet. The rationale behind this alteration in the procedure was to ensure that an identical amount of the sample was plated onto each Braziers agar plate in triplicate. However, this method may not have yielded the greatest number of *C. difficile* colonies, as many of the bacterial cells would have been pelleted under centrifugal force. The latest *C. difficile* recovery rate identified from samples submitted to the CDRN in 2014 to 2015 was 93.7% (Public Health England, 2018). However, in this study, the recovery of *C. difficile* from the primary diagnostic samples was challenging at times and a small number of samples had to be cultured more than once. Therefore, to improve the isolation of *C. difficile* in future, plating out of the pellet should be employed in any further work.

Additionally, this research relied on typical colony morphology observed on the egg yolk-based Braziers agar and ribotyping to confirm the identity of the *C. difficile* colonies isolated from the RAPID stool samples. Although these methods differentiated the *C. difficile* isolates from two *R. peoriensis* isolates, the UK Standards for Microbiology Investigations for the Processing of Faeces for *C. difficile* (Public Health England, 2018) recommends additional identification methods. These include using long-wave UV light to visualise green-yellow florescence, a *C. difficile* somatic antigen latex agglutination kit or sub-culturing onto Fastidious Anaerobic Agar (FAA). This would increase ease of identification and reduce the risk of recovering organisms other than *C. difficile*.

5.2.6.3. RAPID ISOLATE CHARACTERISATION

Unfortunately, Dr Emma Stevenson was unable to complete the ribotyping for the 3rd group of RAPID isolates, due to time limitations, and so 14 strains were not ribotyped. This was regrettable, as some conclusions drawn from the

findings relating to patient outcome in this work had to be speculative or were incomplete, due to the lack of ribotyping data.

The minimum inhibitory concentration (MIC) of the rifaximin resistant RAPID isolates were investigated by using the broth dilution method, stated in the study by (Stevenson *et al.*, 2016). Although this method is recommended for determining the MIC of aerobic microorganisms by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), according to the ISO standard 20776-1 (International Organisation for Standardisation, 2006), there are no guidelines for testing anaerobic bacteria. However, the US organisation, Clinical and Laboratory Standards Institute (CLSI), have published a procedure for anaerobic susceptibility testing, routinely called M11 (Clinical and Laboratory Standards Institute, 2018). They recommend the use of both agar dilution and broth dilution methods for anaerobes, though current recommendations limit the use of the broth dilution assay to testing *Bacteroides* and *Parabacteroides* species. Therefore, to improve the MIC work completed in this study, the use of the agar dilution method would have been a more superior option in this case.

5.2.7. DETECTION OF CDT IN PATIENT CLINICAL SAMPLES

As the importance of *C. difficile* binary toxin-producing strains is recognised, the need to develop an assay which can detect CDT production in patient clinical samples is necessary. A research group (Carman *et al.*, 2011) in association with TechLab developed an in-house enzyme immunoassay (EIA) to detect CdtB production in stool samples. The EIA generated mixed results with seventeen out of fifty-three CDT positive samples producing a negative result in the assay. It would be interesting to determine if an optimised version of this assay could be used to detect binary toxin production in the RAPID isolates, detailed in Chapter 4. This would also perhaps signify those samples which possess the CDT gene locus but do not produce binary toxin, and whether specific identified SNPs were responsible.

5.2.8. PUTATIVE SPORULATION GENE STUDIES

Much of the proposed future research of this work is centred on the role of CDT in *C. difficile* virulence, however, it is also evident that there are numerous uncharacterised genes involved in *C. difficile* sporulation. Firstly, it would be necessary to generate and characterise an allelic exchange in-frame deletion mutant for the CD630_26350 gene. This putative spore envelope protein gene was initially identified using a reverse genetics approach; the methodical identification and knock-out of genes that are expected to play a role in *C. difficile* sporulation, built on sequence homology data of other spore-forming bacteria such as *B. subtilis*. However, subsequent analysis of potential sporulation genes can now be identified through forward genetics studies using a *mariner*-based transposon system (Cartman and Minton, 2010; Zhang *et al.*, 2015) such as transposon directed insertion sequencing (TraDIS) (Dembek *et al.*, 2015; Barquist *et al.*, 2016) for high-throughput screening of transposon mutant libraries. This method has been successfully demonstrated in *C. difficile*, in which 798 potential sporulation genes were identified in the R20291 strain (Dembek *et al.*, 2015).

5.3. CONCLUDING REMARKS

The precise role of certain *C. difficile* virulence and antibiotic resistance factors, and how these relate to patient outcome, is yet to be completely elucidated. However, the work presented in this study has demonstrated, through the use of allelic exchange methodology and clinical isolates, that the CDT gene locus, and indeed the ghost locus, are significant. Nonetheless, many questions remain about the binary toxin synergistic effect with toxin A and B, the effect on colonisation and adherence, and the impact on the immune response.

The importance of sporulation in *C. difficile* persistence and spread is well-defined, however, the exact mechanism and identification of numerous genes which influence sporulation, remain unknown.

One of the most interesting results in this research was the impact of rifaximin treatment on resistance observed in some patient clinical isolates. Given the amount of evidence in the literature (Marchese *et al.*, 2000; Hecht *et al.*, 2007; Johnson *et al.*, 2007; O'Connor *et al.*, 2008; Curry *et al.*, 2009; Johnson *et al.*, 2009; Carman *et al.*, 2012; Obuch-Woszczatyński *et al.*, 2013; Freeman *et al.*, 2015b; Kullin *et al.*, 2017), it *is* likely that exposure to rifaximin increases the risk of developing resistance in certain individuals, however, it is necessary to research other strain and host specific interactions before recommending rifaximin as appropriate treatment for the prevention of *C. difficile* relapse. The data generated from the RAPID clinical trial has been invaluable for expanding our knowledge of *C. difficile* virulence *in vivo*.

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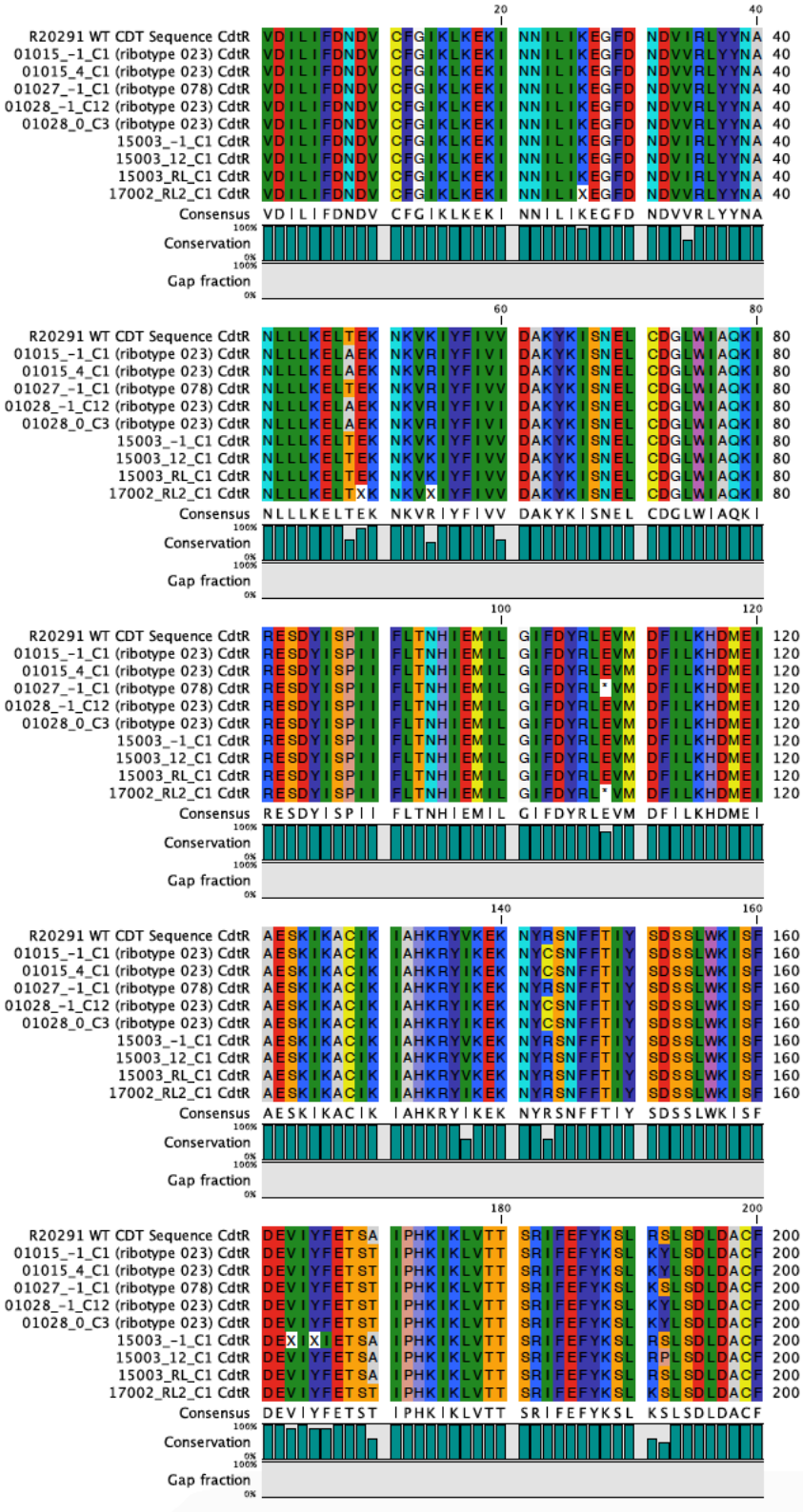
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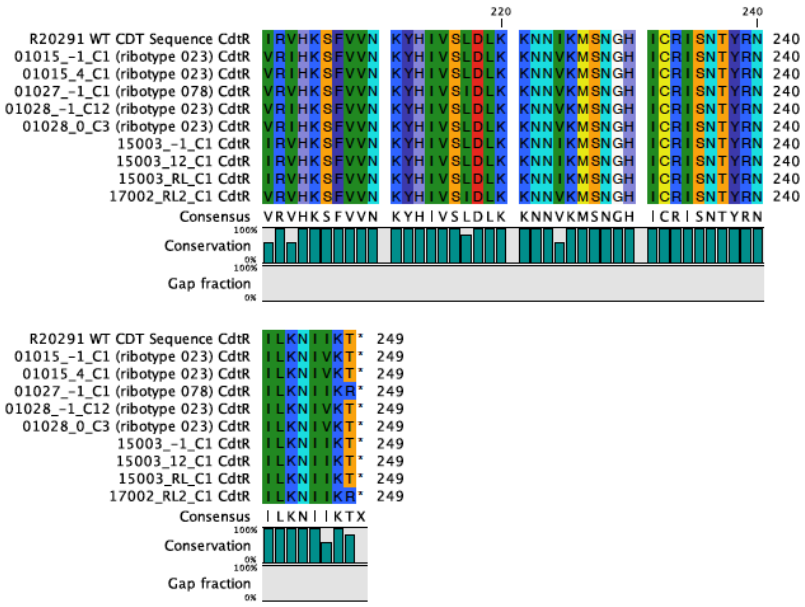
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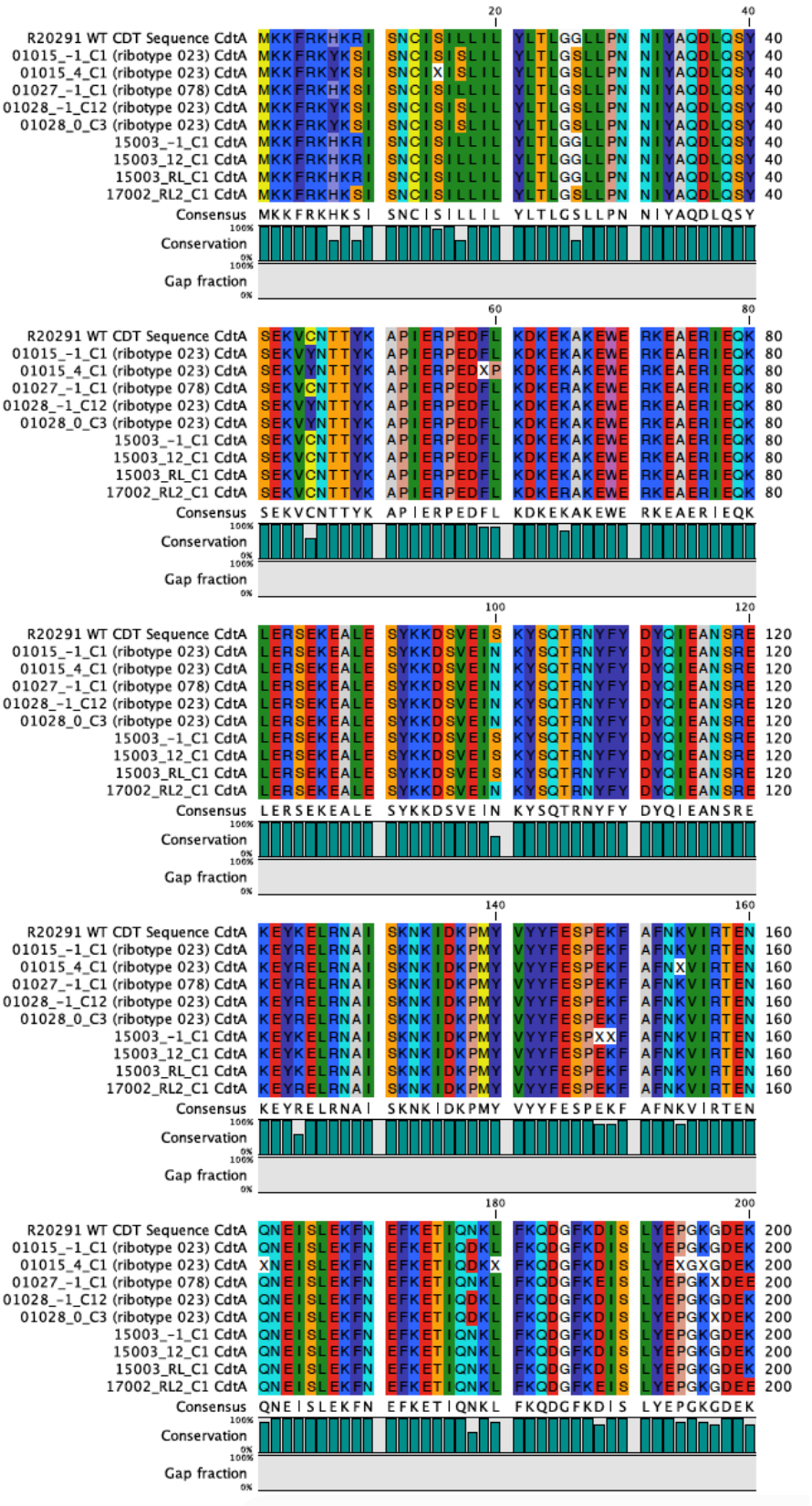
APPENDIX 1: RAPID ISOLATE FULL CdtR SEQUENCING ALIGNMENT

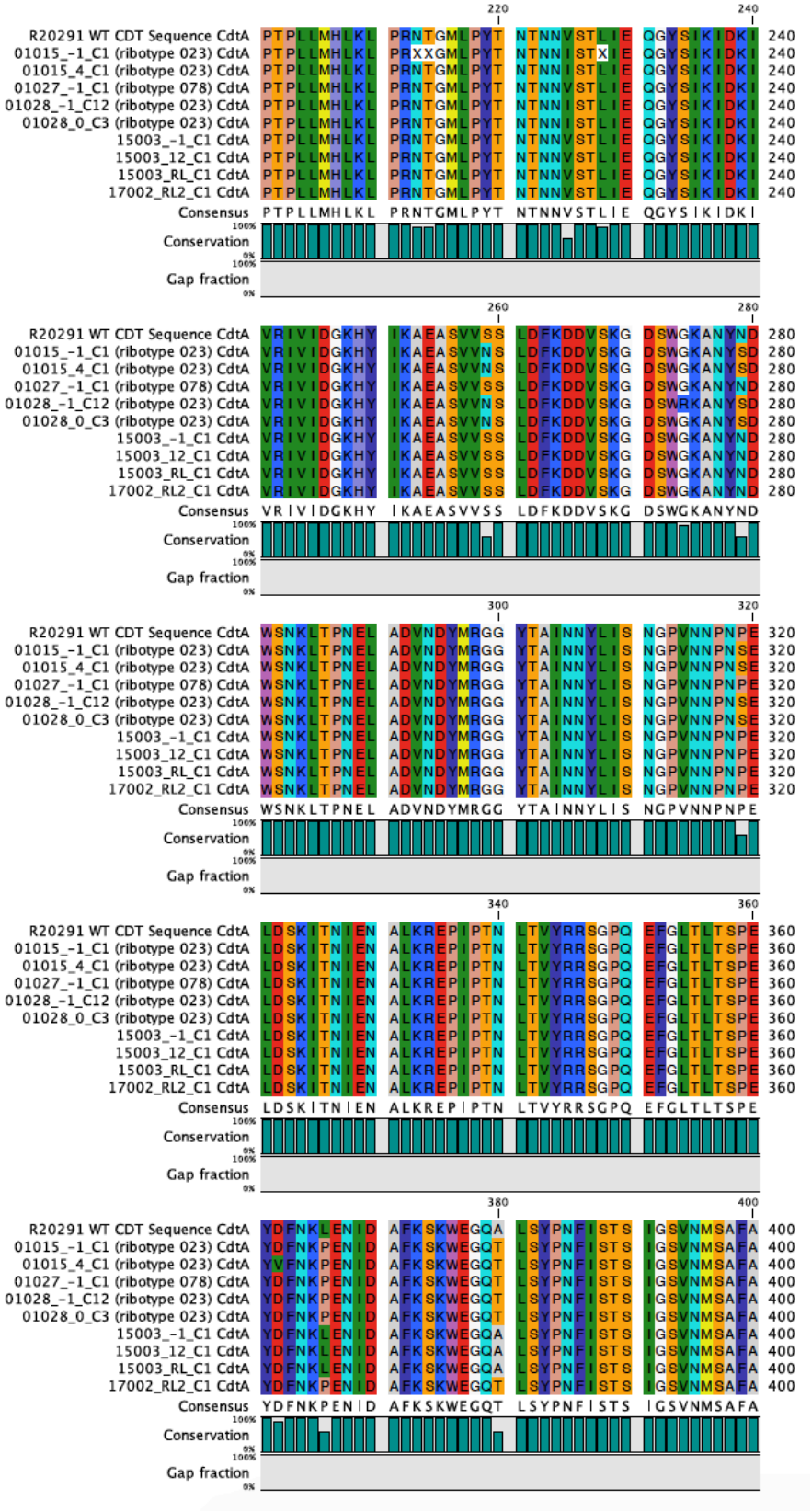


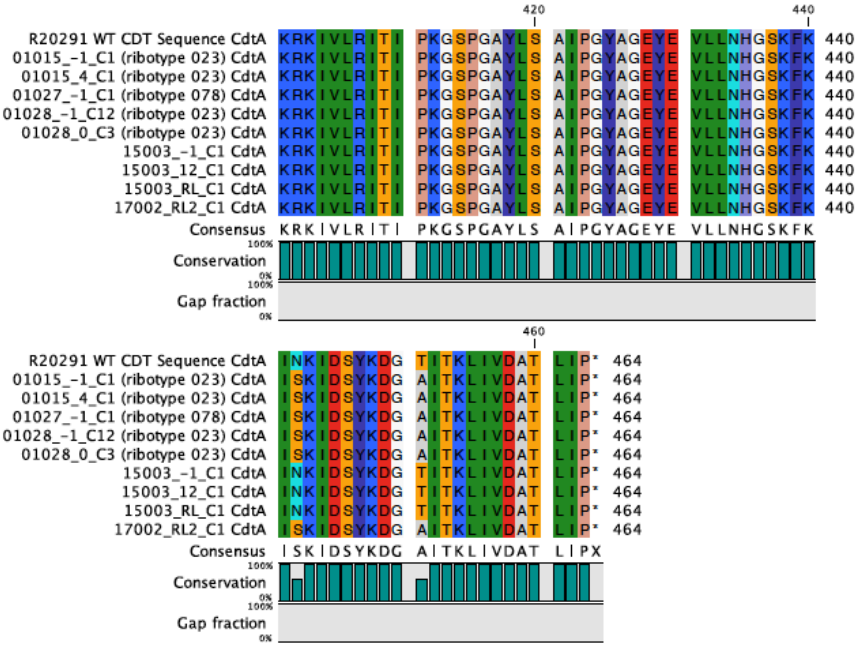

220
240
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01015_4_C1 (ribotype 023) CdtR VRIHKSFVVN KYHIVSLDLK KNNVKMSNGH ICRISNTYRN 240
01027_-1_C1 (ribotype 078) CdtR VRVHKSFVVN KYHIVSIDLK KNNVKMSNGH ICRISNTYRN 240
01028_-1_C12 (ribotype 023) CdtR VRIHKSFVVN KYHIVSLDLK KNNVKMSNGH ICRISNTYRN 240
01028_0_C3 (ribotype 023) CdtR VRIHKSFVVN KYHIVSLDLK KNNVKMSNGH ICRISNTYRN 240
15003_-1_C1 CdtR IRVHKSFVVN KYHIVSLDLK KNNIKMSNGH ICRISNTYRN 240
15003_12_C1 CdtR IRVHKSFVVN KYHIVSLDLK KNNIKMSNGH ICRISNTYRN 240
15003_RL_C1 CdtR IRVHKSFVVN KYHIVSLDLK KNNIKMSNGH ICRISNTYRN 240
17002_RL2_C1 CdtR VRVHKSFVVN KYHIVSIDLK KNNVKMSNGH ICRISNTYRN 240
Consensus VRVHKSFVVN KYHIVSLDLK KNNVKMSNGH ICRISNTYRN
100%
Conservation
0%
100%
Gap fraction
0%

R20291 WT CDT Sequence CdtR ILKNIIKT* 249
01015_-1_C1 (ribotype 023) CdtR ILKNIVKT* 249
01015_4_C1 (ribotype 023) CdtR ILKNIVKT* 249
01027_-1_C1 (ribotype 078) CdtR ILKNIIKR* 249
01028_-1_C12 (ribotype 023) CdtR ILKNIVKT* 249
01028_0_C3 (ribotype 023) CdtR ILKNIVKT* 249
15003_-1_C1 CdtR ILKNIIKT* 249
15003_12_C1 CdtR ILKNIIKT* 249
15003_RL_C1 CdtR ILKNIIKT* 249
17002_RL2_C1 CdtR ILKNIIKR* 249
Consensus ILKNIIKTX
100%
Conservation
0%
100%
Gap fraction
0%

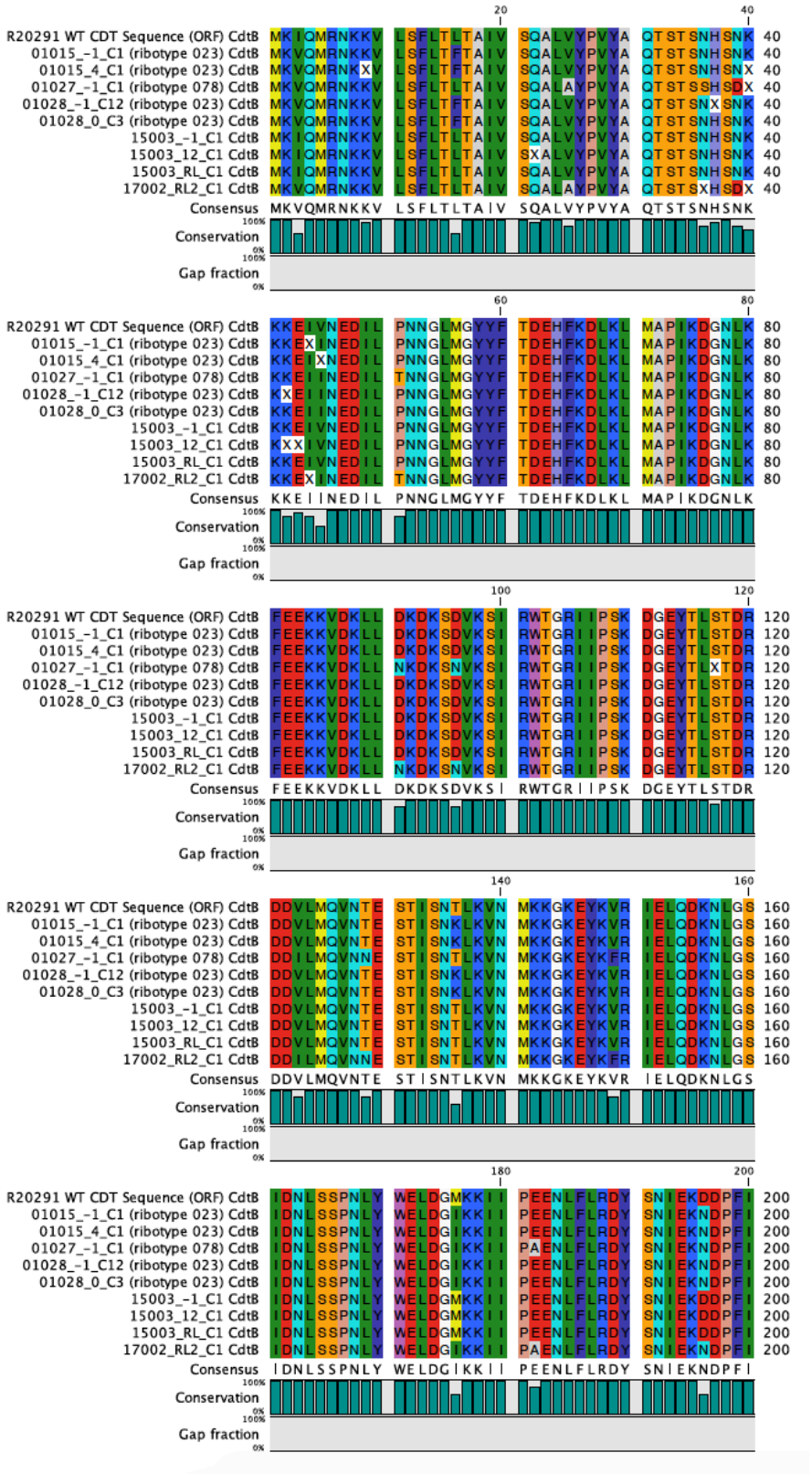
APPENDIX 2: RAPID ISOLATE FULL CdtA SEQUENCING ALIGNMENT

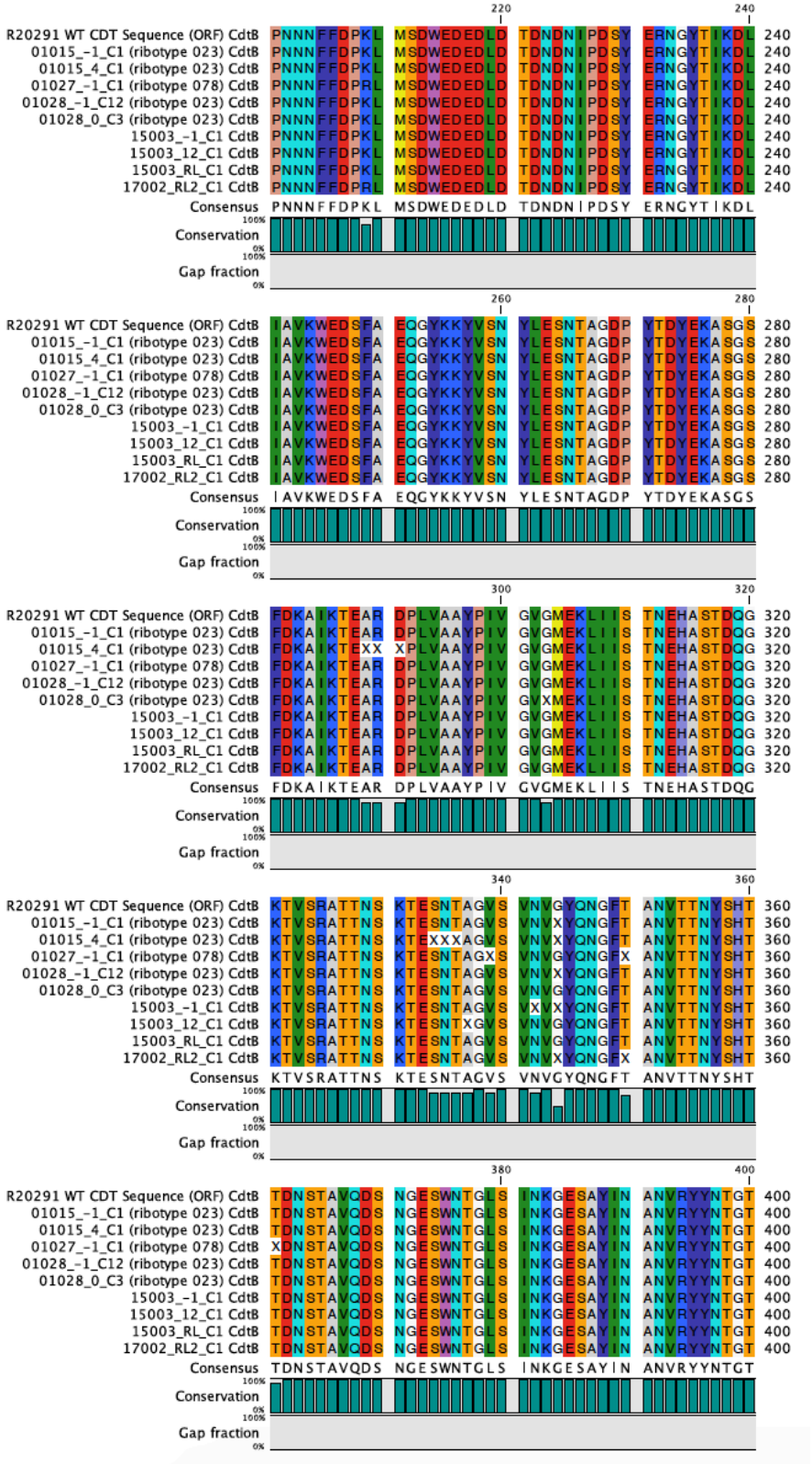


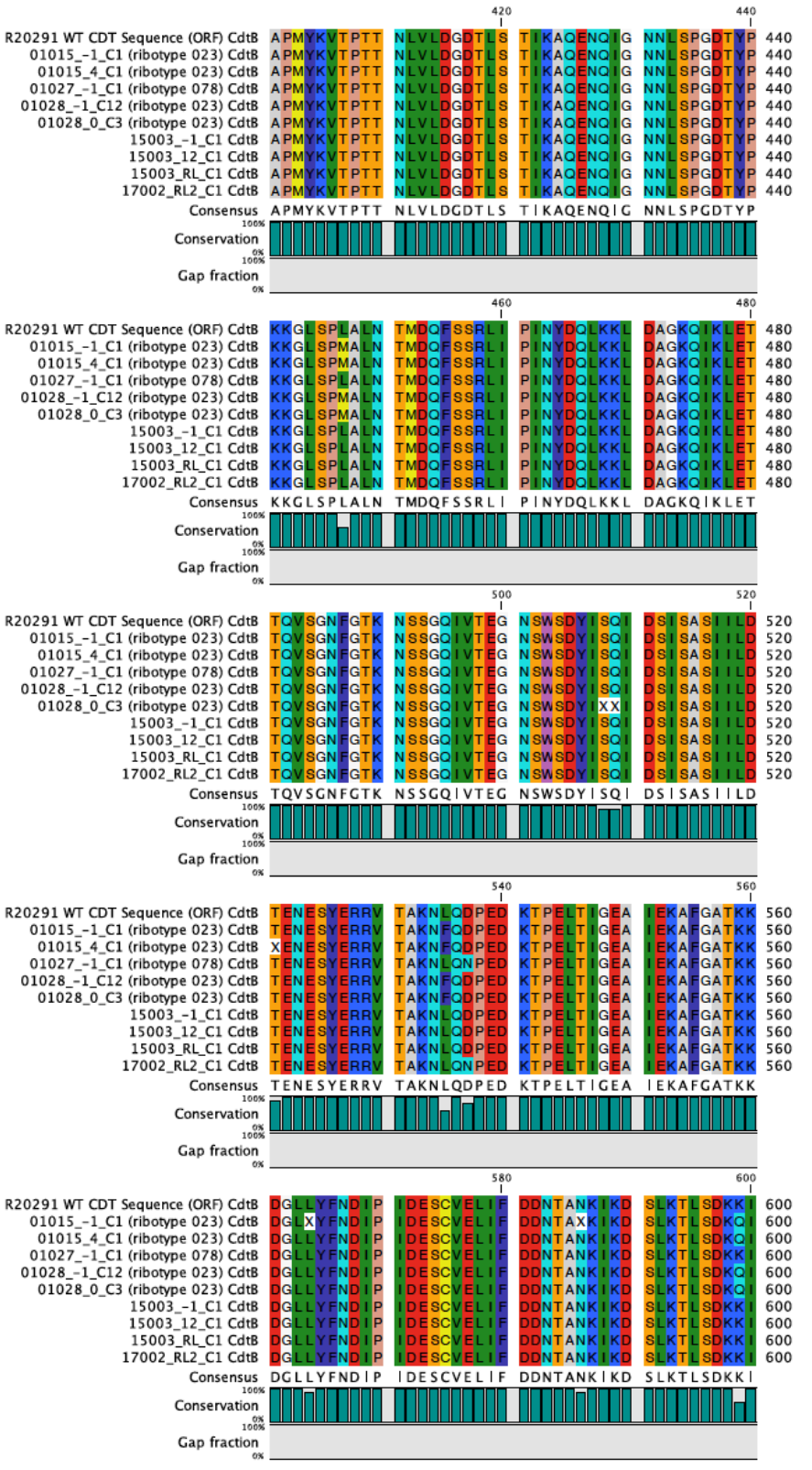

220
240
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01015_4_C1 (ribotype 023) CdtA PTPLLMHLKL PRNTGMLPYT NTNNISTLIE QGYSIKIDKI 240
01027_-1_C1 (ribotype 078) CdtA PTPLLMHLKL PRNTGMLPYT NTNNVSTLIE QGYSIKIDKI 240
01028_-1_C12 (ribotype 023) CdtA PTPLLMHLKL PRNTGMLPYT NTNNISTLIE QGYSIKIDKI 240
01028_0_C3 (ribotype 023) CdtA PTPLLMHLKL PRNTGMLPYT NTNNISTLIE QGYSIKIDKI 240
15003_-1_C1 CdtA PTPLLMHLKL PRNTGMLPYT NTNNVSTLIE QGYSIKIDKI 240
15003_12_C1 CdtA PTPLLMHLKL PRNTGMLPYT NTNNVSTLIE QGYSIKIDKI 240
15003_RL_C1 CdtA PTPLLMHLKL PRNTGMLPYT NTNNVSTLIE QGYSIKIDKI 240
17002_RL2_C1 CdtA PTPLLMHLKL PRNTGMLPYT NTNNVSTLIE QGYSIKIDKI 240
Consensus PTPLLMHLKL PRNTGMLPYT NTNNVSTLIE QGYSIKIDKI
100%
Conservation
0%
100%
Gap fraction
0%
260
280
R20291 WT CDT Sequence CdtA VRIVIDGKHY IKAEASVVSS LDFKDDVSKG DSWGKANYND 280
01015_-1_C1 (ribotype 023) CdtA VRIVIDGKHY IKAEASVVNS LDFKDDVSKG DSWGKANYSD 280
01015_4_C1 (ribotype 023) CdtA VRIVIDGKHY IKAEASVVNS LDFKDDVSKG DSWGKANYSD 280
01027_-1_C1 (ribotype 078) CdtA VRIVIDGKHY IKAEASVVSS LDFKDDVSKG DSWGKANYND 280
01028_-1_C12 (ribotype 023) CdtA VRIVIDGKHY IKAEASVVNS LDFKDDVSKG DSWRKANYSD 280
01028_0_C3 (ribotype 023) CdtA VRIVIDGKHY IKAEASVVNS LDFKDDVSKG DSWGKANYSD 280
15003_-1_C1 CdtA VRIVIDGKHY IKAEASVVSS LDFKDDVSKG DSWGKANYND 280
15003_12_C1 CdtA VRIVIDGKHY IKAEASVVSS LDFKDDVSKG DSWGKANYND 280
15003_RL_C1 CdtA VRIVIDGKHY IKAEASVVSS LDFKDDVSKG DSWGKANYND 280
17002_RL2_C1 CdtA VRIVIDGKHY IKAEASVVSS LDFKDDVSKG DSWGKANYND 280
Consensus VRIVIDGKHY IKAEASVVSS LDFKDDVSKG DSWGKANYND
100%
Conservation
0%
100%
Gap fraction
0%
300
320
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01015_-1_C1 (ribotype 023) CdtA WSNKLTPNEL ADVNDYMRGG YTAINNYLIS NGPVNNPNSE 320
01015_4_C1 (ribotype 023) CdtA WSNKLTPNEL ADVNDYMRGG YTAINNYLIS NGPVNNPNSE 320
01027_-1_C1 (ribotype 078) CdtA WSNKLTPNEL ADVNDYMRGG YTAINNYLIS NGPVNNPNPE 320
01028_-1_C12 (ribotype 023) CdtA WSNKLTPNEL ADVNDYMRGG YTAINNYLIS NGPVNNPNSE 320
01028_0_C3 (ribotype 023) CdtA WSNKLTPNEL ADVNDYMRGG YTAINNYLIS NGPVNNPNSE 320
15003_-1_C1 CdtA WSNKLTPNEL ADVNDYMRGG YTAINNYLIS NGPVNNPNPE 320
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15003_RL_C1 CdtA WSNKLTPNEL ADVNDYMRGG YTAINNYLIS NGPVNNPNPE 320
17002_RL2_C1 CdtA WSNKLTPNEL ADVNDYMRGG YTAINNYLIS NGPVNNPNPE 320
Consensus WSNKLTPNEL ADVNDYMRGG YTAINNYLIS NGPVNNPNPE
100%
Conservation
0%
100%
Gap fraction
0%
340
360
R20291 WT CDT Sequence CdtA LDSKITNIEN ALKREPIPTN LTVYRRSGPQ EFGLTLTSPE 360
01015_-1_C1 (ribotype 023) CdtA LDSKITNIEN ALKREPIPTN LTVYRRSGPQ EFGLTLTSPE 360
01015_4_C1 (ribotype 023) CdtA LDSKITNIEN ALKREPIPTN LTVYRRSGPQ EFGLTLTSPE 360
01027_-1_C1 (ribotype 078) CdtA LDSKITNIEN ALKREPIPTN LTVYRRSGPQ EFGLTLTSPE 360
01028_-1_C12 (ribotype 023) CdtA LDSKITNIEN ALKREPIPTN LTVYRRSGPQ EFGLTLTSPE 360
01028_0_C3 (ribotype 023) CdtA LDSKITNIEN ALKREPIPTN LTVYRRSGPQ EFGLTLTSPE 360
15003_-1_C1 CdtA LDSKITNIEN ALKREPIPTN LTVYRRSGPQ EFGLTLTSPE 360
15003_12_C1 CdtA LDSKITNIEN ALKREPIPTN LTVYRRSGPQ EFGLTLTSPE 360
15003_RL_C1 CdtA LDSKITNIEN ALKREPIPTN LTVYRRSGPQ EFGLTLTSPE 360
17002_RL2_C1 CdtA LDSKITNIEN ALKREPIPTN LTVYRRSGPQ EFGLTLTSPE 360
Consensus LDSKITNIEN ALKREPIPTN LTVYRRSGPQ EFGLTLTSPE
100%
Conservation
0%
100%
Gap fraction
0%
380
400
R20291 WT CDT Sequence CdtA YDFNKLENID AFKSKWEGQA LSYPNFISTS IGSVNMSAFA 400
01015_-1_C1 (ribotype 023) CdtA YDFNKPENID AFKSKWEGQT LSYPNFISTS IGSVNMSAFA 400
01015_4_C1 (ribotype 023) CdtA YVFNKPENID AFKSKWEGQT LSYPNFISTS IGSVNMSAFA 400
01027_-1_C1 (ribotype 078) CdtA YDFNKPENID AFKSKWEGQT LSYPNFISTS IGSVNMSAFA 400
01028_-1_C12 (ribotype 023) CdtA YDFNKPENID AFKSKWEGQT LSYPNFISTS IGSVNMSAFA 400
01028_0_C3 (ribotype 023) CdtA YDFNKPENID AFKSKWEGQT LSYPNFISTS IGSVNMSAFA 400
15003_-1_C1 CdtA YDFNKLENID AFKSKWEGQA LSYPNFISTS IGSVNMSAFA 400
15003_12_C1 CdtA YDFNKLENID AFKSKWEGQA LSYPNFISTS IGSVNMSAFA 400
15003_RL_C1 CdtA YDFNKLENID AFKSKWEGQA LSYPNFISTS IGSVNMSAFA 400
17002_RL2_C1 CdtA YDFNKPENID AFKSKWEGQT LSYPNFISTS IGSVNMSAFA 400
Consensus YDFNKPENID AFKSKWEGQT LSYPNFISTS IGSVNMSAFA
100%
Conservation
0%
100%
Gap fraction
0%

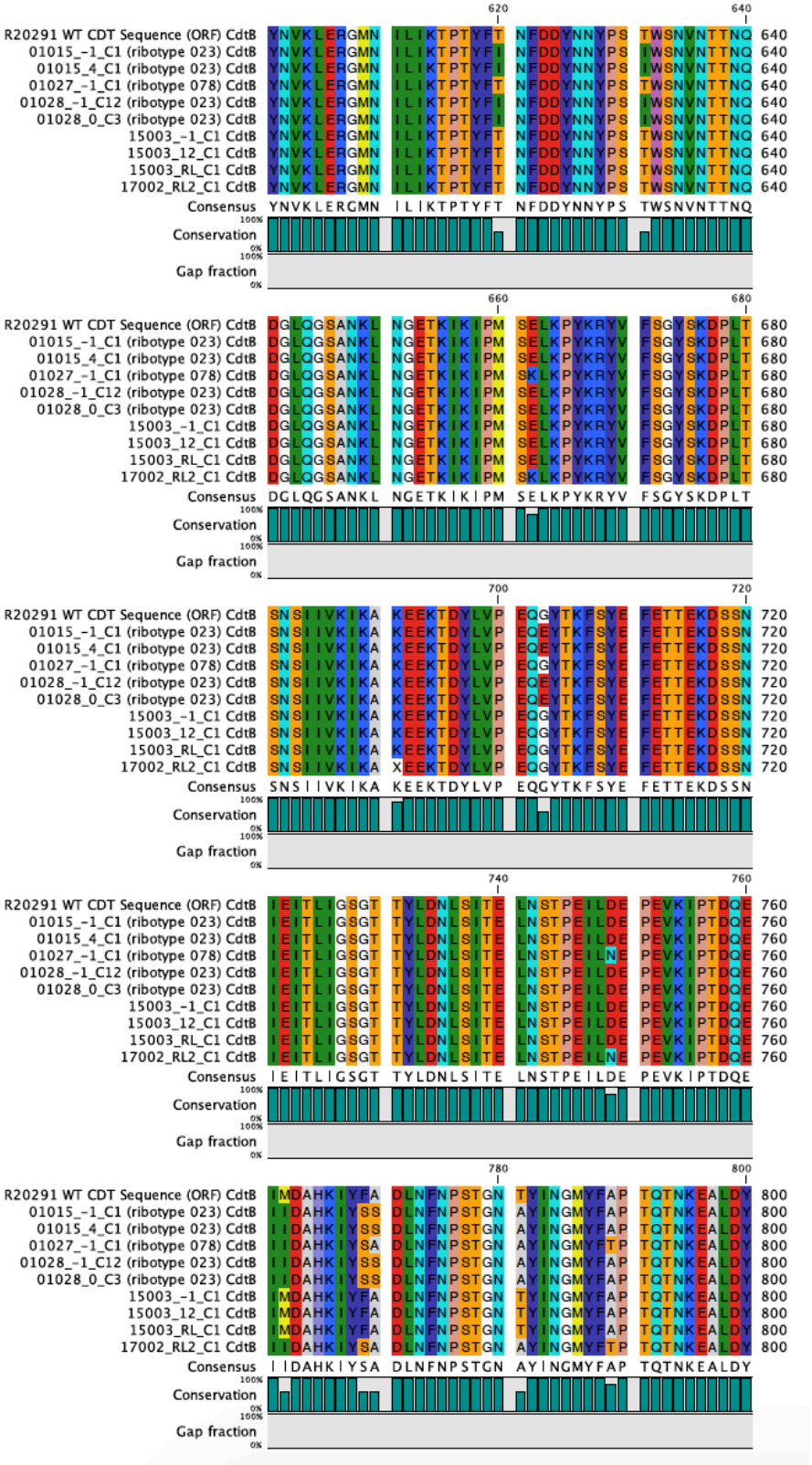

420
440
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01027_-1_C1 (ribotype 078) CdtA KRKIVLRITI PKGSPGAYLS AIPGYAGEYE VLLNHGSKFK 440
01028_-1_C12 (ribotype 023) CdtA KRKIVLRITI PKGSPGAYLS AIPGYAGEYE VLLNHGSKFK 440
01028_0_C3 (ribotype 023) CdtA KRKIVLRITI PKGSPGAYLS AIPGYAGEYE VLLNHGSKFK 440
15003_-1_C1 CdtA KRKIVLRITI PKGSPGAYLS AIPGYAGEYE VLLNHGSKFK 440
15003_12_C1 CdtA KRKIVLRITI PKGSPGAYLS AIPGYAGEYE VLLNHGSKFK 440
15003_RL_C1 CdtA KRKIVLRITI PKGSPGAYLS AIPGYAGEYE VLLNHGSKFK 440
17002_RL2_C1 CdtA KRKIVLRITI PKGSPGAYLS AIPGYAGEYE VLLNHGSKFK 440
Consensus KRKIVLRITI PKGSPGAYLS AIPGYAGEYE VLLNHGSKFK
100%
Conservation
0%
100%
Gap fraction
0%
460
R20291 WT CDT Sequence CdtA INKIDSYKDG TITKLIVDAT LIP* 464
01015_-1_C1 (ribotype 023) CdtA ISKIDSYKDG AITKLIVDAT LIP* 464
01015_4_C1 (ribotype 023) CdtA ISKIDSYKDG AITKLIVDAT LIP* 464
01027_-1_C1 (ribotype 078) CdtA ISKIDSYKDG AITKLIVDAT LIP* 464
01028_-1_C12 (ribotype 023) CdtA ISKIDSYKDG AITKLIVDAT LIP* 464
01028_0_C3 (ribotype 023) CdtA ISKIDSYKDG AITKLIVDAT LIP* 464
15003_-1_C1 CdtA INKIDSYKDG TITKLIVDAT LIP* 464
15003_12_C1 CdtA INKIDSYKDG TITKLIVDAT LIP* 464
15003_RL_C1 CdtA INKIDSYKDG TITKLIVDAT LIP* 464
17002_RL2_C1 CdtA ISKIDSYKDG AITKLIVDAT LIP* 464
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100%
Conservation
0%
100%
Gap fraction
0%

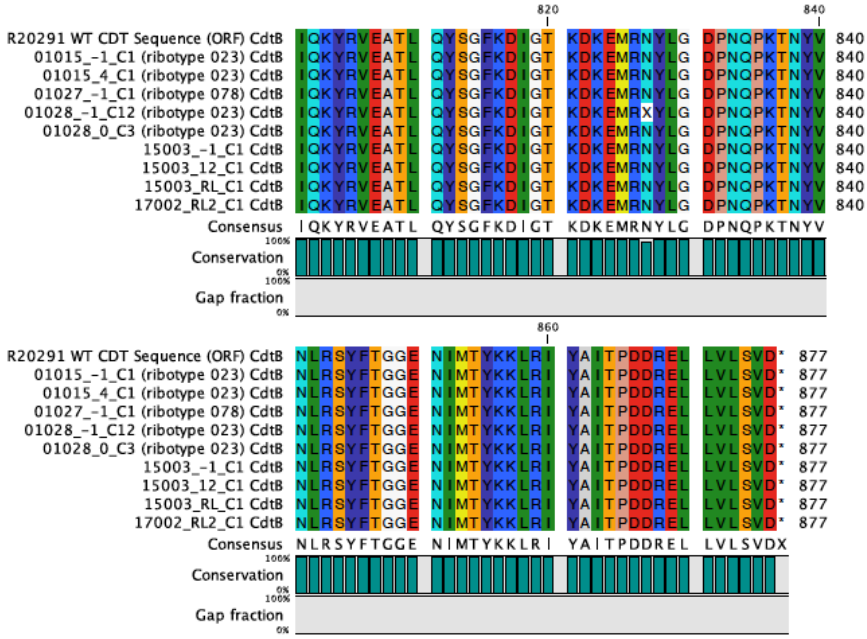
APPENDIX 3: RAPID ISOLATE FULL CdtB SEQUENCING ALIGNMENT



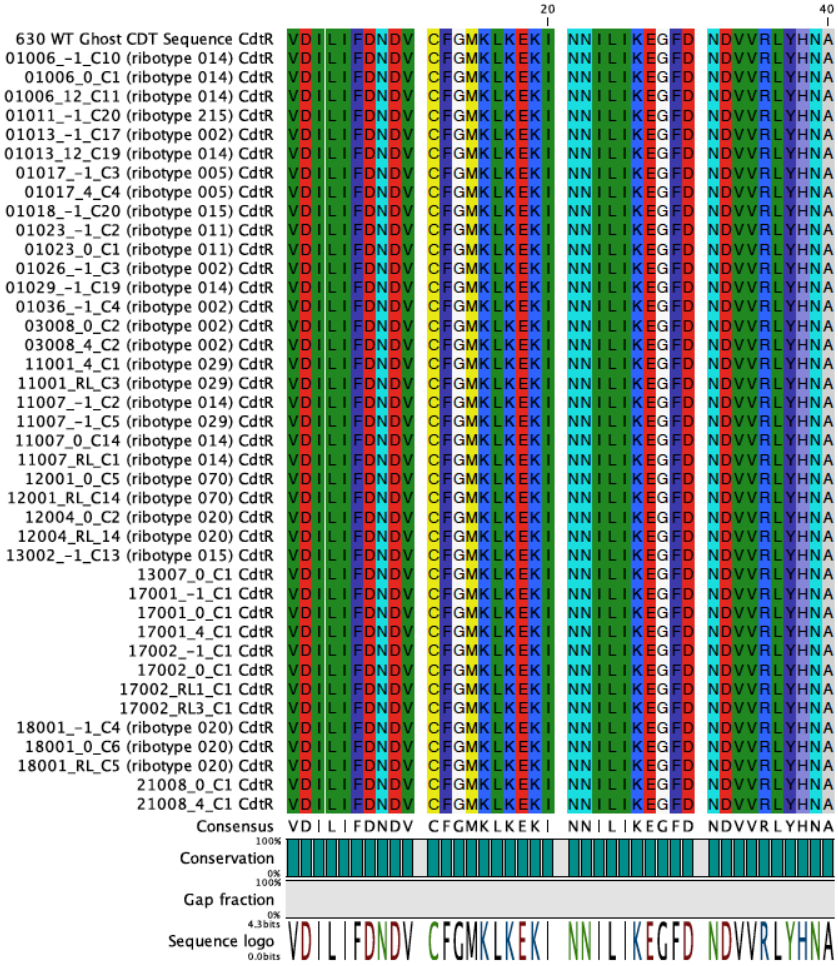

220
240
R20291 WT CDT Sequence (ORF) CdtB PNNNFFDPKL MSDWEDEDLD TDNDNIPDSY ERNGYTIKDL 240
01015_-1_C1 (ribotype 023) CdtB PNNNFFDPKL MSDWEDEDLD TDNDNIPDSY ERNGYTIKDL 240
01015_4_C1 (ribotype 023) CdtB PNNNFFDPKL MSDWEDEDLD TDNDNIPDSY ERNGYTIKDL 240
01027_-1_C1 (ribotype 078) CdtB PNNNFFDPRL MSDWEDEDLD TDNDNIPDSY ERNGYTIKDL 240
01028_-1_C12 (ribotype 023) CdtB PNNNFFDPKL MSDWEDEDLD TDNDNIPDSY ERNGYTIKDL 240
01028_0_C3 (ribotype 023) CdtB PNNNFFDPKL MSDWEDEDLD TDNDNIPDSY ERNGYTIKDL 240
15003_-1_C1 CdtB PNNNFFDPKL MSDWEDEDLD TDNDNIPDSY ERNGYTIKDL 240
15003_12_C1 CdtB PNNNFFDPKL MSDWEDEDLD TDNDNIPDSY ERNGYTIKDL 240
15003_RL_C1 CdtB PNNNFFDPKL MSDWEDEDLD TDNDNIPDSY ERNGYTIKDL 240
17002_RL2_C1 CdtB PNNNFFDPRL MSDWEDEDLD TDNDNIPDSY ERNGYTIKDL 240
Consensus PNNNFFDPKL MSDWEDEDLD TDNDNIPDSY ERNGYTIKDL
100%
Conservation
0%
100%
Gap fraction
0%
260
280
R20291 WT CDT Sequence (ORF) CdtB IAVKWEDSFA EQGYKKYVSN YLESNTAGDP YTDYEKASGS 280
01015_-1_C1 (ribotype 023) CdtB IAVKWEDSFA EQGYKKYVSN YLESNTAGDP YTDYEKASGS 280
01015_4_C1 (ribotype 023) CdtB IAVKWEDSFA EQGYKKYVSN YLESNTAGDP YTDYEKASGS 280
01027_-1_C1 (ribotype 078) CdtB IAVKWEDSFA EQGYKKYVSN YLESNTAGDP YTDYEKASGS 280
01028_-1_C12 (ribotype 023) CdtB IAVKWEDSFA EQGYKKYVSN YLESNTAGDP YTDYEKASGS 280
01028_0_C3 (ribotype 023) CdtB IAVKWEDSFA EQGYKKYVSN YLESNTAGDP YTDYEKASGS 280
15003_-1_C1 CdtB IAVKWEDSFA EQGYKKYVSN YLESNTAGDP YTDYEKASGS 280
15003_12_C1 CdtB IAVKWEDSFA EQGYKKYVSN YLESNTAGDP YTDYEKASGS 280
15003_RL_C1 CdtB IAVKWEDSFA EQGYKKYVSN YLESNTAGDP YTDYEKASGS 280
17002_RL2_C1 CdtB IAVKWEDSFA EQGYKKYVSN YLESNTAGDP YTDYEKASGS 280
Consensus IAVKWEDSFA EQGYKKYVSN YLESNTAGDP YTDYEKASGS
100%
Conservation
0%
100%
Gap fraction
0%
300
320
R20291 WT CDT Sequence (ORF) CdtB FDKAIKTEAR DPLVAAYPIV GVGMEKLIIS TNEHASTDQG 320
01015_-1_C1 (ribotype 023) CdtB FDKAIKTEAR DPLVAAYPIV GVGMEKLIIS TNEHASTDQG 320
01015_4_C1 (ribotype 023) CdtB FDKAIKTEXX XPLVAAYPIV GVGMEKLIIS TNEHASTDQG 320
01027_-1_C1 (ribotype 078) CdtB FDKAIKTEAR DPLVAAYPIV GVGMEKLIIS TNEHASTDQG 320
01028_-1_C12 (ribotype 023) CdtB FDKAIKTEAR DPLVAAYPIV GVGMEKLIIS TNEHASTDQG 320
01028_0_C3 (ribotype 023) CdtB FDKAIKTEAR DPLVAAYPIV GVXMEKLIIS TNEHASTDQG 320
15003_-1_C1 CdtB FDKAIKTEAR DPLVAAYPIV GVGMEKLIIS TNEHASTDQG 320
15003_12_C1 CdtB FDKAIKTEAR DPLVAAYPIV GVGMEKLIIS TNEHASTDQG 320
15003_RL_C1 CdtB FDKAIKTEAR DPLVAAYPIV GVGMEKLIIS TNEHASTDQG 320
17002_RL2_C1 CdtB FDKAIKTEAR DPLVAAYPIV GVGMEKLIIS TNEHASTDQG 320
Consensus FDKAIKTEAR DPLVAAYPIV GVGMEKLIIS TNEHASTDQG
100%
Conservation
0%
100%
Gap fraction
0%
340
360
R20291 WT CDT Sequence (ORF) CdtB KTVSRATTNS KTESNTAGVS VNVGYQNGFT ANVTTNYSHT 360
01015_-1_C1 (ribotype 023) CdtB KTVSRATTNS KTESNTAGVS VNVXYQNGFT ANVTTNYSHT 360
01015_4_C1 (ribotype 023) CdtB KTVSRATTNS KTEXXXAGVS VNVXYQNGFT ANVTTNYSHT 360
01027_-1_C1 (ribotype 078) CdtB KTVSRATTNS KTESNTAGXS VNVGYQNGFX ANVTTNYSHT 360
01028_-1_C12 (ribotype 023) CdtB KTVSRATTNS KTESNTAGVS VNVXYQNGFT ANVTTNYSHT 360
01028_0_C3 (ribotype 023) CdtB KTVSRATTNS KTESNTAGVS VNVGYQNGFT ANVTTNYSHT 360
15003_-1_C1 CdtB KTVSRATTNS KTESNTAGVS VXVXYQNGFT ANVTTNYSHT 360
15003_12_C1 CdtB KTVSRATTNS KTESNTXGVS VNVGYQNGFT ANVTTNYSHT 360
15003_RL_C1 CdtB KTVSRATTNS KTESNTAGVS VNVGYQNGFT ANVTTNYSHT 360
17002_RL2_C1 CdtB KTVSRATTNS KTESNTAGVS VNVXYQNGFX ANVTTNYSHT 360
Consensus KTVSRATTNS KTESNTAGVS VNVGYQNGFT ANVTTNYSHT
100%
Conservation
0%
100%
Gap fraction
0%
380
400
R20291 WT CDT Sequence (ORF) CdtB TDNSTAVQDS NGESWNTGLS INKGESAYIN ANVRYYNTGT 400
01015_-1_C1 (ribotype 023) CdtB TDNSTAVQDS NGESWNTGLS INKGESAYIN ANVRYYNTGT 400
01015_4_C1 (ribotype 023) CdtB TDNSTAVQDS NGESWNTGLS INKGESAYIN ANVRYYNTGT 400
01027_-1_C1 (ribotype 078) CdtB XDNSTAVQDS NGESWNTGLS INKGESAYIN ANVRYYNTGT 400
01028_-1_C12 (ribotype 023) CdtB TDNSTAVQDS NGESWNTGLS INKGESAYIN ANVRYYNTGT 400
01028_0_C3 (ribotype 023) CdtB TDNSTAVQDS NGESWNTGLS INKGESAYIN ANVRYYNTGT 400
15003_-1_C1 CdtB TDNSTAVQDS NGESWNTGLS INKGESAYIN ANVRYYNTGT 400
15003_12_C1 CdtB TDNSTAVQDS NGESWNTGLS INKGESAYIN ANVRYYNTGT 400
15003_RL_C1 CdtB TDNSTAVQDS NGESWNTGLS INKGESAYIN ANVRYYNTGT 400
17002_RL2_C1 CdtB TDNSTAVQDS NGESWNTGLS INKGESAYIN ANVRYYNTGT 400
Consensus TDNSTAVQDS NGESWNTGLS INKGESAYIN ANVRYYNTGT
100%
Conservation
0%
100%
Gap fraction
0%

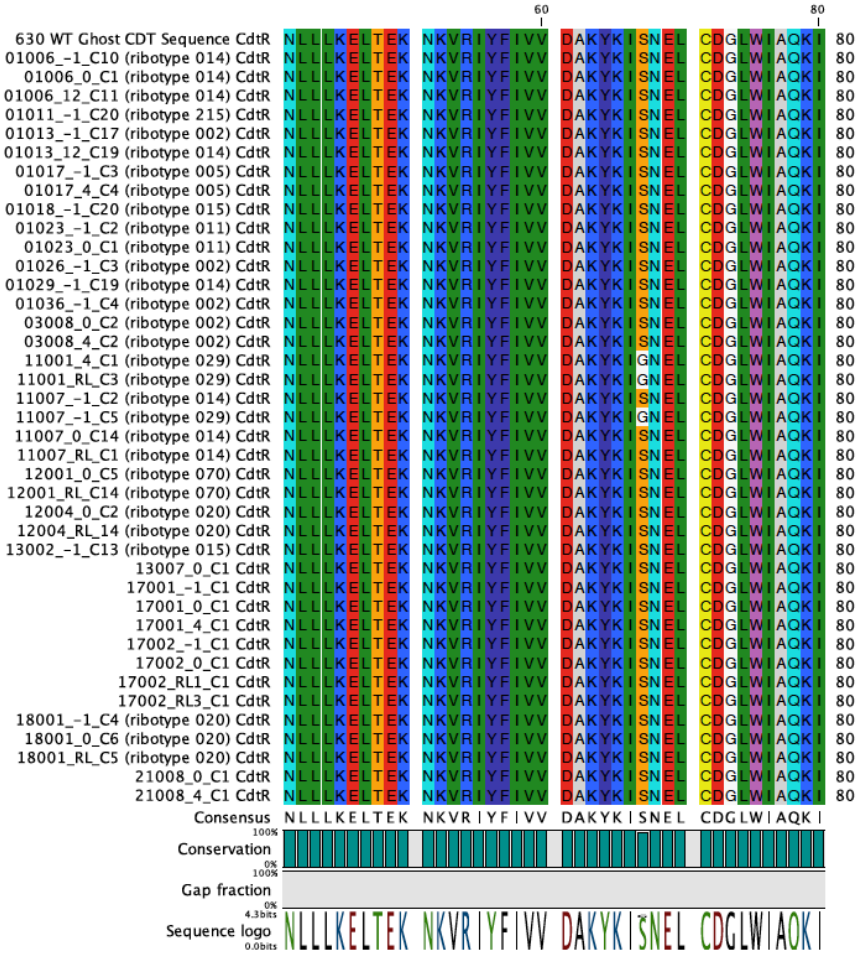

420
440
R20291 WT CDT Sequence (ORF) CdtB APMYKVTPTT NLVLDGDTLS TIKAQENQIG NNLSPGDTYP 440
01015_-1_C1 (ribotype 023) CdtB APMYKVTPTT NLVLDGDTLS TIKAQENQIG NNLSPGDTYP 440
01015_4_C1 (ribotype 023) CdtB APMYKVTPTT NLVLDGDTLS TIKAQENQIG NNLSPGDTYP 440
01027_-1_C1 (ribotype 078) CdtB APMYKVTPTT NLVLDGDTLS TIKAQENQIG NNLSPGDTYP 440
01028_-1_C12 (ribotype 023) CdtB APMYKVTPTT NLVLDGDTLS TIKAQENQIG NNLSPGDTYP 440
01028_0_C3 (ribotype 023) CdtB APMYKVTPTT NLVLDGDTLS TIKAQENQIG NNLSPGDTYP 440
15003_-1_C1 CdtB APMYKVTPTT NLVLDGDTLS TIKAQENQIG NNLSPGDTYP 440
15003_12_C1 CdtB APMYKVTPTT NLVLDGDTLS TIKAQENQIG NNLSPGDTYP 440
15003_RL_C1 CdtB APMYKVTPTT NLVLDGDTLS TIKAQENQIG NNLSPGDTYP 440
17002_RL2_C1 CdtB APMYKVTPTT NLVLDGDTLS TIKAQENQIG NNLSPGDTYP 440
Consensus APMYKVTPTT NLVLDGDTLS TIKAQENQIG NNLSPGDTYP
100%
Conservation
0%
100%
Gap fraction
0%
460
480
R20291 WT CDT Sequence (ORF) CdtB KKGLSPLALN TMDQFSSRLI PINYDQLKKL DAGKQIKLET 480
01015_-1_C1 (ribotype 023) CdtB KKGLSPMALN TMDQFSSRLI PINYDQLKKL DAGKQIKLET 480
01015_4_C1 (ribotype 023) CdtB KKGLSPMALN TMDQFSSRLI PINYDQLKKL DAGKQIKLET 480
01027_-1_C1 (ribotype 078) CdtB KKGLSPLALN TMDQFSSRLI PINYDQLKKL DAGKQIKLET 480
01028_-1_C12 (ribotype 023) CdtB KKGLSPMALN TMDQFSSRLI PINYDQLKKL DAGKQIKLET 480
01028_0_C3 (ribotype 023) CdtB KKGLSPMALN TMDQFSSRLI PINYDQLKKL DAGKQIKLET 480
15003_-1_C1 CdtB KKGLSPLALN TMDQFSSRLI PINYDQLKKL DAGKQIKLET 480
15003_12_C1 CdtB KKGLSPLALN TMDQFSSRLI PINYDQLKKL DAGKQIKLET 480
15003_RL_C1 CdtB KKGLSPLALN TMDQFSSRLI PINYDQLKKL DAGKQIKLET 480
17002_RL2_C1 CdtB KKGLSPLALN TMDQFSSRLI PINYDQLKKL DAGKQIKLET 480
Consensus KKGLSPLALN TMDQFSSRLI PINYDQLKKL DAGKQIKLET
100%
Conservation
0%
100%
Gap fraction
0%
500
520
R20291 WT CDT Sequence (ORF) CdtB TQVSGNFGTK NSSGQIVTEG NSWSDYISQI DSISASIILD 520
01015_-1_C1 (ribotype 023) CdtB TQVSGNFGTK NSSGQIVTEG NSWSDYISQI DSISASIILD 520
01015_4_C1 (ribotype 023) CdtB TQVSGNFGTK NSSGQIVTEG NSWSDYISQI DSISASIILD 520
01027_-1_C1 (ribotype 078) CdtB TQVSGNFGTK NSSGQIVTEG NSWSDYISQI DSISASIILD 520
01028_-1_C12 (ribotype 023) CdtB TQVSGNFGTK NSSGQIVTEG NSWSDYISQI DSISASIILD 520
01028_0_C3 (ribotype 023) CdtB TQVSGNFGTK NSSGQIVTEG NSWSDYIXXI DSISASIILD 520
15003_-1_C1 CdtB TQVSGNFGTK NSSGQIVTEG NSWSDYISQI DSISASIILD 520
15003_12_C1 CdtB TQVSGNFGTK NSSGQIVTEG NSWSDYISQI DSISASIILD 520
15003_RL_C1 CdtB TQVSGNFGTK NSSGQIVTEG NSWSDYISQI DSISASIILD 520
17002_RL2_C1 CdtB TQVSGNFGTK NSSGQIVTEG NSWSDYISQI DSISASIILD 520
Consensus TQVSGNFGTK NSSGQIVTEG NSWSDYISQI DSISASIILD
100%
Conservation
0%
100%
Gap fraction
0%
540
560
R20291 WT CDT Sequence (ORF) CdtB TENESYERRV TAKNLQDPED KTPELTIGEA IEKAFGATKK 560
01015_-1_C1 (ribotype 023) CdtB TENESYERRV TAKNFQDPED KTPELTIGEA IEKAFGATKK 560
01015_4_C1 (ribotype 023) CdtB XENESYERRV TAKNFQDPED KTPELTIGEA IEKAFGATKK 560
01027_-1_C1 (ribotype 078) CdtB TENESYERRV TAKNLQNPED KTPELTIGEA IEKAFGATKK 560
01028_-1_C12 (ribotype 023) CdtB TENESYERRV TAKNFQDPED KTPELTIGEA IEKAFGATKK 560
01028_0_C3 (ribotype 023) CdtB TENESYERRV TAKNFQDPED KTPELTIGEA IEKAFGATKK 560
15003_-1_C1 CdtB TENESYERRV TAKNLQDPED KTPELTIGEA IEKAFGATKK 560
15003_12_C1 CdtB TENESYERRV TAKNLQDPED KTPELTIGEA IEKAFGATKK 560
15003_RL_C1 CdtB TENESYERRV TAKNLQDPED KTPELTIGEA IEKAFGATKK 560
17002_RL2_C1 CdtB TENESYERRV TAKNLQNPED KTPELTIGEA IEKAFGATKK 560
Consensus TENESYERRV TAKNLQDPED KTPELTIGEA IEKAFGATKK
100%
Conservation
0%
100%
Gap fraction
0%
580
600
R20291 WT CDT Sequence (ORF) CdtB DGLLYFNDIP IDESCVELIF DDNTANKIKD SLKTLSDKKI 600
01015_-1_C1 (ribotype 023) CdtB DGLXYFNDIP IDESCVELIF DDNTAXKIKD SLKTLSDKQI 600
01015_4_C1 (ribotype 023) CdtB DGLLYFNDIP IDESCVELIF DDNTANKIKD SLKTLSDKQI 600
01027_-1_C1 (ribotype 078) CdtB DGLLYFNDIP IDESCVELIF DDNTANKIKD SLKTLSDKKI 600
01028_-1_C12 (ribotype 023) CdtB DGLLYFNDIP IDESCVELIF DDNTANKIKD SLKTLSDKQI 600
01028_0_C3 (ribotype 023) CdtB DGLLYFNDIP IDESCVELIF DDNTANKIKD SLKTLSDKQI 600
15003_-1_C1 CdtB DGLLYFNDIP IDESCVELIF DDNTANKIKD SLKTLSDKKI 600
15003_12_C1 CdtB DGLLYFNDIP IDESCVELIF DDNTANKIKD SLKTLSDKKI 600
15003_RL_C1 CdtB DGLLYFNDIP IDESCVELIF DDNTANKIKD SLKTLSDKKI 600
17002_RL2_C1 CdtB DGLLYFNDIP IDESCVELIF DDNTANKIKD SLKTLSDKKI 600
Consensus DGLLYFNDIP IDESCVELIF DDNTANKIKD SLKTLSDKKI
100%
Conservation
0%
100%
Gap fraction
0%


620
640
R20291 WT CDT Sequence (ORF) CdtB YNVKLERGMN ILIKTPTYFT NFDDYNNYPS TWSNVNTTNQ 640
01015_-1_C1 (ribotype 023) CdtB YNVKLERGMN ILIKTPTYFI NFDDYNNYPS IWSNVNTTNQ 640
01015_4_C1 (ribotype 023) CdtB YNVKLERGMN ILIKTPTYFI NFDDYNNYPS IWSNVNTTNQ 640
01027_-1_C1 (ribotype 078) CdtB YNVKLERGMN ILIKTPTYFT NFDDYNNYPS TWSNVNTTNQ 640
01028_-1_C12 (ribotype 023) CdtB YNVKLERGMN ILIKTPTYFI NFDDYNNYPS IWSNVNTTNQ 640
01028_0_C3 (ribotype 023) CdtB YNVKLERGMN ILIKTPTYFI NFDDYNNYPS IWSNVNTTNQ 640
15003_-1_C1 CdtB YNVKLERGMN ILIKTPTYFT NFDDYNNYPS TWSNVNTTNQ 640
15003_12_C1 CdtB YNVKLERGMN ILIKTPTYFT NFDDYNNYPS TWSNVNTTNQ 640
15003_RL_C1 CdtB YNVKLERGMN ILIKTPTYFT NFDDYNNYPS TWSNVNTTNQ 640
17002_RL2_C1 CdtB YNVKLERGMN ILIKTPTYFT NFDDYNNYPS TWSNVNTTNQ 640
Consensus YNVKLERGMN ILIKTPTYFT NFDDYNNYPS TWSNVNTTNQ
100%
Conservation
0%
100%
Gap fraction
0%
660
680
R20291 WT CDT Sequence (ORF) CdtB DGLQGSANKL NGETKIKIPM SELKPYKRYV FSGYSKDPLT 680
01015_-1_C1 (ribotype 023) CdtB DGLQGSANKL NGETKIKIPM SELKPYKRYV FSGYSKDPLT 680
01015_4_C1 (ribotype 023) CdtB DGLQGSANKL NGETKIKIPM SELKPYKRYV FSGYSKDPLT 680
01027_-1_C1 (ribotype 078) CdtB DGLQGSANKL NGETKIKIPM SKLKPYKRYV FSGYSKDPLT 680
01028_-1_C12 (ribotype 023) CdtB DGLQGSANKL NGETKIKIPM SELKPYKRYV FSGYSKDPLT 680
01028_0_C3 (ribotype 023) CdtB DGLQGSANKL NGETKIKIPM SELKPYKRYV FSGYSKDPLT 680
15003_-1_C1 CdtB DGLQGSANKL NGETKIKIPM SELKPYKRYV FSGYSKDPLT 680
15003_12_C1 CdtB DGLQGSANKL NGETKIKIPM SELKPYKRYV FSGYSKDPLT 680
15003_RL_C1 CdtB DGLQGSANKL NGETKIKIPM SELKPYKRYV FSGYSKDPLT 680
17002_RL2_C1 CdtB DGLQGSANKL NGETKIKIPM SKLKPYKRYV FSGYSKDPLT 680
Consensus DGLQGSANKL NGETKIKIPM SELKPYKRYV FSGYSKDPLT
100%
Conservation
0%
100%
Gap fraction
0%
700
720
R20291 WT CDT Sequence (ORF) CdtB SNSIIVKIKA KEEKTDYLVP EQGYTKFSYE FETTEKDSSN 720
01015_-1_C1 (ribotype 023) CdtB SNSIIVKIKA KEEKTDYLVP EQEYTKFSYE FETTEKDSSN 720
01015_4_C1 (ribotype 023) CdtB SNSIIVKIKA KEEKTDYLVP EQEYTKFSYE FETTEKDSSN 720
01027_-1_C1 (ribotype 078) CdtB SNSIIVKIKA KEEKTDYLVP EQGYTKFSYE FETTEKDSSN 720
01028_-1_C12 (ribotype 023) CdtB SNSIIVKIKA KEEKTDYLVP EQEYTKFSYE FETTEKDSSN 720
01028_0_C3 (ribotype 023) CdtB SNSIIVKIKA KEEKTDYLVP EQEYTKFSYE FETTEKDSSN 720
15003_-1_C1 CdtB SNSIIVKIKA KEEKTDYLVP EQGYTKFSYE FETTEKDSSN 720
15003_12_C1 CdtB SNSIIVKIKA KEEKTDYLVP EQGYTKFSYE FETTEKDSSN 720
15003_RL_C1 CdtB SNSIIVKIKA KEEKTDYLVP EQGYTKFSYE FETTEKDSSN 720
17002_RL2_C1 CdtB SNSIIVKIKA XEEKTDYLVP EQGYTKFSYE FETTEKDSSN 720
Consensus SNSIIVKIKA KEEKTDYLVP EQGYTKFSYE FETTEKDSSN
100%
Conservation
0%
100%
Gap fraction
0%
740
760
R20291 WT CDT Sequence (ORF) CdtB IEITLIGSGT TYLDNLSITE LNSTPEILDE PEVKIPTDQE 760
01015_-1_C1 (ribotype 023) CdtB IEITLIGSGT TYLDNLSITE LNSTPEILDE PEVKIPTDQE 760
01015_4_C1 (ribotype 023) CdtB IEITLIGSGT TYLDNLSITE LNSTPEILDE PEVKIPTDQE 760
01027_-1_C1 (ribotype 078) CdtB IEITLIGSGT TYLDNLSITE LNSTPEILNE PEVKIPTDQE 760
01028_-1_C12 (ribotype 023) CdtB IEITLIGSGT TYLDNLSITE LNSTPEILDE PEVKIPTDQE 760
01028_0_C3 (ribotype 023) CdtB IEITLIGSGT TYLDNLSITE LNSTPEILDE PEVKIPTDQE 760
15003_-1_C1 CdtB IEITLIGSGT TYLDNLSITE LNSTPEILDE PEVKIPTDQE 760
15003_12_C1 CdtB IEITLIGSGT TYLDNLSITE LNSTPEILDE PEVKIPTDQE 760
15003_RL_C1 CdtB IEITLIGSGT TYLDNLSITE LNSTPEILDE PEVKIPTDQE 760
17002_RL2_C1 CdtB IEITLIGSGT TYLDNLSITE LNSTPEILNE PEVKIPTDQE 760
Consensus IEITLIGSGT TYLDNLSITE LNSTPEILDE PEVKIPTDQE
100%
Conservation
0%
100%
Gap fraction
0%
780
800
R20291 WT CDT Sequence (ORF) CdtB IMDAHKIYFA DLNFNPSTGN TYINGMYFAP TQTNKEALDY 800
01015_-1_C1 (ribotype 023) CdtB IIDAHKIYSS DLNFNPSTGN AYINGMYFAP TQTNKEALDY 800
01015_4_C1 (ribotype 023) CdtB IIDAHKIYSS DLNFNPSTGN AYINGMYFAP TQTNKEALDY 800
01027_-1_C1 (ribotype 078) CdtB IIDAHKIYSA DLNFNPSTGN AYINGMYFTP TQTNKEALDY 800
01028_-1_C12 (ribotype 023) CdtB IIDAHKIYSS DLNFNPSTGN AYINGMYFAP TQTNKEALDY 800
01028_0_C3 (ribotype 023) CdtB IIDAHKIYSS DLNFNPSTGN AYINGMYFAP TQTNKEALDY 800
15003_-1_C1 CdtB IMDAHKIYFA DLNFNPSTGN TYINGMYFAP TQTNKEALDY 800
15003_12_C1 CdtB IMDAHKIYFA DLNFNPSTGN TYINGMYFAP TQTNKEALDY 800
15003_RL_C1 CdtB IMDAHKIYFA DLNFNPSTGN TYINGMYFAP TQTNKEALDY 800
17002_RL2_C1 CdtB ITDAHKIYSA DLNFNPSTGN AYINGMYFTP TQTNKEALDY 800
Consensus IIDAHKIYSA DLNFNPSTGN AYINGMYFAP TQTNKEALDY
100%
Conservation
0%
100%
Gap fraction
0%


820
840
R20291 WT CDT Sequence (ORF) CdtB IQKYRVEATL QYSGFKDIGT KDKEMRNYLG DPNQPKTNYV 840
01015_-1_C1 (ribotype 023) CdtB IQKYRVEATL QYSGFKDIGT KDKEMRNYLG DPNQPKTNYV 840
01015_4_C1 (ribotype 023) CdtB IQKYRVEATL QYSGFKDIGT KDKEMRNYLG DPNQPKTNYV 840
01027_-1_C1 (ribotype 078) CdtB IQKYRVEATL QYSGFKDIGT KDKEMRNYLG DPNQPKTNYV 840
01028_-1_C12 (ribotype 023) CdtB IQKYRVEATL QYSGFKDIGT KDKEMRXYLG DPNQPKTNYV 840
01028_0_C3 (ribotype 023) CdtB IQKYRVEATL QYSGFKDIGT KDKEMRNYLG DPNQPKTNYV 840
15003_-1_C1 CdtB IQKYRVEATL QYSGFKDIGT KDKEMRNYLG DPNQPKTNYV 840
15003_12_C1 CdtB IQKYRVEATL QYSGFKDIGT KDKEMRNYLG DPNQPKTNYV 840
15003_RL_C1 CdtB IQKYRVEATL QYSGFKDIGT KDKEMRNYLG DPNQPKTNYV 840
17002_RL2_C1 CdtB IQKYRVEATL QYSGFKDIGT KDKEMRNYLG DPNQPKTNYV 840
Consensus IQKYRVEATL QYSGFKDIGT KDKEMRNYLG DPNQPKTNYV
100%
Conservation
0%
100%
Gap fraction
0%
860
R20291 WT CDT Sequence (ORF) CdtB NLRSYFTGGE NIMTYKKLRI YAITPDDREL LVLSVD* 877
01015_-1_C1 (ribotype 023) CdtB NLRSYFTGGE NIMTYKKLRI YAITPDDREL LVLSVD* 877
01015_4_C1 (ribotype 023) CdtB NLRSYFTGGE NIMTYKKLRI YAITPDDREL LVLSVD* 877
01027_-1_C1 (ribotype 078) CdtB NLRSYFTGGE NIMTYKKLRI YAITPDDREL LVLSVD* 877
01028_-1_C12 (ribotype 023) CdtB NLRSYFTGGE NIMTYKKLRI YAITPDDREL LVLSVD* 877
01028_0_C3 (ribotype 023) CdtB NLRSYFTGGE NIMTYKKLRI YAITPDDREL LVLSVD* 877
15003_-1_C1 CdtB NLRSYFTGGE NIMTYKKLRI YAITPDDREL LVLSVD* 877
15003_12_C1 CdtB NLRSYFTGGE NIMTYKKLRI YAITPDDREL LVLSVD* 877
15003_RL_C1 CdtB NLRSYFTGGE NIMTYKKLRI YAITPDDREL LVLSVD* 877
17002_RL2_C1 CdtB NLRSYFTGGE NIMTYKKLRI YAITPDDREL LVLSVD* 877
Consensus NLRSYFTGGE NIMTYKKLRI YAITPDDREL LVLSVDX
100%
Conservation
0%
100%
Gap fraction
0%

APPENDIX 4: RAPID ISOLATE GHOST CdtR SEQUENCING ALIGNMENT




60
80
630 WT Ghost CDT Sequence CdtR NLLLKELTEK NKVRIYFIVV DAKYKISNEL CDGLWIAQKI 80
01006_-1_C10 (ribotype 014) CdtR NLLLKELTEK NKVRIYFIVV DAKYKISNEL CDGLWIAQKI 80
01006_0_C1 (ribotype 014) CdtR NLLLKELTEK NKVRIYFIVV DAKYKISNEL CDGLWIAQKI 80
01006_12_C11 (ribotype 014) CdtR NLLLKELTEK NKVRIYFIVV DAKYKISNEL CDGLWIAQKI 80
01011_-1_C20 (ribotype 215) CdtR NLLLKELTEK NKVRIYFIVV DAKYKISNEL CDGLWIAQKI 80
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01036_-1_C4 (ribotype 002) CdtR NLLLKELTEK NKVRIYFIVV DAKYKISNEL CDGLWIAQKI 80
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11007_-1_C5 (ribotype 029) CdtR NLLLKELTEK NKVRIYFIVV DAKYKIGNEL CDGLWIAQKI 80
11007_0_C14 (ribotype 014) CdtR NLLLKELTEK NKVRIYFIVV DAKYKISNEL CDGLWIAQKI 80
11007_RL_C1 (ribotype 014) CdtR NLLLKELTEK NKVRIYFIVV DAKYKISNEL CDGLWIAQKI 80
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18001_RL_C5 (ribotype 020) CdtR NLLLKELTEK NKVRIYFIVV DAKYKISNEL CDGLWIAQKI 80
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APPENDIX 5: RAPID CLINICAL TRIAL EXPERIMENTAL PROTOCOL AND ETHICAL APPROVAL

A randomised placebo controlled trial of "follow on" Rifaximin for the prevention of relapse of Clostridium associated diarrhoea

Final Version 2.2
30 October 2012

Short title:	Rifaxamin for preventing relapse of Clostridium associated Diarrhoea
Acronym:	RAPID
EudraCT number:	2012-003205-10
Trial Registration:	NCT01670149
ISRCTN:	65163992
CTA reference:	03057/0055
NRES reference:	12/EM/0292
Trial Sponsor:	University of Nottingham
Sponsor reference:	12072
Funding Source:	NIHR RfPB Grant No: PB-PG-0110-21041

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SYNOPSIS

Title	A randomised placebo controlled trial of "follow on" Rifaximin for the prevention of relapse of Clostridium associated diarrhoea.
Acronym	RAPID
Short title	Rifaximin for preventing relapse of Clostridium associated Diarrhoea
Chief Investigator	Professor Robin Spiller
Objectives	i) To examine efficacy of a follow-on course of Rifaximin given after a successful initial course of standard treatment, in the prevention of relapse in *C. difficile* associated diarrhoea (CDAD). ii) To examine changes in faecal microbiota in patients given Rifaximin vs. Placebo.
Trial Configuration	A randomised placebo controlled trial.
Setting	A Multi-site study across 4+ hospitals. Identifying participants from both primary and secondary care
Sample size estimate	Prior data indicate that the failure rate among controls is 30% and in Rifaximin treated patients as low as 5%. If the true failure rate for subjects receiving Rifaximin is reduced to 10% which would be a clinically important difference, we will need to study, 72 experimental subjects and 72 control subjects to be able to reject the null hypothesis that the failure rates for experimental and control subjects are equal with probability (power) 0.8. We will allow for 20% drop out rate and plan to recruit 180 patients. The Type I error probability associated with this test of this null hypothesis is 0.05. We will use a continuity-corrected chi-squared statistic or Fisher's exact test to evaluate this null hypothesis.
Number of participants	180
Eligibility criteria	**Inclusion criteria** 1) Men / Women aged 18 and over (We will also include those adults who lack mental capacity for whom we have a legal representative) 2) Successful treatment of clinically diagnosed CDAD using standard therapy (metronidazole or vancomycin given according to standard local hospital guidelines) 3) Able to swallow tablets 4) Able to stop chronic antibiotic use 5) Women of child bearing potential willing and able to use at least one highly effective contraceptive method

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	throughout the study. Acceptable contraceptive methods include: established use of oral, injected or implanted hormonal methods; placement of an intrauterine device (IUD) or intrauterine system (IUS); condom or occlusive cap (diaphragm or cervical/vault caps) with spermicide; true abstinence (when this is in line with the preferred and usual lifestyle of the participant); or vasectomised partner. Definitions Clinically diagnosed CDAD as defined by the Health Protection Agency[1] namely an episode of loose stools that occurs at the same time as a positive toxin assay (GDH +ive and toxin EIA +ive with or without a positive *C. difficile* culture) and/or endoscopic evidence of pseudomembranous colitis Successful treatment is defined as cessation of diarrhoea for 2 or more days with no loose stools. Loose stools for the purposes of this trial are defined as stools that take the shape of a container used to sample it or have a Bristol Stool Form Score (BSFS) of 6–7. Other rifamycins (e.g. rifampicin or rifabutin) are contraindicated. Patients will continue to receive all their normal medication for other conditions as prescribed by their physician. Concomitant medication including any antibiotics prescribed will be documented but will not constitute a protocol violation. **Exclusion criteria** 1) Pregnant or breast feeding 2) Hypersensitivity to the active substance, to any rifamycin (e.g. rifampicin or rifabutin) or to any of its excipients (*Tablet core:* Sodium starch glycolate type A, glycerol distearate, colloidal anhydrous, silica, talc and microcrystalline cellulose. *Tablet coating:* hypromellose, titanium dioxide (E171), disodium edentate, propylene glycol and red iron oxide E172) 3) >5 days post standard therapy (metronidazole or vancomycin) for clinically diagnosed CDAD
Description of interventions	4 weeks treatment with Rifaximin or Placebo tablets. Tapering dose starting with 2 x 200mg tablets three times a day (total = 1.2g per day) for the 1st 2 weeks, reduced to 1 x 200mg tablet three times a day (total = 0.6g per day) for the 2nd 2 weeks.

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Duration of study	Recruitment will be carried out over 24 months. Each participant will be in the trial for 12 weeks, with a follow-up contact at 6 months.
Randomisation and blinding	The Nottingham Clinical Trials Unit will use a web-based block randomisation system to allocate treatment and ensure equal numbers receive active treatment in all centres.
Outcome measures	**Primary endpoint:** The difference in % relapse between Rifaximin and placebo at 12 weeks will be assessed using a Generalised Linear Model (GLM) of Binomial family and identity Link, adjusting for centre. **Secondary endpoints**: Clinical: 1) Bowel symptoms -Average stool frequency during weeks 1-4 and weeks 11-12 -Average stool consistency during weeks 1-4 and weeks 11-12 2) Length of stay on active versus placebo 3) The difference in relapse of CDAD within 6 months of start of therapy will be assessed using a Generalised Linear Model (GLM) of Binomial family and identity Link, adjusting for centre. **Assessment of safety** Given the excellent safety record of the active there are no specific adverse events we will enquire about. At each contact, up to visit 4 for AEs and the 6 month follow-up visit for SAEs, patients will be asked whether there have been any changes in their condition since the last contact and any spontaneous adverse events will be recorded.
Statistical methods	For Descriptive purposes continuous data will be summarised in terms of the mean, standard deviation, median, lower & upper quartiles, minimum, maximum and number of observations. Categorical data will be summarised in terms of frequency counts and percentages. Tests will be two-tailed, and results will be declared "statistically significant" if $p<0.05$ and 95% confidence intervals will be presented. No formal adjustment for multiple significance testing will be applied. Full details will be given in a separate Statistical Analysis Plan to be approved before data lock.

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ABBREVIATIONS

ADR	Adverse Drug Reaction
AE	Adverse Event
BRU	Biomedical Research Unit
CDAD	Clostridium Difficile - Associated Diarrhoea
CF	Informed Consent Form
CI	Chief Investigator overall
eCRF	Electronic Case Report Form
DAP	Data Analysis Plan
DMC	Data Monitoring Committee
EOT	End of Trial
GCP	Good Clinical Practice
LOCF	Last Observation Carried Forward
MHRA	Medicines and Healthcare products Regulatory Agency
MIC	Minimum Inhibitory Concentration
NDDC	Nottingham Digestive Diseases Centre
NHS	National Health Service
NCTU	Nottingham Clinical Trials Unit (NCTU)
PI	Principal Investigator at a local centre
PIS	Participant Information Sheet
REC	Research Ethics Committee
R&D	Research and Development department
RfPB	Research for Patient Benefit
SAE	Serious Adverse Event
SAR	Serious Adverse Reaction
SCFA	Short Chain Fatty Acid
SmPC	Summary of Product Characteristics
SUSAR	Suspected Unexpected Serious Adverse Reaction
TMG	Trial Management Group
TSC	Trial Steering Committee
VRE	Vancomycin resistant enterococcus

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Rifaximin is an intestinal antibiotic used to treat traveller's diarrhoea. It is poorly absorbed and targets the microbiota of the colon. 14
Modified Xifaxanta™ (rifaximin film-coated tablet) manufactured by Alfa Wasermann (AW), Italy will be used in this trial. They will be referred to as rifaximin 200mg tablets throughout the protocol. AW will also manufacture a matching placebo formulation. In order to ensure distribution of adequate stock across the sites AW bulk supply will include a 25% overage. In order to maintain blinding active and placebo tablets will be packaged in matching de-identified trial specific containers. Final labelling and release of blinded trial treatment packs will be carried out by Norgine Limited. All sites involved have appropriate licenses in place. 14
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TRIAL / STUDY BACKGROUND INFORMATION AND RATIONALE

Clostridium difficile infection is the main cause of antibiotic associated diarrhoea. It mainly affects frail and elderly hospitalised patients, although more recently a rising incidence of a more virulent strain has been associated with infection in younger patients and those in the community. It is a significant cause of morbidity, and mortality of up to 10% has been described. *Clostridium difficile* infection is characterised by a high incidence of recurrent infection which can have debilitating consequences on already weakened patients. There are few well designed clinical trials in this condition and treatment is largely based on experience and consensus opinion. Rifaximin is a poorly absorbed antibiotic which has been used for many years in Italy and the USA for treatment of traveller's diarrhoea and Irritable Bowel Syndrome (IBS). It has an excellent safety record and has been shown to achieve high concentrations in the bowel. It has been used effectively to treat *Clostridium difficile* infection and has a low rate of resistance development. It has also been suggested as beneficial when used after an effective course of treatment with metronidazole since it is said to disturb the normal colonic bacteria less than metronidazole and vancomycin and hence might be predicted to reduce the incidence of relapse. We propose to test this hypothesis in our study. A reduction in recurrence rate of *C. difficile* from 30% to 10% will significantly reduce the burden of this disease in hospitals and the community and provide an inexpensive solution to this serious and debilitating illness.

Changes in Colonic Microbiota predisposing to *Clostridium difficile* associated diarrhoea (CDAD)

The normal colon is host to over 500 different bacteria, occupying every ecological niche, a complexity which is thought to make it difficult for pathogens to establish themselves and cause disease, a phenomenon described as “colonisation resistance. Disruption of this protective bacterial community by broad spectrum antibiotics is the commonest predisposing factor to CDAD [2]. This allows pathogenic *C. difficile* strains to establish them, causing disease with diarrhoea and colonic inflammation, mediated through secreted toxins A and B. The epidemic strains of *C. difficile* e.g. NAP1/027 produce high levels of these toxins in addition to Binary toxin which may contribute to more severe disease which is being seen more frequently[3].

Hypothesis and rationale for use of Rifaximin

Our hypothesis is that rifaximin reduces relapse of *C. difficile* by suppressing *C. difficile* growth while allowing regrowth of normal microbiota, hence restoring colonisation resistance. Placebo should allow regrowth of the normal microbiota but also *C. difficile* and hence relapse. We suspect that those that relapse have a more depleted microbiota and impaired bacterial metabolism as shown by less SCFA production

Effect of Rifaximin on *C. difficile* and Colonic microbiota

Acute infectious colitis and its associated inflammatory changes induces a shift in the dominant species away from strict anaerobes towards facultative aerobes [4] Over the following few days or weeks after cessation of antibiotic therapy with Metronidazole or Vancomycin we predict a restoration of anaerobic fermentation with

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a rise in SCFA concentration and rebalancing of the microbiota towards anaerobic organisms. SCFA levels can provide a biomarker of restoration of normal metabolic activity of colonic bacteria. We also expect that Rifaximin will continue to suppress *C. difficile* while allowing the restoration towards normal of the faecal bacteria.

Whilst *Clostridium difficile* is a major clinical problem in UK hospitals, the evidence base on which to recommend treatment is deficient. A recent Cochrane review concludes that it is impossible to establish the efficacy of antibiotic therapy for *Clostridium difficile* associated diarrhoea as the only placebo controlled trial is inadequate[5]. The reviewer made recommendations that more adequately controlled trials were urgently needed. Several recent reviews have suggested the attractiveness of rifaximin as a treatment to prevent recurrence when given after a successful initial treatment. Its advantages include its long safety record and very high luminal concentrations exceeding the MIC for *C.difficile* by several hundred fold, together with a low rate of resistance development[6, 7].
It has also been suggested as beneficial when used after an effective course of treatment with metronidazole[8] or vancomycin [9]. The rationale is that it disturbs the normal microbiota less than metronidazole and vancomycin and hence might be predicted to reduce the incidence of relapse. A recent study reported a reduced diversity of faecal microbiota in patients with recurrent *C. difficile* infection, which would be predicted to reduce colonisation resistance[10].

Evidence of Efficacy of Rifaximin in CDAD

Clinical trials of CDAD treatments

Trials have shown that in mild CDAD, specific antimicrobial treatment may not be required if the offending antibiotics can be discontinued [11] . In this study, diarrhoea resolved in 30% of patients on cessation of offending antibiotics. In a prospective randomised trial of metronidazole versus vancomycin, in 22.8% of patients with CDAD, the diarrhoea resolved during the 48-72 hours observation period before recruitment into the study [12].
Previous trials have demonstrated efficacy of vancomycin and metronidazole in treatment of CDAD and these two drugs are currently widely used in clinical practice. Many guidelines recommend Metronidazole as first line due to reported equivalence of both drugs in clinical trials, risk of colonisation with VRE, and cost of vancomycin. Recent studies have reported higher failure rates with metronidazole and higher mean duration of symptoms compared to vancomycin [13, 14,15] Data also exist for efficacy of Teicoplanin which is reported to be equivalent to Vancomycin in efficacy. Other drugs which have shown some efficacy in CDAD are Bacitracin, fusidic acid, Rifampicin, Nitazoxanide, and PAR101[16] and more recently fidaxomicin[14,15].

Recurrent CDAD

Recurrent CDAD has been reported in 15-65% of patients[13, 15] (for review see [17]). The risk of recurrence is higher in those who have already had more than one episode of CDAD[18] . This has been shown to be related to the host immune response with recurrence often being caused by a different *C. difficile* strain. There is some suggestion that relapse rate is increasing recently[15]. Treatment options for CDAD

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include tapered and pulsed regimens of vancomycin [19, 20]. The rationale for such treatment is that antibiotic resistant spores may convert to antibiotic sensitive vegetative forms during gradual withdrawal of antibiotics (tapered) and given on alternate days (pulsed) [17].
In an uncontrolled study, Rifaximin administered for 2 weeks and given immediately after vancomycin and before recurrence of symptoms, was effective in preventing predictable recurrences in 4 out of 6 chronically relapsing patients[9], the relapsed patient responded to a second course of Rifaximin but resistant *C. difficile* organisms were recovered after treatment. Results of controlled trials are still awaited.

Defining a relapse:
A relapse will be defined as the onset of >3 loose bowel movements per day for at least 2 consecutive days combined with a positive toxin assay (with or without a positive *C. difficile* culture).

Potential impact
The health protection agency's data in the UK demonstrate an increasingly serious clinical problem with *C. difficile* infection with increasing morbidity and mortality associated with more virulent epidemic strains. One of the most challenging aspects of this infection is the tendency for recurrence of disease after successful initial therapy is completed with reported recurrence rates of 30% usually within 4 weeks of completion of therapy. This is thought to be due to either re-infection or persistence of the original strain in a susceptible host. A variety of clinical strategies are currently in use for prevention of recurrence but none of these are evidence based. Clinical trials demonstrating efficacy of treatment protocols in this condition are urgently needed.

Impact of Research on NHS
Although *C. difficile* associated diarrhoea is an increasing difficult public health problem it is a neglected area of research and there are scarce evidence based data on its management. If successful these results will be widely presented and published and should be incorporated into local and National guidelines for treatment of CDAD. Implementation is easy since Rifaximin is an easily available licensed drug. Since patients with CDAD are keen to avoid recurrence we believe they will be enthusiastic for a new treatment to reduce this risk. As it is so inexpensive unlike most new treatments there should be little reason why this should not be rapidly implemented throughout the NHS.

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DETAILS OF INVESTIGATIONAL MEDICINAL PRODUCT(S)

Description and Manufacture

Rifaximin is an intestinal antibiotic used to treat traveller's diarrhoea. It is poorly absorbed and targets the microbiota of the colon.

Rifaximin 200mg tablets and placebo details.

Modified Xifaxanta™ (rifaximin film-coated tablet) manufactured by Alfa Wassermann (AW), Italy will be used in this trial. They will be referred to as rifaximin 200mg tablets throughout the protocol. AW will also manufacture a matching placebo formulation. In order to ensure distribution of adequate stock across the sites AW bulk supply will include a 25% overage. In order to maintain blinding active and placebo tablets will be packaged in matching de-identified trial specific containers. Final labelling and release of blinded trial treatment packs will be carried out by Norgine Limited. All sites involved have appropriate licenses in place.

Dosage

Treatment is for 4 weeks. The initial trial daily dose will be 2 x 200mg tablets three times a day for first 2 weeks then, 1 x 200mg tablet three times a day for the final 2 weeks.

Excipients

Tablet core: Sodium starch glycolate type A, glycerol distearate, colloidal anhydrous, silica, talc and microcrystalline cellulose
Tablet coating: hypromellose, titanium dioxide (E171), disodium edentate, propylene glycol and red iron oxide E172

Packaging and labelling

Norgine Limited (MIA(IMP) 322) will receive bulk active and placebo blister packs (12 tablets / blister pack) supplies from AW (IMP license no: aM-16/2012), Italy. They will then package the 10 complete blister packs and a single ½ blister pack into final blinded trial treatment packs. These will contain 126 blinded tablets for use by an individual participant over a 4 week period.

They will label the primary blisters according to annex 13, assuming that primary and secondary packaging remain together throughout. In particular blister packs will be labelled with a container number that will facilitate identification of the contents. Groups of 10 + ½ blister packs, all with active or placebo, will be assigned the same container number. These groups will be placed in secondary boxes labelled with the same container number as the primary packs. Again the secondary box will be labelled according to annex 13.

All participants will be given a dose instruction card describing when to take the medicine, how to take the medicine and the dose.

The final product will be QP released by the designated person to provide blinded trial treatment packs for use in the trial.

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Norgine will ship all trial supplies to Nottingham University Hospitals NHS Trust (MIA(IMP) 19162). Blinded supplies will be stored under temperature controlled conditions (<30°C) at Nottingham for distribution to trial sites.

Storage, dispensing and return

The trial treatment will be stored at Nottingham University Hospitals NHS Trust (MIA(IMP) 19162) for distribution to participating centres using a web-based system control. Each participating site will take receipt of numbered supplies from the central distribution site.

The web-based system operates as follows. Participating centres will be allocated a batch of trial treatment. The container numbers for these batches are tracked by the web-based system to the participating site and once receipt has been confirmed they are released for use in the trial. When the supplies at the participating centre reach a pre-determined level then a re-order is triggered and a further supply of trial treatment is sent to the corresponding participating site.

At site the trial treatment will be stored below 30°C. Storage will be in a secure location, in a temperature controlled environment, with a temperature log maintained daily, and may be dispensed only by specifically authorised personnel.

The local site investigator is responsible for ensuring trial treatment accountability, including reconciliation of trial treatment and maintenance of trial treatment records, throughout the course of the study in accordance with UK regulatory requirements. Responsibility can be delegated to the site pharmacy clinical trials staff.

Prescription will be issued at visit 1 for 4 weeks supply of trial treatment i.e. 1 container. Details will be transferred to a trial specific prescription and the pharmacy will complete the dispensing process by addition of the participant's name, date of dispensing and participant number to each allocated container.

Dispensing will be recorded on the appropriate trial specific accountability forms. Trial treatment must not be used for any other purpose than the present study. Returned trial treatment that has been dispensed to a participant must not be re-dispensed to a different participant. Unused trial treatment with the original container will be collected by the local study team at visit 3 and returned to the local pharmacy for destruction. There is enough treatment for 4 weeks protocol defined dose.

Residual numbers of tablets will be recorded and accountability completed before local destruction to assess compliance (see compliance section for more details).

Placebo

The placebo will be manufactured, supplied, packaged, labelled and QP released to provide blinded treatment packs and distributed as for the active IMP. The placebo formulation will match that of the rifaximin formulation in packaging, appearance and composition, except for the active drug. However the placebo is not matched for tablet core colour and unblinding could occur if both tablets are crushed.

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Known Side Effects
Since less than 3% of the drug is absorbed there are few common side effects.

Frequency	Side Effect
Common (≥1/100 to 1/10)	Dizziness, Headache, Abdominal pain, Constipation, Defecation urgency, Diarrhoea, Flatulence, bloating and distension, Nausea and vomiting symptoms, Rectal tenesmus, Pyrexia
Uncommon (≥ 1/1000 to < 1/100)	Candidiasis, Herpes simplex, Nasopharyngitis, Pharyngitis, Upper respiratory tract infection, lymphocytosis, Monocytosis, Neutropenia, Decreased appetite, Dehydration, Abnormal dreams, Depressed mood, Insomnia, Nervousness Hypoesthesia, Migraine, Paraesthesia, Sinus headache, Somnolence, Diplopia Ear pain, Vertigo, Palpitations, Blood pressure increased, Hot flush, Cough, Dry throat, Dyspnoea, Nasal congestion, Oropharyngeal pain, Rhinorrhea, Abdominal pain upper, Dry lips, Dyspepsia, Gastrointestinal motility disorder, Faeces hard, Haematochezia, Mucous stools, Taste disorders, Aspartate aminotransferase increased, rashes, eruptions and exanthemas NEC, Sunburn, Back pain, Muscle spasms, Muscular weakness, Myalgia, Neck pain, Blood in urine present, Glycosuria, Pollakiuria, Polyuria, Proteinuria, Polymenorrhoea, Asthenic conditions, Chills, Cold sweat, Hyperhidrosis, Influenza like illness, Oedema peripheral, Pain and discomfort, NEC
Unknown	Clostridial infections, Thrombocytopenia Anaphylactic responses, Angioedemas, Hypersensitivity, Presyncope, Liver function tests abnormalities, Dermatitis, Eczema, Erythemas, Pruritus NEC, Urticarias, International normalised ratio abnormalities.

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TRIAL / STUDY OBJECTIVES AND PURPOSE

PURPOSE
A clinical and microbiological investigation into the value of the broad spectrum poorly absorbed antibiotic, Rifaximin, used as a prophylactic measure to reduce the relapse rate of *Clostridium difficile* associated diarrhoea (CDAD) after initial successful standard antibiotic therapy with metronidazole or vancomycin.

PRIMARY OBJECTIVE
To assess whether rifaximin can reduce the relapse rate of CDAD, following clinical resolution of CDAD with standard therapy (metronidazole or vancomycin).

SECONDARY OBJECTIVES
Effect of "follow on rifaximin" on:

a) bowel symptoms during 12 weeks after start of treatment,
b) length of stay
c) stool microbiota
d) risk of relapse at 6 months

In addition qualitative data will be collected on:

e) Patient experience in taking part in trial to inform future larger trials.

TRIAL / STUDY DESIGN

TRIAL / STUDY CONFIGURATION

This is a multi-centre, two-arm, parallel group, double-blind, randomised placebo controlled trial comparing rifaximin with placebo in patients with clinically resolved CDAD.

Primary endpoint
This is the proportion of patients who have relapsed within 12 weeks on the active versus placebo.

Definition of relapse:
A relapse will be defined as the onset of >3 loose bowel movements per day for at least 2 days combined with a positive toxin assay (with or without a positive *C. difficile* culture). Loose stools for the purposes of this trial are defined as stools that take the shape of a container used to sample it or have a Bristol Stool Form Score (BSFS) of 6–7.

Secondary endpoint
Clinical:

1) Stool frequency and consistency during 12 weeks after start of treatment
2) Rehospitalisation for CDAD within 6 months
3) Length of stay where relevant

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Microbiological: These exploratory assessments will be made at 0, 4 and 12 (and on residual diagnostic and relapse samples) weeks after starting rifaximin or placebo. They will include:

1) Assessment of metabolic activity from pH and SCFA concentrations
2) % *C. difficile* positive (cultured earlier if relapse).
3) Number of *C. difficile* spores as assessed from colony counts after heat shock treatment of stool.
4) Differences in microbiological profile as assessed using the HIT chip which provides quantification for over 400 separate microbial species.
5) Antibody response to *C. difficile*

Safety endpoints

1) Adverse events, as per study specific definition

Stopping rules and discontinuation

Participants may withdraw themselves from the study at any point. This will not affect their normal clinical care in any way. Adverse events are likely to be minor but will be assessed by the supervising doctor who will decide whether they should be allowed to continue in the trial. Non-compliance with treatment regime may also lead to a participant discontinuing in the study.

Any participant who does not continue to meet the inclusion/exclusion criteria will be withdrawn from the trial immediately.

In the unlikely event of recurrent SAE's then the study may be stopped on advice from the Data Monitoring Committee if it is considered that the safety of the participant is being put at unacceptable risk.

RANDOMIZATION AND BLINDING

This is a randomized double blind placebo controlled study. Participants, supervising doctor, study nurses and the research team (including NCTU) will be blinded to treatment allocation.

The randomisation will be based on a computer generated pseudo-random code using random permuted blocks of randomly varying size, created by the Nottingham Clinical Trials Unit (NCTU) in accordance with their standard operating procedure (SOP) and held on a secure server. The randomisation will be stratified by site.

The supervising doctor or study nurse will obtain a randomisation reference number for each participant by means of a remote, internet-based randomisation system developed and maintained by the NCTU.

The sequence and decode of treatment allocations will be concealed until interventions have all been assigned and recruitment, data collection, and all other trial-related assessments are complete.

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Maintenance of randomisation codes and procedures for breaking code
Access to the master sequence of treatment allocations will be confined to the NCTU IT Manager and the Nottingham University Hospitals NHS Trust Clinical Trials pharmacy.

In the event of the need to break the code, usually due to clinical need, the date and reason for breaking the code will be recorded in the eCRF. The code can only be broken by authorised personnel via the web-based eCRF. In the event the system being inaccessible, the Nottingham University Hospitals NHS Trust Clinical Trials pharmacy (24hr contact number) will keep a copy of the treatment allocations.

The need for code breaking will, if possible, be discussed with the Chief Investigator or designee or local Principle Investigator who will authorise the code break and decide on further management including the need to inform regulatory authorities. For the vast majority of cases unblinding will not be required because there is no antidote to the investigational treatments, and the medical care and usually the management of the patient would not be any different even if the treatment group assignment of the patient were known.

TRIAL MANAGEMENT

The Sponsor is the University of Nottingham. The CI, delegated by the Sponsor, is responsible for the proper conduct and management of the trial. A number of committees will be assembled to help ensure the proper management and conduct of the trial, and to ensure the safety and well-being of the patients enrolled.

The following committees have been formed:

Trial Steering Committee (TSC)
Data monitoring committee (DMC)
Trial Management Group (TMG)

The general purpose, responsibilities and structures of the committees are described below. However, it is assumed that the TSC and TMG will develop their own rules and procedures which may evolve with time, during the preparation and conduct of the trial. The DMC will use the Sponsor contract (containing the terms and conditions) and standard charter as a basis for their rules and procedures.

Trial Management Group
The TMG will oversee the operational aspects of the trial, which include the processes and procedures employed, and the day-to-day activities involved in study conduct. Day to day management of the trial will be undertaken by the research fellow based in Nottingham and the Nottingham Clinical Trials Unit. The TMG will meet regularly to review the progress of the trial and to address any urgent issues. The TMG will provide recommendations and suggestions to the TSC.

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Trial Steering Committee (TSC)
The TSC is composed of representatives from the academic and medical communities, and non-voting members from the TMG. The TSC has the overall responsibility for ensuring a scientifically sound study design, a well-executed trial, and accurate reporting of the study results. The TSC must address and resolve scientific, medical, and practical issues encountered during the trial. The TSC will draw up its own guidelines, and will review the criteria and guidelines of the other committees in order to provide advice and suggestions if necessary. The TSC will convene, either in person or via teleconference, as often as deemed necessary to carry out its responsibilities, but at least once per year.

Data Monitoring Committee (DMC)
An independent DMC will evaluate the outcome and safety data in the context of the overall trial and the currently existing information about the study drugs. The DMC will consider the appropriate timeframe for reviewing the data during the course of the study and develop the Sponsor Charter delineating their guidelines for operating, and stopping rules for terminating a portion or all of the trial prematurely.

The DMC will have access to all trial data. It may request and will be provided with whatever data it deems necessary or useful for it to carry out its duties, including the provision of unblinded data. The DMC will provide their recommendations to the TSC over the course of the trial.

Protocol deviations and violations.
A protocol deviation is an unanticipated or unintentional divergence or departure from the expected conduct of a study inconsistent with the protocol, consent document or other study procedures. All protocol deviations shall be recorded on the eCRF by local investigator staff.

Protocol violations of eligibility criteria will be discussed by the TMG and may lead to withdrawal of those participants. These and other deviations from protocol will be assessed by TMG and discussed with the TSC during study evaluation before data lock and unblinding.

DURATION OF THE TRIAL / STUDY AND PARTICIPANT INVOLVEMENT

Participant recruitment will start as soon as all relevant approvals are in place, and is anticipated to continue for approximately 2 years, though this may be adjusted according to the rate of recruitment. The study is funded for 3 years.

End of the Trial
The end of the trial will be defined as the last participant last visit. The last scheduled visit is 6 months post-randomisation.

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SELECTION AND WITHDRAWAL OF PARTICIPANTS

Recruitment

Participants will be recruited from both Primary care (GP Practices) and Secondary Care (Hospitals).

Patients testing positive for *C. difficile* while in secondary care will be identified through the central testing laboratory. They will inform a Specialist Nurse who also forms part of their usual care team and whose designated role is that of improving management of patients with *C. difficile* associated diarrhoea (CDAD), or a local research nurse. Once each patient has commenced their standard hospital treatment for CDAD they will be approached by the Nurse who will explain the study and give them a copy of the information sheet, with a view to the patient being recruited onto the study once their CDAD has clinically resolved. If a patient later decides that they would wish to take part in the study, then an appointment will be made for a screening visit, where the study will be explained in more detail. They will have the opportunity to have their questions answered and if they decide to go ahead with the study, they can sign a consent form.

Patients testing positive for *C. difficile* while in primary care will be identified through the central testing laboratory. The Specialist Nurse or the laboratory reporting team will send the results of the stool sample test to the patients GP. They will also include a GP invitation letter, a patient invitation letter and a patient Information sheet. The invitation will ask the GP or their practice nurse if they would provide the study information to the patient, and request if the patient would allow the research team to contact them. The patient will be under no-obligation and will have the opportunity to opt-out prior to the contact from the research team. This approach is necessary due to the time restricted nature in recruiting these patients i.e. we need to recruit them as they complete their course of treatment for their CDAD. Alternatively the patient can be provided with the information and contact the research team direct, if the patient does not agree to the research team contacting them direct.

The investigator or their nominee, e.g. from the research team or a member of the participant's usual care team, will inform the participant or their nominated representative (other individual or other body with appropriate jurisdiction), of all aspects pertaining to participation in the study.

If needed, the usual hospital interpreter and translator services will be available to assist with discussion of the trial, the participant information sheets, and consent forms but the consent forms and information sheets will not be available printed in other languages.

It will be explained to the potential participant that entry into the trial is entirely voluntary and that their treatment and care will not be affected by their decision. It will also be explained that they can withdraw at any time. In the event of their withdrawal it will be explained that their data collected so far cannot be erased and we will seek consent to use the data in the final analyses where appropriate.

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Inclusion criteria

1) Men / Women aged 18 and over (We will also include those adults who lack mental capacity for whom we have a legal representative)
2) Successful treatment of clinically diagnosed CDAD using standard therapy (metronidazole or vancomycin given according to standard local hospital guidelines).
3) Able to swallow tablets
4) Able to stop chronic antibiotic use
5) Women of child bearing potential willing and able to use at least one highly effective contraceptive method throughout the study. Acceptable contraceptive methods include: established use of oral, injected or implanted hormonal methods; placement of an intrauterine device (IUD) or intrauterine system (IUS); condom or occlusive cap (diaphragm or cervical/vault caps) with spermicide; true abstinence (when this is in line with the preferred and usual lifestyle of the participant); or vasectomised partner.

Definitions

Clinically diagnosed CDAD is defined by the Health Protection Agency[1] namely an episode of loose stools that occurs at the same time as a positive toxin assay (GDH +ive and toxin EIA +ive with or without a positive *C. difficile* culture) and/or endoscopic evidence of pseudomembranous colitis

Successful treatment is defined as cessation of diarrhoea for 2 or more days with no loose stools, assessed, for secondary care patients, from clinical notes or, for primary care patients, patient reported.

Loose stools for the purposes of this trial are defined as stools that take the shape of a container used to sample it or have a Bristol Stool Form Score (BSFS) of 6–7, though this is not a specific entry criteria for all participants as this information may not be recorded in primary care.

Other rifamycins (e.g. rifampicin or rifabutin) are contraindicated. Patients will continue to receive all their normal medication for other conditions as prescribed by their physician. Concomitant medication including any antibiotics prescribed will be documented but will not constitute a protocol violation.

Exclusion criteria

1) Pregnant or breast feeding
2) Hypersensitivity to the active substance, to any rifamycin (e.g. rifampicin or rifabutin) or to any of its excipients (*Tablet core:* Sodium starch glycolate type A, glycerol distearate, colloidal anhydrous, silica, talc and microcrystalline cellulose. *Tablet coating:* hypromellose, titanium dioxide (E171), disodium edentate, propylene glycol and red iron oxide E172)
3) >5 days post standard therapy (metronidazole or vancomycin) for clinically diagnosed CDAD

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Expected duration of participant participation
Study participants will be participating in the study for 12 weeks, with a follow-up contact at 6 months.

Removal of participants from therapy or assessments
The following subject withdrawal criteria apply:

1) Non-compliance - if less than 75% of IMP doses are taken between visits, at the investigator's discretion.
2) Adverse reaction (serious and non-serious) with clear contraindications.
3) Participant withdraws consent.
4) Safety reasons e.g. pregnancy*
5) Lost to follow up.
6) Participant develops an excluded/contraindicated condition.
7) Investigator discretion. (e.g. Protocol violations)
8) Unblinding, at the discretion of the PI in conjunction with the CI.

* In the event of a pregnancy occurring in a trial participant or the partner of a trial participant monitoring shall occur during the pregnancy and after delivery to ascertain any trial related adverse events in the mother or the offspring. Where it is the partner of a trial participant, consent will be obtained for this observation from both the partner and her medical practitioner.

Participants withdrawn from the study will not be replaced. The participants will be made aware that withdrawal will not affect their future care. Participants will also be made aware (via the information sheet and consent form) that should they withdraw, the data collected up to their withdrawal cannot be erased and may still be used in the final analysis.

Due to the fact that patients entered into the study will have already successfully received first line treatment for their *C. difficile* infection prior to entry onto this study; we do not expect that any abrupt termination of study treatment will have any effect on participant safety.

Informed consent
All participants who are able to consent for themselves will provide written informed consent. We also intend to recruit patients who lack capacity. Where there is lack of capacity or there is suspicion of a high risk of diminished capacity then a mental capacity assessment (according to normal local procedures) will also be carried out to determine the patient's ability to give informed consent. Where the patient is found to lack capacity, arrangements will be made to meet with next of kin, relatives or carer's to discuss the trial and whether they would be willing to act as Legal Representative on behalf of the patient and give consent on their behalf, should they believe that the patient would have agreed to take part in the trial if they were able to give consent.

The investigator or their nominee, e.g. from the research team or a member of the participant's usual care team, will inform the participant and/or their relative/carer of

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all aspects pertaining to participation in the study. The Investigator will answer any questions that the participant and/or relative/carer has concerning study participation. If needed, the usual hospital interpreter and translator services will be available to assist with discussion of the study, the participant information sheets, and consent forms, but the consent forms and information sheets will not be available printed in other languages.

Potential patients identified as being suitable for the study will be told about the study during their course of standard clinical care eradication treatment for *C. difficile*; so will have some time to decide whether they wish to take part in the study or not. On successful completion of their treatment they will be asked for their consent to take part in the study.

CDAD is predominant in older patients and the numbers of patients affected over the age of 65 have progressively increased. A significant proportion of this patient group lack capacity either on a permanent basis due to dementia or due to toxic confusional state in the context of acute illness. Given the importance of improving the evidence base for clinical treatment in this potentially life threatening disease in this patient group, we feel that it is important to include patients lacking capacity. We will provide information sheets written in a simplified and abbreviated form to help patients understand as best possible the issues around the trial and what would be expected of them on trial entry. Next of kin/relative or carer will be approached to act as Legal Representative on behalf of any patient who lacks capacity. Where no next of kin are available to act as legal representative, a health professional not involved in the research will be nominated to act as a Professional legal representative. In this instance research nurses will work closely with carers and or nursing staff on inpatients hospital wards to assist with the trial to include, diary completion, stool collection and administration of study medication.

We will provide information sheets for legal representative. Participants consented via this route will not be requested to provide a blood sample at week 4.

If at any point the patient regains capacity, then the original consent will remain valid but the participant will be informed and his/her wishes respected.

Informed consent form will be signed and dated by each participant (or their legal representative) before they undergo any trial procedures. It will be explained that entry into the study is entirely voluntary and that their treatment and care will not be affected by their decision. It will also be explained that they can withdraw at any time but attempts will be made to avoid this occurrence. In the event of their withdrawal it will have be explained that their data collected so far cannot be erased and we may use the data in the final analyses where appropriate.

The investigator or their nominee and the participant or their legal representative must both sign and date the consent form. One copy of this will be kept by the participant, one will be kept by the investigator, and a third will be retained in the patient's hospital records.

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Should there be any subsequent amendment to the final protocol, which might affect their participation in the trial, then these will be discussed with the participant and, if applicable, continuing consent will be obtained using an amended consent form.

The process for obtaining informed consent will be in accordance with the REC guidance, and GCP and any other applicable regulatory requirements, which may be introduced.

TRIAL / STUDY TREATMENT AND REGIMEN

Table 1 and figure 1 summarise the participant involvement and timelines.

Participants should complete all scheduled visits, within proposed visit windows. In the event of a participant withdrawing a discontinuation visit should be arranged, if possible. Participants will be asked to carry a trial identification card in the unlikely event that their treating physician will need to know their treatment allocation.

Randomisation occurs at visit 1. The start of the trial treatment (T=0) should start as close to randomisation as possible. Visits 2 & 4 have a window of ±1 week, visits 3 & 5 have a window of +1 week (in order to ensure timely dose change, trial medication is complete and stool diaries are complete), and the follow-up visit has a window of ±2 weeks, within which it is acceptable to complete the visit. Each participant's actual visit schedule should be determined taking into account practical considerations, the allowed visit window and completion of the treatment period. Visits schedules are in relation to T=0 date. Participants or their carer's will complete a stool diary during weeks 1-4, and for the 7 days prior to the week 12 visit.

There are 5 visits in total with an additional follow-up visit at 6 months.

Visit 1 = informed consent against eligibility criteria, basic demographics, medical history, concurrent medications and stool sample collected. Randomisation, to either rifaximin or placebo. (1x prescription, for 4 weeks treatment). Stool diary for weeks 1-4 provided with instructions. Pregnancy test, if applicable.

Visit 2 (week 2) = telephone contact to check diary completion, IMP compliance and dose change reminder, concurrent medications, adverse events / SAEs and CDAD recurrence / hospitalisation.

Visit 3 (week 4) = check IMP compliance, concurrent medications, adverse events / SAEs and CDAD recurrence / hospitalisation. Collect IMP containers and any remaining IMP and return to pharmacy if necessary. Stool and blood sample (serum to assess antibody response to *C. difficile* toxins, apart from participant with legal representative consent) collected. Stool diary for weeks 1-4 collected. Stool diary for week 12 provided.

Visit 4 (week 8) = telephone contact to check concurrent medications, adverse events / SAEs and CDAD recurrence / hospitalisation.

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Visit 5 (week 12) = check concurrent medications, SAEs and CDAD recurrence. Stool sample collected. Stool diary for week 12 collected (must be completed for 7 days prior to this visit).

Follow-up visit = telephone contact at 6 months will be made with the patient or their carer to determine if there has been any recurrence of CDAD and any resulting rehospitalisation. Check concurrent medications and SAEs. The participant's hospital records will be checked prior to this contact to confirm health status.

Telephone visits at weeks 2 & 8 (and for the 6 month follow-up) can, if convenient, be completed face-to-face in the hospital.

Stool samples will be collected, if possible, following any relapse within the first 3 months. This will either be from the standard clinical sample sent to the central testing laboratory (which they will consent for) or a separate sample specifically collected for the trial.

Details of stool collection

In-patients will be asked to provide a stool sample using a cardboard bed pan or if incontinent the sample will be collected by the ward nurse looking after the patient. Outpatients will be given labelled routine stool sample screw top 20ml collection tubes with integral spatula and instructed to put a cherry sized sample of stool into three containers, screwed shut and placed in a cool gel pack container which will keep the samples cool overnight before being delivered to the BRU the next day. Carers or attending nurses will be asked to assist those unable to perform these tasks.

Completion of stool diaries and medication check

Patients or their carers will be asked to complete the stool diaries and instructed in the Bristol Stool Form Scoring system as is routine practice on wards and in outpatients. The diary will include reminder to take tablets which will be taken by the patient or administered by their carers if necessary along with any other regular medication.

We aim to recruit over a 24 month period or until we have recruited 180 patients whichever is the sooner, after which the study will close to recruitment.

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Table 1: Study assessments and visit schedule

Procedure (Time (T) in wks)		Visit 1 Week 0	Visit 2 ☎ Week 2	Visit 3 Week 4	Visit 4 ☎ Week 8	Visit 5 Week 12	Follow-up visit ☎ 6 months
Check eligibility		●					
Informed consent		●					
Demographics and CDAD History		●					
Concurrent medication recording		●	●	●	●	●	●
Prescribed antibiotic check		●	●	●	●	●	●
Collect Daily symptom and stool diary				●		●	
Pregnancy test		●					
Randomisation		●					
Blood sample				●			
Stool Sample		●		●		●	
IMP	Dispense	●					
	Return			●			
Adverse event recording			●	●	●		
SAE recording			●	●	●	●	●
Check CDAD Recurrence			●	●	●	●	●
End of Trial							●

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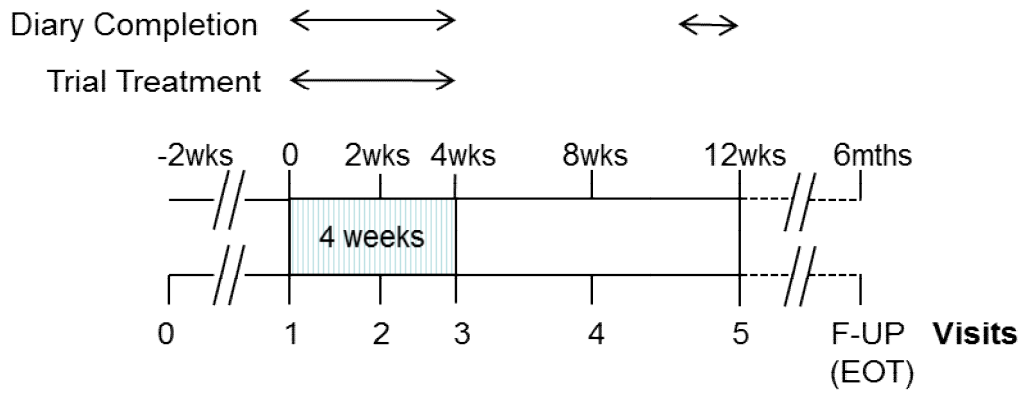


Figure 1 Participant flow chart

Visit	Visits 2 & 4 have a **±1 week** window. Visits 3 & 5 have a **+1 week** window. The follow-up visit has a **±2 week** window. T=0 is date of first trial dose and all visits are based from this date. Concurrent medications are recorded throughout trial participation.
0	Potential participants identified (central testing laboratory) and information about the trial given.
1	Review eligibility, Informed consent & randomisation. Stool sample. Trial treatment dispensed.
2	Telephone visit at 2 weeks to check trial treatment in relation to the planned decrease in dose, diary completion, CDAD recurrence and AEs/SAEs.
3	Clinic visit at 4 weeks to check CDAD recurrence & AEs/SAEs. Stool and blood sample. Collect diaries and remaining trial treatment.
4	Telephone visit at 8 weeks. Check CDAD recurrence & AEs/SAEs.
5 (EOT)	Clinic visit at 12 weeks to check CDAD recurrence & SAEs. Collect week 12 diary. Stool sample.
Follow-up	Telephone visit at 6 months to check CDAD recurrence & SAEs.

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Compliance
Compliance for hospitalised patients will be assessed from their medication chart and stool diary which nursing staff will complete. Patients receiving treatment at home will be asked to bring back unused medication or the nurse will collect and compliance will be assessed by tablet count of remaining tablets. Tablets will be counted and checked for compliance at visit 3. In addition the participants will record their daily dose on the stool diary's during the treatment phase. Consumption of less than 75% of medication will be a protocol violation.

Concurrent Medications
Rifymacins (e.g. rifampicin or rifabutin) are contraindicated and therefore should not be administered concomitantly with rifaximin.

Patients will continue to receive all their normal medication for other conditions as prescribed by their physician. Changes to baseline concomitant medication will be recorded up to and including the follow-up visit at 6 months. Any antibiotics prescribed will be recorded up to and including the follow-up visit at 6 months, but will not constitute a protocol violation. In particular if, during the trial, the participant starts chronic antibiotic use (which is part of the eligibility criteria) we will record this but will not withdraw the participant.

Accountability for drugs & placebos
Residual numbers of tablets will be recorded and accountability forms completed, before local destruction. An on-going record of daily dose will also be kept on the daily stool diaries.

Management of study drug overdose
There are no specific measures needed and no expected toxicity. Patients will merely be observed if in hospital or if at home they will be contacted daily for the next 4 days to confirm no untoward events occur.

Criteria for terminating trial
It is not anticipated that the trial will be stopped before recruitment is complete as there is no formal interim analysis planned for the primary endpoint. In the event any major safety concerns are identified then the TSC will consider terminating the trial.

Recruitment at individual sites may be terminated due to poor recruitment or serious breaches of GCP.

The Data Management Committee will meet 6 monthly to advise if there is any reason to stop the trial owing to imbalance of deaths within the two treatment groups.

Sample storage and disposal

Stool sample
All stool samples (weeks 0, 4 and 12, residual diagnostic sample and relapse samples) will be analysed for the following:

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1) Assessment of metabolic activity from pH and SCFA concentrations
2) % *C. difficile* positive (cultured earlier if relapse).
3) Number of *C. difficile* spores as assessed from colony counts after heat shock treatment of stool.
4) Differences in microbiological profile as assessed using the HIT chip which provides quantification for over 400 separate microbial species.
5) Antibody response to *C. difficile*

Stool samples will be collected from participants at all research sites and temporarily stored locally at -70^{o}C. Sites will receive detailed procedures relating to storage and shipment of samples. All stool samples will be analysed centrally in Nottingham, however results will not be available during the trial and will not influence the clinical management of study participants. Regular frozen shipments will be arranged to Dr Diggle's laboratory, University of Nottingham for long term storage and later analysis. Analysis of bacterial DNA will be performed on anonymous samples which may also be sent to outside laboratories.

Blood sample at 4 weeks

Serum isolated from 20ml of blood will be processed locally and stored at -70^{o}C at the respective sites. Sites will receive detailed procedures relating to processing, storage and shipment of samples. Regular frozen shipments of serum will be arranged to the Nottingham Digestive Diseases Centre (NDDC) for long term storage and analysis. Additional analyses on anonymous samples may be sent to outside laboratories with the appropriate expertise.

Any remaining blood or stool sample at the NDDC will be stored for future analysis under the University of Nottingham Human Tissue License 12265, if applicable, and in accordance with the Human Tissue Act 2004, once ethical approval covering the samples expires. These samples will be coded for study use but the link between the patient and the code will be deleted for future studies.

Where participants do not agree to the future use of the samples they will be destroyed in accordance with the Human Tissue Act, 2006.

Disposal will be according to local laboratory procedures.

TRANSPORT AND STORAGE OF THE TISSUES

Stool and blood samples will be stored in linked anonymised format in the microbiology laboratories at Nottingham University Hospital and the NIHR Biomedical Research Unit (BRU) labelled with a study number to permit accurate linkage to the consent form.
The master database will be held by the CI in a password encrypted file.

The analysis of samples will take place in the Nottingham University Hospitals NHS Trust within the Department of Microbiology, and also in the University of Nottingham, Department of Molecular Microbiology and samples may be sent in anonymised form to outside laboratories for further specialised analysis.

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Stool and blood samples will be transferred from the BRU to the respective collaborators in batch shipments by courier as frequently as required. All shipments will contain a complete inventory of all samples, along with the name of the person responsible for sending the samples.

STATISTICS

Methods

For Descriptive purposes continuous data will be summarised in terms of the mean, standard deviation, median, lower & upper quartiles, minimum, maximum and number of observations. Categorical data will be summarised in terms of frequency counts and percentages.

Tests will be two-tailed, and results will be declared "statistically significant" if $p<0.05$ and 95% confidence intervals will be presented.

No formal adjustment for multiple significance testing will be applied.

Full details will be given in a separate Statistical Analysis Plan to be approved before data lock.

Sample size and justification

Prior data indicate that the failure rate among controls is 30% and in Rifaximin treated patients as low as 5%. If the true failure rate for subjects receiving Rifaximin is reduced to 10% which would be a clinically important difference of 20% (i.e. 30-10), we will require 72 participants per arm (144 in total, though will recruit 180 in total to allow for up to 20% drop-out) to be able to reject the null hypothesis that the failure rates for Rifaximin group and control group are equal. The sample size was estimated using 80% power and 5% significance level (type I error). After allowing for 20% attrition rate, our sample size totals to 180.

Assessment of efficacy

Primary endpoint: The difference in % relapse between Rifaximin and placebo at 12 weeks will be assessed using a Generalised Linear Model (GLM) of Binomial family and identity Link, adjusting for centre.

Secondary endpoints:

Clinical:

1) Bowel symptoms

- Average stool frequency during weeks 1-4 and weeks 11-12
- Average stool consistency during weeks 1-4 and weeks 11-12

2) Length of stay on active versus placebo

3) The difference in relapse of CDAD within 6 months of start of therapy will be assessed using a Generalised Linear Model (GLM) of Binomial family and identity Link, adjusting for centre (similar to primary analysis).

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Further details regarding the analysis will be documented in a separate statistical analysis plan.

Microbiological:
These exploratory assessments will be made at 0, 4 and 12 weeks (and on residual diagnostic and relapse samples) after starting rifaximin or placebo. They will include 1) assessment of metabolic activity from pH and SCFA concentrations which will be compared using either the t test or Mann-Whitney U depending on their distribution 2) % *C. difficile* positive (cultured earlier if relapse) which will be compared using a continuity-corrected chi-squared statistic or Fisher's exact test 3) Average number of spores as assessed from colony counts after heat shock treatment of stool will be compared for active versus placebo using Students t Test. 4) Differences in microbiological profile as assessed using the HIT chip will be assessed using hierarchial clustering analysis using Pearson's correlation to calculate the distance among samples and Ward's minimum variance agglomeration method. as previously described Simpson's reciprocal index of diversity will be used to express the diversity of microbial communities, calculated using the equation I=1/S Pi2, where Pi is the proportion of the ith taxon. The proportion of each taxon was calculated as the proportion of each probe signal compared to the total signal. A higher value of Simpson index corresponds to a more diverse community. Comparisons of the average Simpson's Index between active and placebo will be made using Students t-Test or in the event of a non-normal distribution the Mann-Whitney U test will be used.

Assessment of safety
Given the excellent safety record of the active[21,22] there are no specific adverse events we will enquire about.
At each contact up to visit 4 for AEs and the 6 month follow-up visit 4 for SAEs, patients will be asked whether there have been any changes in their condition since the last contact and any spontaneous adverse events will be recorded.

Procedures for missing, unused and spurious data
Where patients cannot be contacted we will make every effort to ascertain whether they have had a further episode of CDAD by contacting their GP, checking the Public Health Laboratory records and by examining their online hospital record.
It is unlikely that a recurrence would be missed using this approach and given this, we will conduct an available case data analysis. If there happens to be any missing data, then these will be explored and as a sensitivity analysis, the LOCF approach will be used.

Definition of populations analysed
Safety set: All randomised participants who receive at least one dose of the study drug.
Full Analysis set: All randomised participants, who take at least one dose of study medication and for whom at least one post-baseline assessment of the primary endpoint is available.
Per protocol set: All participants in the Full Analysis set who are deemed to have no major protocol violations that could interfere with the objectives of the study.

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ADVERSE EVENTS

Definitions

In general terms an adverse event is any unfavourable and unintended sign, symptom, syndrome or illness that develops or worsens during the period of observation in the study.

An AE does include a / an:

1. exacerbation of a pre-existing illness.

2. increase in frequency or intensity of a pre-existing episodic event or condition.

3. condition detected or diagnosed after medicinal product administration even though it may have been present prior to the start of the study.

4. continuous persistent disease or symptoms present at baseline that worsen following the start of the study.

An AE does not include a / an:

1. medical or surgical procedure (e.g., surgery, endoscopy, tooth extraction, transfusion); but the condition that lead to the procedure is an AE.

2. pre-existing disease or conditions present or detected at the start of the study that did not worsen.

3. situations where an untoward medical occurrence has not occurred (e.g., hospitalisations for cosmetic elective surgery, social and / or convenience admissions).

4. disease or disorder being studied or sign or symptom associated with the disease or disorder unless more severe than expected for the participant's condition.

5. overdose of concurrent medication without any signs or symptoms.

A Serious Adverse Event (SAE) is any adverse event occurring following study mandated procedures, having received the IMP or placebo that results in any of the following outcomes:

1. Death

2. A life-threatening adverse event

3. Inpatient hospitalisation or prolongation of existing hospitalisation

4. A disability / incapacity

5. A congenital anomaly in the offspring of a participant

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Important medical events that may not result in death, be life-threatening, or require hospitalisation may be considered a serious adverse event when, based upon appropriate medical judgment, they may jeopardize the patient or participant and may require medical or surgical intervention to prevent one of the outcomes listed in this definition

All adverse events will be assessed for seriousness, expectedness and causality:

A distinction is drawn between serious and severe AEs. Severity is a measure of intensity whereas seriousness is defined using the criteria above. Hence, a severe AE need not necessarily be serious.

Causality
Not related or improbable: a clinical event including laboratory test abnormality with temporal relationship to trial treatment administration which makes a causal relationship incompatible or for which other drugs, chemicals or disease provide a plausible explanation**.** This will be counted as "unrelated" for notification purposes.

Possible: a clinical event, including laboratory test abnormality, with temporal relationship to trial treatment administration which makes a causal relationship a reasonable possibility, but which could also be explained by other drugs, chemicals or concurrent disease. This will be counted as "related" for notification purposes.

Probable: a clinical event, including laboratory test abnormality, with temporal relationship to trial treatment administration which makes a causal relationship a reasonable possibility, and is unlikely to be due to other drugs, chemicals or concurrent disease. This will be counted as "related" for notification purposes.

Definite: a clinical event, including laboratory test abnormality, with temporal relationship to trial treatment administration which makes a causal relationship a reasonable possibility, and which can definitely not be attributed to other causes. This will be counted as "related" for notification purposes.

An AE whose causal relationship to the study IMP is assessed by the Chief Investigator as "possible", "probable", or "definite" is an Adverse Drug Reaction.

With regard to the criteria above, medical and scientific judgment shall be used in deciding whether prompt reporting is appropriate in that situation.

Reporting of adverse events
All AEs will be documented following the first dose of IMP, up to and including visit 4 at week 8.

All SAEs, including rehospitalisation due to CDAD, will be reported and documented following the first dose of IMP, up to and including the 6 month follow-up visit.

Elective surgery or pre-planned hospitalisation will not be recorded.

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All available sources of information will be used to collect information on adverse events including hospital notes, the participant diary and discussion with the participant.

Participants will also be asked to contact the study site immediately in the event of any serious adverse event. All adverse events will be recorded and closely monitored until resolution, stabilisation, or until it has been shown that the study medication will not be stopped. The Chief Investigator (delegated the sponsor's responsibilities) shall be informed immediately of any serious adverse events and shall determine seriousness and causality in conjunction with any treating medical practitioners.

In order to assess the relatedness or causality of an SAE the investigator should review the list of known side effects and referred to in the SPC and decide if the SAE is possibly, probably or definitely related to the trial treatment. Events considered unrelated to the trial treatment include those due to progression of an existing disease, development of new diseases, treatments, procedures or hospitalisation for these diseases, or known adverse effects of concomitant therapies, other than the trial treatment, received by the participants.

In the event of a pregnancy occurring in a trial participant or the partner of a trial participant monitoring shall occur during the pregnancy and after delivery to ascertain any trial related adverse events in the mother or the offspring. Where it is the partner of a trial participant consent will be obtained for this observation from both the partner and her medical practitioner. All pregnancy will be reported as an AE and followed up accordingly.

All serious adverse events will be recorded and reported to the MHRA and REC as part of the annual reports. SUSARs will be reported within the statutory timeframes to the MHRA and REC as stated below. The Chief Investigator shall be responsible for all adverse event reporting.

SUSARs

A serious adverse event that is either sudden in its onset, unexpected in its severity and seriousness or not a known side effect of the IMP *and* related or suspected to be related to the IMP is classed as Suspected Unexpected Serious Adverse Reaction and requires expedited reporting as per the clinical trials regulations.

All serious adverse events that fall or are suspected to fall within these criteria shall be treated as a SUSAR until deemed otherwise.

The event shall be reported immediately of knowledge of its occurrence to the Chief Investigator.

The Chief Investigator will:

- Assess the event for seriousness, expectedness and relatedness to the study IMP

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- Take appropriate medical action, which may include halting the trial and inform the Sponsor of such action
- If the event is deemed a SUSAR, shall, within seven days, enter the required data on the MHRA's eSUSAR web site.
- Shall inform the REC using the reporting form found on the NRES web page within 7 days of knowledge of the event
- Shall, within a further eight days send any follow-up information and reports to the MHRA and REC.
- Make any amendments as required to the study protocol and inform the ethics and regulatory authorities as required

Participant removal from the study due to adverse events
Any participant who experiences an adverse event, serious or non-serious, may be withdrawn from the study at the discretion of the Investigator.

ETHICAL AND REGULATORY ASPECTS

ETHICS COMMITTEE AND REGULATORY APPROVALS

The trial will not be initiated before the protocol, informed consent forms and participant and GP information sheets have received approval / favourable opinion from the Medicines and Healthcare products Regulatory Agency (MHRA), Research Ethics Committee (REC), and the respective National Health Service (NHS) Research & Development (R&D) department. Should a protocol amendment be made that requires REC approval, the changes in the protocol will not be instituted until the amendment and revised informed consent forms and participant and GP information sheets (if appropriate) have been reviewed and received approval / favourable opinion from the REC and R&D departments. A protocol amendment intended to eliminate an apparent immediate hazard to participants may be implemented immediately providing that the MHRA, R&D and REC are notified as soon as possible and an approval is requested. Minor protocol amendments only for logistical or administrative changes may be implemented immediately; and the REC will be informed.

The trial will be conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki, 1996; the principles of Good Clinical Practice, in accordance with the Medicines for Human Use Regulations, Statutory Instrument 2004, 1031 and its subsequent amendments and the Department of Health Research Governance Framework for Health and Social care, 2005.

INFORMED CONSENT AND PARTICIPANT INFORMATION

The process for obtaining participant informed consent or consultee informed consent will be in accordance with the REC guidance, and Good Clinical Practice (GCP) and any other regulatory requirements that might be introduced. The investigator or their nominee and the participant or other legally authorised

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representative shall both sign and date the Consent Form before the person can participate in the study.

The participant will receive a copy of the signed and dated forms and the original will be retained in the Trial Master File. A second copy will be filed in the participant's medical notes and a signed and dated note made in the notes that informed consent was obtained for the trial.

The decision regarding participation in the study is entirely voluntary. The investigator or their nominee shall emphasize to them that consent regarding study participation may be withdrawn at any time without penalty or affecting the quality or quantity of their future medical care, or loss of benefits to which the participant is otherwise entitled. No trial-specific interventions will be done before informed consent has been obtained.

The investigator will inform the participant or their consultee of any relevant information that becomes available during the course of the study, and will discuss with them, whether they wish to continue with the study. If applicable they will be asked to sign revised consent forms.

If the Informed Consent Form is amended during the study, the investigator shall follow all applicable regulatory requirements pertaining to approval of the amended Informed Consent Form by the REC and use of the amended form (including for ongoing participants).

RECORDS

Drug accountability

Drug supplies will be kept in a secure, limited access storage area under the storage conditions specified by Pharmacy.
The investigator and the local site pharmacist shall maintain records of the study drug's delivery to the pharmacy, an inventory at the site, the distribution to each participant, and the return to the pharmacy or alternative disposition of unused study drugs. These records will include dates, quantities received, batch / serial numbers, expiration dates, and the unique code numbers (patient trial number) assigned to the trial participant. Investigators and /or the local site pharmacists will maintain records that document adequately that the participants were provided with the correct study medication. All study medication packs and bottles received by the pharmacy shall be accounted for.

Electronic Case Report Forms

Access to the online eCRF will be provided by the Nottingham Clinical Trials Unit and paper worksheets will be provided to sites where online access is not available. All paper worksheet pages which have entries will be transcribed to the online eCRF, and any changes on the paper worksheets must also be made online, where all data will be stored in a secure dedicated server with access

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restricted by use to appropriate identified password holders. Original paper worksheets where utilised, will remain with the investigator as a permanent record. The Principal Investigator at each centre must ensure that their worksheets are kept in a secure location (i.e. in a locked cabinet or cupboard, or a locked room).

Each participant will be assigned a trial identity code number, allocated at consent, for use on eCRFs and other trial documents. The documents and database will also use their initials (of first and last names separated by a hyphen or a middle name initial when available) and date of birth (dd/mmm/yy).

The investigator will make a separate confidential record of the participant's name, date of birth, local hospital number or NHS number, and Participant Trial Number (the Trial Recruitment Log), to permit identification of all participants enrolled in the trial in accordance with regulatory requirements and for follow-up as required.
eCRFs shall be restricted to those personnel approved by the Chief or local Principal Investigator and recorded on the 'Trial Delegation Log.'

All paper forms shall be filled in using black ballpoint pen. Errors shall be lined out but not obliterated by using correction fluid and the correction inserted, initialled and dated.

The Chief or local Principal Investigator shall sign a declaration ensuring accuracy of data recorded in the eCRF.

Source documents
Source documents shall be filed at the investigator's site and may include but are not limited to, consent forms, current medical records, laboratory results and pharmacy records. Only trial staff as listed on the Delegation Log shall have access to trial documentation other than the regulatory requirements listed below.

Direct access to source data / documents
The eCRF and all source documents, including progress notes and copies of laboratory and medical test results shall made be available at all times for review by the Chief Investigator, Sponsor's designee and inspection by relevant regulatory authorities (MHRA).

DATA PROTECTION

All trial staff and investigators will endeavour to protect the rights of the trial's participants to privacy and informed consent, and will adhere to the Data Protection Act, 1998. The eCRF will only collect the minimum required information for the purposes of the trial. Access to the information will be limited to the trial staff and investigators and relevant regulatory authorities (see above). ECRF data will be held securely and password protected. All data will be stored on a secure dedicated web server. Access will be restricted by user identifiers and passwords (encrypted using a one way encryption method).
Information about the trial in the participant's medical records / hospital notes will be treated confidentially in the same way as all other confidential medical information.

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Electronic data will be backed up every 24 hours to both local and remote media in encrypted format.

QUALITY ASSURANCE & AUDIT

INSURANCE AND INDEMNITY

Insurance and indemnity for trial participants and trial staff is covered within the NHS Indemnity Arrangements for clinical negligence claims in the NHS, issued under cover of HSG (96)48. There are no special compensation arrangements, but trial participants may have recourse through the NHS complaints procedures.

The University of Nottingham as research Sponsor indemnifies its staff, research participants and research protocols with both public liability insurance and clinical trials insurance. These policies include provision for indemnity in the event of a successful litigious claim for proven non-negligent harm.

TRIAL CONDUCT

Trial conduct will be subject to systems audit of the Trial Master File for inclusion of essential documents; permissions to conduct the trial; Trial Delegation Log; CVs of trial staff and training received; local document control procedures; consent procedures and recruitment logs; adherence to procedures defined in the protocol (e.g. inclusion / exclusion criteria, correct randomisation, timeliness of visits); adverse event recording and reporting; drug accountability, pharmacy records and equipment calibration logs.

TRIAL DATA

Monitoring of trial data shall include confirmation of informed consent; source data verification; data storage and data transfer procedures; local quality control checks and procedures, back-up and disaster recovery of any local databases and validation of data manipulation. The Trial Manager, or where required, a nominated designee of the Sponsor, shall carry out monitoring of trial data as an on-going activity.

Entries on eCRFs will be verified by inspection against the source data according to the Monitoring Plan. In addition the subsequent capture of the data on the trial database will be checked. Where corrections are required these will carry a full audit trail and justification.

Trial data and evidence of monitoring and systems audits will be made available for inspection by the regulatory authority as required.

RECORD RETENTION AND ARCHIVING

In compliance with the ICH/GCP guidelines, regulations and in accordance with the University of Nottingham Code of Research Conduct and Research Ethics, the Chief or local Principal Investigator will maintain all records and documents regarding the

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conduct of the study. These will be retained for at least 7 years or for longer if required. If the responsible investigator is no longer able to maintain the study records, a second person will be nominated to take over this responsibility.
The Trial Master File and trial documents held by the Chief Investigator on behalf of the Sponsor shall be finally archived at secure archive facilities at the University of Nottingham. This archive shall include all trial databases and associated meta-data encryption codes.

DISCONTINUATION OF THE TRIAL BY THE SPONSOR

The Sponsor reserves the right to discontinue this trial at any time for failure to meet expected enrolment goals, for safety or any other administrative reasons. The Sponsor shall take advice from the Trial Steering Committee and Data Monitoring Committee as appropriate in making this decision.

STATEMENT OF CONFIDENTIALITY

Individual participant medical information obtained as a result of this study are considered confidential and disclosure to third parties is prohibited with the exceptions noted above.
Participant confidentiality will be further ensured by utilising identification code numbers to correspond to treatment data in the computer files.

Such medical information may be given to the participant's medical team and all appropriate medical personnel responsible for the participant's welfare.

Data generated as a result of this trial will be available for inspection on request by the participating physicians, the University of Nottingham representatives, the REC, local R&D Departments and the regulatory authorities.

PUBLICATION AND DISSEMINATION POLICY

The trial will be written up and presented at international Gastroenterology meetings and submitted for publication in a peer reviewed journal. No participants will be identified in any publications.

USER AND PUBLIC INVOLVEMENT

The protocol has been reviewed by the NIHR Biomedical Research Unit Patient Advisory Group and their comments incorporated into the final proposal.

STUDY FINANCES

Funding source

A Research for Patient Benefit (RfPB) grant has been awarded to carry out this research study.

Participant stipends and payments

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Participants will not be paid to participate in the trial. Travel expenses will be offered for any hospital visits in excess of usual care.

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SIGNATURE PAGES

Signatories to Protocol:

Chief Investigator: (name) Prof R. Spiller

Signature: R Spiller

Date: 7/11/12

Trial Statistician: (name) SAMIR MEHTA

Signature: S.K. Mehta

Date: 07/11/12.

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31 August 2012

Robin Spiller
University of Nottingham
Derby Road
E Floor, West Block,
NG7 2UH

Dear Spiller

Study title:	**A randomised placebo controlled trial of follow on Rifaximin for the prevention of relapse of Clostridium difficile associated diarrhoea.**
REC reference:	**12/EM/0292**
Protocol number:	**12072**
EudraCT number:	**2012-003205-10**

Thank you for your letter of 14 August 2012, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites listed in the application, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. We will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at non-NHS sites.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of

the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

Clinical trial authorisation must be obtained from the Medicines and Healthcare products Regulatory Agency (MHRA).

The sponsor is asked to provide the Committee with a copy of the notice from the MHRA, either confirming clinical trial authorisation or giving grounds for non-acceptance, as soon as this is available.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering Letter		11 July 2012
Evidence of insurance or indemnity		17 August 2011
Investigator CV		10 July 2012
Letter from Sponsor		10 July 2012
Letter of invitation to participant	1.0	01 June 2012
Other: GP Invitation Letter	1.0	01 June 2012
Other: GP Letter	1.0	01 June 2012
Other: Stool Diary	1.0	01 June 2012
Other: Stool Collection Advice	1.0	01 June 2012
Other: Dose Instruction Card	1.0	01 June 2012
Other: Trial Identification Card	1.0	01 June 2012
Other: SmPC		01 June 2011
Other: Supplementary IMP-Dossier	1.4	06 July 2012
Other: IMPD	1	14 June 2012
Participant Consent Form: Legal Representative	1.1	14 August 2012
Participant Consent Form: Participant	1.1	14 August 2012
Participant Information Sheet: Legal Representative	1.0	01 June 2012

Participant Information Sheet: Short PIS	1.1	14 August 2012
Participant Information Sheet: Full PIS	1.1	14 August 2012
Protocol	1.0	06 July 2012
REC application	67974/341845/1/152	10 July 2012
Response to Request for Further Information		14 August 2012

Statement of compliance

This Committee is recognised by the United Kingdom Ethics Committee Authority under the Medicines for Human Use (Clinical Trials) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

The Committee is fully compliant with the Regulations as they relate to ethics committees and the conditions and principles of good clinical practice.

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/EM/0292	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely



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Chair

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Enclosures: "After ethical review – guidance for researchers"

Copy to: *Mr Paul Cartledge*

Ms Maria Koufali, NHS R&D Office