Characterisation of a putative quorum sensing system in *Clostridium difficile*

A thesis submitted by

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Declaration

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

Manisha Patel

October 2010

For Sandip, there's so much I want to say but not enough words to say it all.

Thank you, without you none of this would have been possible.

Abstract

Clostridium difficile is the major cause of health-care associated infections. However the factors that participate in *C. difficile* infection and the processes that regulate their expression remains poorly understood.

In *Staphylococcus aureus*, quorum sensing (QS) plays a central role in the regulation of virulence factors. The QS system of *S. aureus* is encoded by the accessory gene regulator (*agr*) locus and comprises of four genes. The pre-peptide (AgrD) is processed by AgrB and the mature auto-inducing peptide (AIP) is released into the medium. Free AIP binds to the histidine-sensor kinase AgrC, causing it to autophosphorylate. In turn, AgrC phosphorylates the response regulator AgrA, triggering an intracellular signal-transduction cascade which results in altered expression of several target genes.

Homologues of genes involved in QS have been identified in the genome sequence of *C. difficile* 630, a virulent multidrug resistant strain. These homologues have also been identified in *C. difficile* R20291, a ribotype 027 epidemic strain, which has been characterised to produce increased quantities of Toxin A and Toxin B. *C. difficile* R20291 has two *agr* loci, whereas *C. difficile* 630 only has one *agr* locus. The first *agr* locus present in both strains contains *agrBD* homologues, although there are no apparent *agrA*/*agrC* homologues. However, the second *agr* locus in *C.*

difficile R20291 contains homologues of all four *agr* genes. Insertional inactivation of the QS homologues were made in the two *C. difficile* strains and the effects on virulence assessed. Cytotoxicity assays indicated that there is a substantial reduction in toxin B production in *C. difficile* R20291 strains deficient in the second *agr* system compared to the wild-type strain. Sporulation assays revealed that the onset of sporulation in the agr mutants vary greatly in comparison to the parental strain. The work presented in this study suggests that the *agr* system may be involved in toxin production and sporulation in *C. difficile*.

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List of Abbreviations

aa	Amino acid
agr	Accessory gene regulator
AHL	*N*-acylhomoserine lactone
AI-2	Auto inducer 2
AIP	Autoinducing peptide
BHIS	Supplemented brain heart infusion media
BLAST	Basic local alignment search tool
°C	Degrees centigrade
CDAD	*Clostridium difficile* associated disease
cfu	Colony forming unit
Cm	Chloramphenicol
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
dH_2O	Demineralised water
DNA	Deoxyribonucleotide triphosphate
dNTP	Deoxyribose nucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
Erm	Erythromycin
g	Gram
g	Gravitational force
gDNA	Genomic DNA
h	Hour
HPK	Histidine protein kinase

kb	Kilobase
kV	Kilovolt
l	Litre
LB	Luria Bertani
M	Molar
µg	Microgram
mg	Milligram
min	Minutes
µl	Microlitre
ml	Millilitre
µm	Micrometer
µM	Micromolar
mM	Millimolar
mRNA	Messenger ribonucleic acid
ng	Nanogram
no	Number
OD_{600}	Optical density at a wavelength of 600 nanometers
ORF	Open reading frame
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pH	Potential of hydrogen
QS	Quorum sensing
RNA	Ribonucleic acid
rpm	Revolutions per minute
RR	Response regulator
RT-PCR	Reverse transcriptase polymerase chain reaction

s	Seconds
TAE	Tris-acetate-EDTA
TCS	Two component system
TSST-1	Toxic shock syndrome toxin 1
UV	Ultraviolet
V	Volt
v/v	Percent volume per volume
w/v	Percent weight per volume
WT	Wild type

Chapter 1

Introduction

1.1 Introduction to clostridia

Species that belong to the genus *Clostridium* are characterised as Gram-positive, anaerobic, catalase-negative, spore forming bacilli. *Clostridium* is one of the largest prokaryotic genera, containing over 300 species and is well known for its clinical importance and biotechnological potential (Andreesen *et al.*, 1989).

The species that make up *Clostridium* are highly diverse and the vast majority of them are entirely benign. Clostridia have a huge biotechnological and medical potential which will be industrially, medically and scientifically important in the future (Durre, 2001). The non-pathogenic *Clostridium acetobutylicum* is becoming of great interest because of its role in bio-butanol production (Jones and Woods, 1986; Lee *et al.*, 2008). As genome sequence data and DNA mutagenesis tools are now readily available, recombinant strains with superior biobutanol-producing ability can be engineered (Durre, 2007; 2008). Furthermore, the use of clostridial spores in cancer therapy has also been described for *Clostridium sporogenes* and *Clostridium novyi* (Minton *et al.*, 1995; Minton 2003; Theys *et al.*, 2006 Mengesha *et al*, 2010).

Even though there are a number clostridial species which have shown to have many beneficial properties, on the other hand the pathogenic clostridia continue to overshadow this. Pathogenic clostridia cause disease by the release of potent toxins; enterotoxins by *Clostridium perfringens*, cytotoxins by *Clostridium difficile*, and neurotoxins by *Clostridium botulinum* and *Clostridium tetani*. In fact tetanus and botulinum neurotoxins, produced by *Clostridium tetani* and *Clostridium botulinum*

respectively, are two of the most harmful natural compounds known to man. Both produce neurotoxins that are lethal in very low concentrations (Durre, 2001). The estimated lethal dose of *C. botulinum* neurotoxin type A for a human of 70 kg is approximately 70 μg orally and 0.7-0.9 μg if inhaled (Arnon *et al.*, 2001).

C. perfringens produces a number of extracellular toxins and enzymes associated with gas gangrene and clostridial myonecrosis in humans and animals (Collie *et al.*, 1998). *C. botulinum* is widely used in the cosmetics industry and is of importance for a number of medical applications. However, it has long been associated with food poisoning and bio-terrorism concerns due to its production of the most potent natural toxin known to man (Schantz and Johnson, 1992). The most recent publicised pathogenic clostridia is *Clostridium difficile*; the causative agent of pseudomembranous colitis and antibiotic-associated diarrhoea.

1.2 *Clostridium difficile*

Clostridium difficile was first discovered in 1935 (Hall and Toole, 1935), however it was only in 1978 that *C. difficile* was identified as an etiological agent of antibiotic-associated diarrhoea (AAD) and pseudomembranous colitis (PMC) (Larson *et al.*, 1978). *C. difficile* is now recognised as the leading cause of antibiotic associated diarrhoea (AAD) in hospitals worldwide and has a huge impact on healthcare services (Sebaihia *et al.*, 2006).

1.2.1 Epidemiology

The impact of *Clostridium difficile*-associated disease (CDAD) in European hospitals and healthcare settings is considerable. During outbreaks of CDAD in a healthcare setting comprehensive infection control measures need to be taken, patients need to be isolated and require alternate therapy for underlying diseases as well as specific therapy for CDAD, wards are closed, and environmental decontamination is required (Wilcox *et al.*, 1996; Zafar *et a.l*, 1998; Oldfield, 2004; Song *et al.*, 2008).

In 1990, a voluntary surveillance scheme was introduced whereby the number of positive *C. difficile* laboratory samples in England and Wales were reported. Over the following 15 years after the introduction of the surveillance scheme, the incidence of CDAD rose dramatically (**Figure 1.1**). Subsequently in 2004, a mandatory surveillance scheme was introduced which required all National Health Service Trusts in England to report cases of CDAD in patients aged 65 and over. Following this, similar mandatory surveillance schemes have been implemented across the United Kingdom. These schemes cover all patients from the age of 2 years or over, enabling an accurate assessment of the incidence of CDAD to be achieved. In 2008, it was reported that *C. difficile* was the underlying cause of more than 2500 deaths in England and Wales alone (**Figure 1.2**), ten times more deaths were caused by *C. difficile* than by methicillin-resistant *Staphylococcus aureus* (Office for National Statistics, UK: http://www.statistics.gov.uk).

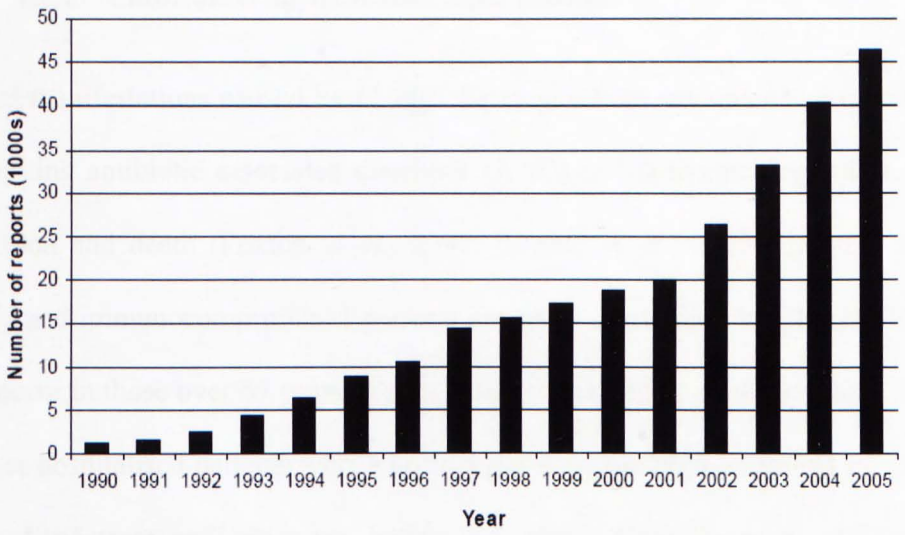


Figure 1.1. Reports of positive *C. difficile* laboratory samples isolated from faecal specimens under the voluntary reporting scheme in England between 1990 and 2005. From the website of the Health Protection Agency (http://www.hpa.org.uk).

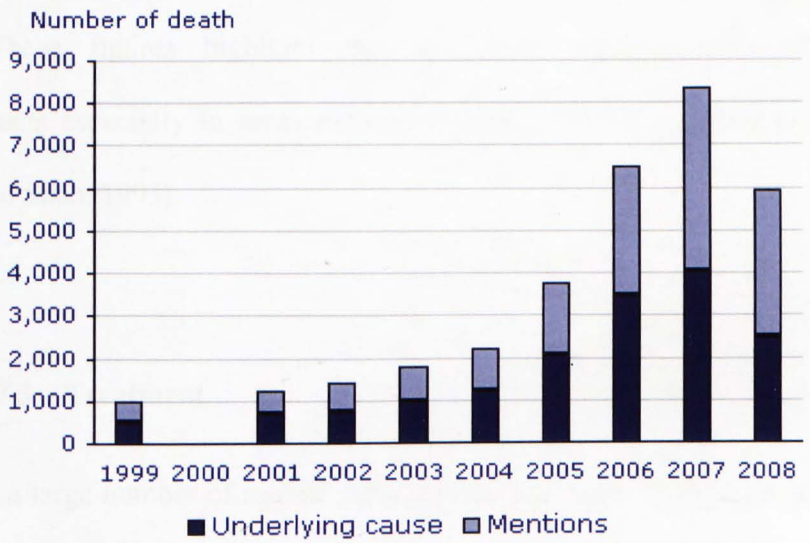


Figure 1.2. Number of death certificates mentioning *C. difficile* in comparison to where *C. difficile* was noted as the underlying cause of death in England and Wales between 1999 and 2008. Data was unavailable for 2000. A mandatory surveillance scheme was introduced between 2004 and 2005, which may partly account for the large increase in *C. difficile* incidence in the following years. From the website for the Office of National Statistics (http://www.statistics.gov.uk).

1.2.2 *Clostridium difficile*-associated disease

Clinical manifestations caused by *C. difficile* ranges from asymptomatic carriage to self-limiting antibiotic associated diarrhoea (AAD) to life-threatening colitis, toxic megacolon and death (Poxton *et al.*, 2001; Sambol *et al.*, 2000; Gerding, 1989). Elderly and immunocompromised patients are most susceptible to CDAD; 80% of cases occur in those over 65 years of age. Healthy individuals rarely develop CDAD, however hospitalised patients whose normal gut flora has been disturbed by the use of broad-spectrum antibiotics are highly susceptible (Johnson *et al.*, 1999). The spores produced by *C. difficile* are extremely difficult to remove from institutional settings (i.e. hospitals and nursing homes), and a new source of infection is nearly always present. Past studies have shown that there is a 20-30% colonisation rate of hospitalised adults compared to 2-3% colonisation rate in healthy adults (Kyne *et al.*, 2002). These figures highlight the widespread contamination of hospital environments especially in areas associated with infection (Gerding *et al.*, 1995; Fekety and Shah, 1993).

1.2.3 Treatment

There are a large number of mobile genetic elements in the *C. difficile* genome that carry antibiotic resistance genes making treatment of CDAD far from straightforward (Sebaihia, *et al.*, 2006). As CDAD development is associated with antibiotic disruption of the gut flora, mild disease may be treated by withdrawing the previously administered antibiotics and providing further supportive therapy (Hedge, *et al.*, 2008). The antibiotics recommended to treat CDAD are metronidazole in the

first instance, followed by vancomycin if the patient does not respond. The pharmacological properties of vancomycin make it the ideal drug to treat pathogens that act in the colonic lumen, yet metronidazole is the recommended agent due to its lower cost and the assumption that vancomycin promotes colonization of vancomycin-resistant enterococci (Gerding, 1997). The antibiotics, metronidazole and vancomycin, combined with patient isolation procedures, remain the principal therapeutic option for the treatment of CDAD.

One of the complications facing antibiotic treatment of CDAD is relapse of infection once antibiotic treatment is terminated (Teasley *et al.*, 1983). Although some studies have suggested an increased failure rate of metronidazole in CDAD, it remains the recommended first line treatment for uncomplicated cases (Cloud and Kelly, 2007). These observations illustrate the contradiction in terms that oral vancomycin and metronidazole both cause and cure antibiotic-associated diarrhoea. This highlights the need of alternative approaches, more effective measures to treat CDAD (McFarland, 2005).

1.2.4 *Clostridium difficile* virulence factors

Whilst the toxins of *C. difficile* are clearly the principal virulence factors, the role of other virulence factors is much more speculative. The unique surface layer proteins of *C. difficile* vegetative cells are potential virulence factors and are thought to be involved in adherence of *C. difficile* to host cells, and eliciting inflammatory and antibody responses (Drudy, *et al.*, 2004; Ausiello, *et al.*, 2006; Rupnik, *et al.*, 2009).

In addition, it has been suggested that FliC and FliD, key components of the *C. difficile* flagella, are involved in adherence and gut colonisation (Tasteyre, *et al.*, 2001).

In addition to the toxins, another important virulence factor of *C. difficile* is its ability to produce endospores, which are thought to be key for transmission of CDAD. Bacterial spores are extremely resistant to all kinds of chemical and physical agents and provide the mechanism by which *C. difficile* can evade the potentially fatal consequences of exposure to heat, oxygen, alcohol and certain disinfectants (Setlow, 2007). The spores shed in faecal matter are very difficult to eradicate and can persist on contaminated surfaces in healthcare facilities for extended periods of time (Setlow, 2007). This leads to infection or re-infection of cohabiting individuals through inadvertent ingestion of contaminated material (Riggs, *et al.*, 2007; Gerding, *et al.*, 2008). Once in the anaerobic environment of the gut, spores are able to germinate yielding the characteristic toxin-producing vegetative cells and, in susceptible individuals, diarrhoeal disease.

1.2.5 Toxins

The two major toxins produced by virulent strains of *C. difficile*, toxin A (TcdA) and toxin B (TcdB), are recognised as the main virulent factors (Borriello *et al.*, 1990). These toxins belong to the group of bacterial toxins called Large Clostridial cytoToxins (LCT's) (Just and Gerhard, 2004). Their structure is organised into three regions; the N-terminal region contains the catalytically active glucosyltransferase

site (Hoffman *et al.*, 1997), the hydrophobic central region, which is involved in translocation of the toxins across the cell membrane (Pfeifer *et al.*, 2003), and the C-terminal region which is responsible for receptor binding (von Eichel-Streiber *et al.*, 1992).

The mode of action of toxins A and B have been extensively studied and shown to be functionally similar. They are both endocytosed by the host cell and target the Ras super family of GTPases for modification via glycosylation. Both toxin A and B of *C. difficile* act by causing rearrangement and disruption of the actin cytoskeleton through monoglucosylation of small Ras-like GTP binding-proteins Rho, Rac and Cdc42 (Just *et al.*, 1994; 1995). The disruption of the actin cytoskeleton and tight junctions results in excessive fluid accumulation and destruction of the intestinal epithelium (Thelestam and Chaves-Olarte, 2000; Poxton, *et al.*, 2001; Voth and Ballard, 2005; Rupnik, *et al.*, 2009).

In the hamster animal model, the two toxins are thought to exhibit distinct effects; hence, TcdA is occasionally referred to as an 'entertoxin' and TcdB as a 'cytotoxin' (Lyerly *et al.*, 1985; Kim *et al.*, 1987; Boriello and Barclay, 1985).

Previously, it was generally accepted that toxin A was the principal virulence factor responsible for CDAD. Yet, a recent study concluded that toxin B is required for disease in Golden Syrian hamsters (Lyras, *et al.*, 2009). However, further research into this area is clearly needed, as data from our laboratory indicates that both toxin

A and toxin B alone are sufficient for disease in the hamster model (Kuehne, *et al.*, 2010).

It has also been shown in the past that some *C. difficile* types also produce an actin-specific ADP-ribosylating toxin, a binary toxin encoded by *cdtA-cdtB* (Carter, *et al.*, 2007; Rupnik, *et al.*, 2009). Interestingly, this binary toxin is produced by all emerging *C. difficile* types that have been associated with outbreaks of increased disease severity, and it has been suggested that this toxin induces morphological changes in host intestinal epithelial cells, which facilitates increased adherence of these *C. difficile* types (Schwan, *et al.*, 2009).

The amount of toxin produced by different toxigenic strains varies and is thought to be highly influenced by environmental conditions. In the laboratory, toxin expression is at its maximum when cells enter stationary phase and is reliant on growth temperature (Hundsberger *et al.*, 1997; Dupuy and Sonenshein, 1998; Karrisson *et al.*, 2003). Other factors also influence toxin expression such as the presence of certain amino acids in the growth media, butyric acid and butanol, and also carbon sources that are rapidly metabolised (Karlsson *et al.*, 1999; Yamakawa *et al.*, 1994; Karlsson *et al.*, 2000; Dupuy and Sonenshein, 1998). It has also been observed that addition of certain antibiotics and growth-limiting levels of the vitamin biotin also stimulate toxin production (Nakamura *et al.*, 1982; Yamakawa *et al.*, 1996).

The toxin genes (*tcdA* and *tcdB*) along with three accessory genes (*tcdR*, *tcdE* and *tcdC*) are encoded within a 19.6kb chromosomal unit called the pathogenicity locus (PaLoc) (Cohen *et al.*, 2000) (**Figure 1.3**). TcdR is an alternative RNA polymerase σ factor that activates toxin gene expression (Mani and Dupuy, 2001). It is thought that toxin expression is negatively regulated by TcdC (Matamouros *et al.*, 2007). This notion first arose when it was observed that *tcdC* is highly expressing during rapid exponential growth but repressed in stationary phase when toxin expression is at its maximum (Govind *et al.*, 2006; Hundsberger *et al.*, 1997).

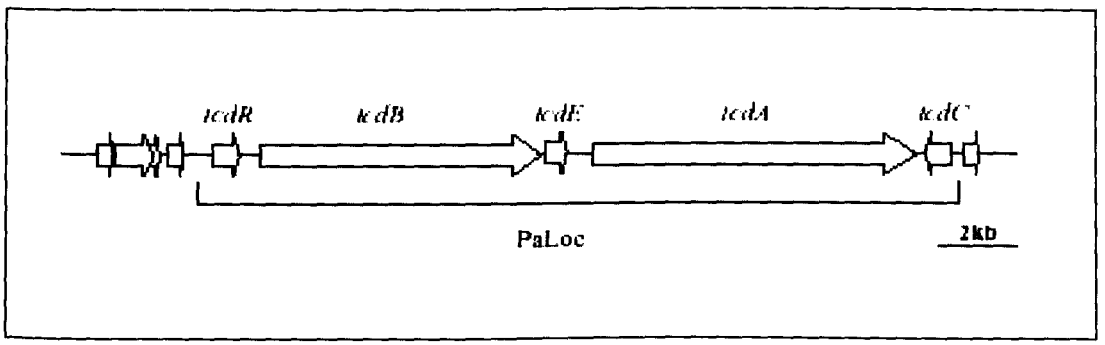


Figure 1.3 Genetic organisation of the *C. difficile* pathogenicity locus (PaLoc). Taken from Matamorous *et al.*, 2007

The variation in the severity of CDAD has been attributed to the level of toxin produced by the infecting strain of *C. difficile* (Borriello, 1990). However, there is little knowledge known of the exact molecular mechanism that play key roles in the disease process. For example, regulation of the factors that causes an increase in toxin synthesis are poorly understood. In other bacterial pathogens quorum sensing (QS) plays a central role in the regulation of virulence factors.

1.3 Quorum Sensing in Bacterial Pathogens

1.3.1 The principle of quorum sensing

Quorum sensing describes a form of cell-cell communication among bacteria (Lerat & Moran, 2004). It is utilised by bacterial populations as a means of co-ordinately responding to external stimuli in a cell density-dependent manner. Quorum sensing occurs via signalling molecules known as autoinducers, and results in the regulation of specific genes (Fuqua *et al.*, 1994). These signal molecules are released so that bacteria can detect the density of a population in a particular environment. The molecules are released during growth and accumulate in the environment. Once the concentration of the signal molecule reaches a threshold, or 'quorum', this triggers a response in the bacterial population (Winzer *et al.*, 2003). This usually involves a cascade of events which ultimately leads to changes in gene regulation (Winzer *et al.*, 2003). Genes regulated by quorum sensing include those involved in virulence (Winzer *et al.*, 2003), cell migration, formation of biofilms (Lerat & Moran, 2004), sporulation, and the production of toxins (Kleerebezem & Quadri, 2001).

It may be favourable for individual bacterial cells to suppress the expression of certain genes when the population density falls below a threshold. Synchronising the expression of virulence factors, once a minimal cell population density has been achieved enables a "united attack" against the host (Dong *et al.*, 2005). This type of multicellular coordination is vital for the successful colonisation/infection of the host by many pathogenic bacteria (Williams *et al.*, 2000; Swift *et al.*, 2001; Cámara *et al.*, 2002).

Quorum sensing was first described in the regulation of bioluminescence in *Vibrio fischeri* and *Vibrio harveyi* (Nealson *et al.*, 1970; Nealson and Hastings, 1979). This Gram-negative bacterium *Vibrio fischeri* lives on marine eukaryotes and produces light for its hosts. Light emission only occurs when the bacterial cell density reaches a threshold, and the luciferase operon is transcribed. The autoinducer molecule is an acylated homoserine lactone (AHL) (Lerat & Moran, 2004). AHLs accumulate, and once a threshold is reached, activate a transcriptional regulator of the LuxR family (Winzer *et al.*, 2003).

Vibrio harveyi is another marine bacterium whose luminescence is regulated by quorum sensing. However, this organism has two quorum sensing systems and produces two autoinducers; autoinducer 1 (AI-1) and autoinducer 2 (AI-2) (Bassler *et al.*, 1993). The first quorum sensing system is similar to that of *Vibrio fischeri* and uses an AHL signal molecule; however AI-1 production is not reliant on a LuxI-type molecule but instead is produced by the *luxLM* locus (Bassler *et al.*, 1993). The second system relies on the accumulation of a molecule called autoinducer 2 (AI-2), which is believed to be a furanosyl borate diester (Chen *et al.*, 2002). AI-2 is generated via the LuxS enzyme (Schauder *et al.*, 2001). AI-1 is sensed by the periplasmic protein LuxN. AI-2 binds to the periplasmic protein LuxP which then interacts with a membrane-bound histidine protein kinase, LuxQ. A series of dephosphorylation reactions are commenced resulting in the transduction of information to a shared integrator protein, LuxU. In turn, LuxU sends a signal to the response regulator protein LuxO, a σ^{54} –dependent transcriptional activator. The function of LuxO is to control the transcription of a putative repressor protein and a transcriptional activator protein called LuxR, which is required for the expression of

the luciferase operon (*luxCDABE*) (Bassler *et al.*, 1993). Once the signal has been passed to LuxO, it can no longer activate transcription of the repressor gene, and therefore the *lux* operon is consequently transcribed (Winzer *et al.*, 2003).

1.3.2 LuxS/AI-2 quorum sensing systems

A variety of Gram-positive and Gram-negative bacteria possess *luxS* homologues, similar to those found in *V. harveyi* (Winzer *et al.*, 2003). In fact, it has been claimed that BLAST searches have shown evidence of *luxS* homologues in the genomes of 'most bacterial species for which complete sequence data are available' (Hilgers & Ludwig, 2001). In addition, Xavier and Bassler (2003) found that the *luxS* gene exists in 35 of the 89 currently available complete bacterial genomes by database analysis. PCR, along with AI-2 activity assays, have allowed the identification of *luxS* in a number of species for which no genome sequences are available (Xavier & Bassler, 2003).

In some cases, signalling molecules can be recognised by different bacterial species. This is known as interspecies cross-talk and can either promote or inhibit gene expression (Sturme *et al.*, 2002). As *luxS* homologues have been found in many different bacteria, it has recently been proposed that AI-2 could serve as a 'universal signal' for interspecies cross-talk (Xavier & Bassler, 2003). However, this is yet to be proven in species other than *Vibrio*.

Despite the proposed role of LuxS in quorum sensing, it is speculated that LuxS is in fact a metabolic side product produced during the activated methyl cycle (a recycling pathway involved in the metabolism of methionine) (Winzer *et al.*, 2003). Indeed, Doherty *et al.* (2006) demonstrated that the inactivation of *luxS* in *Staphylococcus aureus* had no effect on virulence-associated traits. It is possible that the phenotypic changes observed in some bacteria following inactivation of *luxS*, may be due to the absence of the AI-2 signal molecule, but they could also be attributed to the disruption of the activated methyl cycle (Winzer *et al.*, 2003).

1.3.3 Quorum sensing in Gram-positive bacteria

Quorum sensing in Gram-positive bacteria occurs via a range of different signals but all systems involve a two-component regulatory signal transduction system, consisting of a membrane-bound receptor histidine protein kinase, and an intracellular response regulator (Sturme *et al.*, 2002). When the response regulator is phosphorylated by the receptor kinase, transcription of target genes is activated or repressed. Transcription of genes involved in the production of the autoinducing peptides is also activated (Sturme *et al.*, 2002). Most Gram-positive bacteria use signalling molecules that are created from large precursors and post-translationally modified (Fuqua & Greenberg, 1998). They are then exported using ATP binding cassette (ABC) export systems (van der Meer *et al.*, 1993). Some bind to sensor kinases in the cell membrane, which transduce the signal across the membrane. Others bind to intracellular receptors after being transported into the cell by oligopeptide permeases.

1.3.4 The *agr* system of *Staphylococcus aureus*

In *S. aureus* the accessory gene regulator (*agr*) system regulates the temporal expression of cell-surface colonisation factors and exoproteins, which contribute to virulence (Novick and Muir, 1999). This is the only known quorum sensing system in the staphylococci (Kies *et al.*, 2003) and was first identified in *S. aureus*. The expression of many secreted virulence factors is up-regulated by the *agr* system in the transition from late-exponential growth to stationary phase, whereas the expression of the surface proteins, for example fibronectin binding proteins A and B, is down-regulated (Ji *et al.*, 1995, Lyon *et al.*, 2000; Novick *et al.*, 1993; Yarwood & Schlievert, 2003). The system is able to switch on in the later parts of the growth phase by sensing the cell density of its surroundings (Otto *et al.*, 1998). The virulence factors controlled by the *agr* system include α, β and δ cytotoxins (haemolysins), serine protease, DNase, fibrinolysin, enterotoxins A-D, toxic shock syndrome (TSS) toxin 1 (Otto *et al.*, 1998), and leucocidins (Novick *et al.*, 2008). Other proteins whose production is regulated by the *agr* system in staphylococci include staphopain, lipase, phospholipase C, nuclease, hyaluronidase, coagulase, protein A (Novick, 2003), arginase, peptidase and chitinase B (Dunman *et al.*, 2001). In addition, the *agr* system is also involved in the invasion and apoptosis of epithelial cells (Yarwood & Schlievert, 2003).

The *agr* locus is approximately 3.5 Kb consists of two divergent operons, RNAII and RNAIII, which are transcribed from neighbouring but non-overlapping promoters, P2 and P3 respectively. The P2 operon contains four open reading frames *agrA*, *agrC*, *agrD*, and *agrB*, of which *agrA* and *agrC* form a two-component system for signal transduction (Novick *et al.*, 1995). The P3 generated transcript, RNAIII, is the

actual intracellular effector of the *agr* response and controls the expression of target genes (Novick *et al.*, 1993). It also encodes staphylococcal δ toxin (Janzon and Arvind, 1990; Novick *et al.*, 1995). The P2 operon is doubly autocatalytic since transcription of P2 requires P2 operon products, and is therefore suited to the need for rapid expression of exoproteins required when overall growth is coming to a halt (Morfeldt *et al.*, 1988).

The roles of the components of the *agr* system are outlined below and illustrated in Figure 1.4.

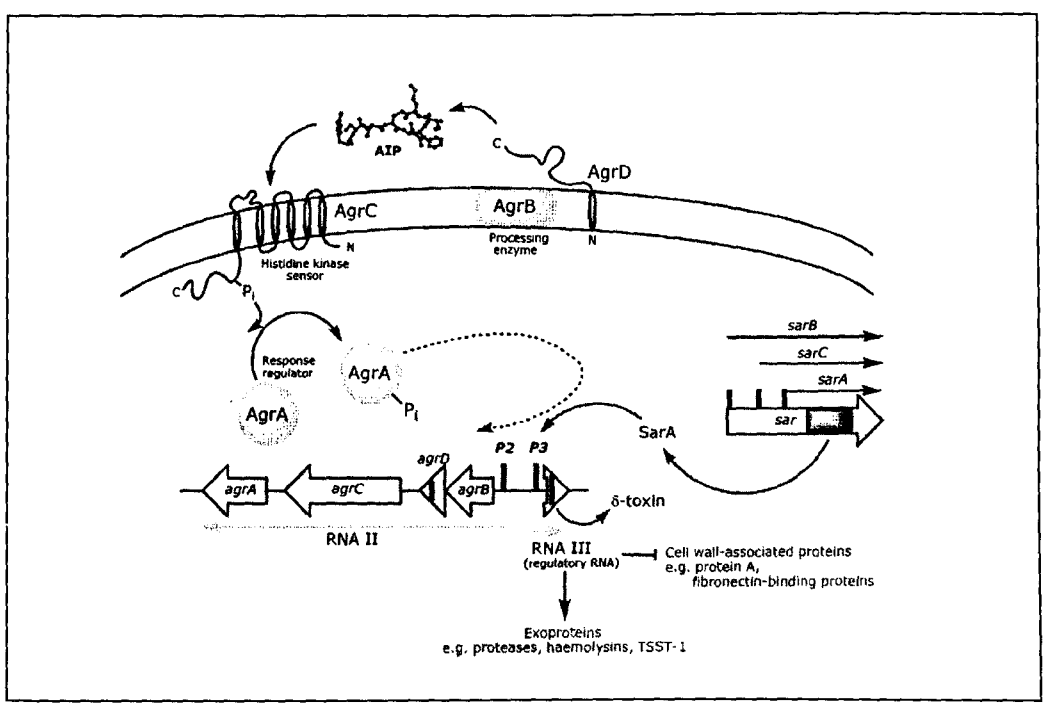


Figure 1.4. A schematic diagram of the *agr* quorum sensing system in *Staphylococcus aureus* taken from Chan *et al.* (2004). The AgrD propeptide is modified and secreted from the cell by AgrB and possibly another peptidase. This results in the formation of the mature AIP, which binds to the AgrC sensor kinase. Subsequent phosphorylation of AgrC activates the AgrA response regulator which promotes transcription of the *agrBDCA* operon. It also increases transcription of RNAIII, the effector molecule of the system.

AgrC is the transmembrane sensor kinase of a two-component system, whose N terminal interacts with a signal molecule (Lina *et al.*, 1998). Activation of AgrC then occurs via a phosphorylation event. It was originally proposed that the C-terminal was autophosphorylated at a histidine residue when stimulated by the signal molecule (Ji *et al.*, 1995; Novick *et al.*, 1995). However, subsequent investigations have found that AgrC is partially phosphorylated in the absence of any AIP. It has been suggested that the cytoplasmic domain of AgrC is dimeric, containing a four-helix bundle, and that autophosphorylation would occur as a *trans* process (Novick, 2003).

AgrA is the response regulator, which is required for the activation of the P2 and P3 promoters and is phosphorylated by AgrC. It binds with high affinity to the RNAIII-*agr* intergenic region, with this binding being localised to a pair of direct repeats in the P2 and P3 promoter regions (Koenig *et al.*, 2004).

AgrD is the propeptide, which is synthesised within the cell, processed and then secreted. The *agr*-activating ligand is a post-translationally modified peptide (auto-inducing peptide-AIP) which is processed from a pre-peptide encoded by *agrD*. The transmembrane protein, AgrB, is involved in processing of the pre-peptide, and secretion of the post-translationally modified auto-inducing peptide (AIP) (Ji *et al.*, 1995). The AgrD protein is processed at both the N- and C-terminal ends. A cyclisation reaction occurs, where a thioester linkage is formed between a conserved cysteine residue and the C terminal carboxyl group, in order to form the mature thiolactone AIP (Novick, 2003; Lyon and Novick, 2004).

AgrB is a transmembrane protein essential for the generation and secretion of the AIP. In *S. aureus* the peptide export system is not an ABC transporter, and it has been suggested that this function may be carried out by AgrB (Sturme *et al.*, 2002). It is also required for the proteolytic modification of AgrD (Yarwood & Schlievert, 2003). This modification of AgrD results in the production and secretion of the AIP (Zhang *et al.*, 2004)

AIP, the autoinducing peptide of the two-component system, is a cyclic thiolactone of seven to nine amino acids, and is crucial for *agr* activity (Zhang *et al.*, 2002). The *agr* genes are constitutively expressed at low cell densities, but when the AIP level reaches a threshold, the *agr* response is activated (Ji *et al.*, 1995). The signalling transduction cascade produced by the AgrA and AgrC two component system results in an increase in the transcription of RNAII and RNAIII in a positive feedback manner (Yarwood & Schlievert, 2003).

Signal molecules produced by the *agr* system in *S. aureus* have been shown to stimulate *agr* activation in cells of their own population, but to inhibit expression of the *agr* system in cells of a different population (Kies *et al.*, 2003). This is largely due to the fact that variations in specific regions of *agrB*, *agrD* and *agrC* have resulted in the allocation of four *agr* specificity groups in *S. aureus* (Novick, 2003). The groups were determined by assessing the ability of their AIP molecules to inhibit the *agr* response in other groups (Ji *et al.*, 1997). These AIP groups show extremely high specificity, and just a single amino acid change can transform the AIP to a different group (Novick, 2003). Cross inhibition occurs across these four

AIP groups, but the AIPs from groups I and IV are able to activate each other's *agr* system due to their strong similarity (Chan *et al.*, 2004). The structures of the AIPs from the four *S. aureus agr* groups are illustrated in Figure 1.5.

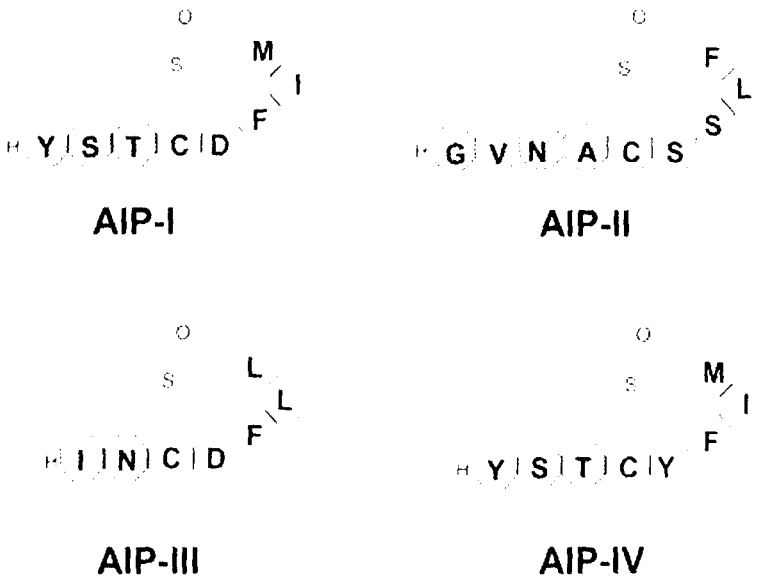


Figure 1.5 – Structures of the AIPs from the four *S. aureus agr* groups. Letters refer to amino acid residues. Exocyclic tail residues are shown in grey; endocyclic residues are shown in white. Taken from Lyon *et al.* (2002).

RNAIII has two functions. It encodes the δ haemolysin toxin via the *hld* gene, but is also responsible for the increase in transcription of extracellular virulence factors, such as toxins, and the decrease in transcription of cell surface proteins (Yarwood & Schlievert, 2003). It has been demonstrated that RNAIII exerts these effects by its 3' domain acting as an antisense RNA, and repressing translation of mRNAs encoding virulence factors acting early in the infection process (Boisset *et al.*, 2007). In

addition, the increase in transcription of secreted virulence factors observed in the later stages of infection was shown to occur by the antisense down-regulation of Rot (repressor of toxins) mRNA by RNAIII. Since Rot has been identified as an antagonist to the *agr* response, this de-repression permits the translation of many of the secreted virulence factors associated with the later stages of infection. It has also been demonstrated that the 3' domain of RNAIII by itself is able to promote the synthesis of several exoproteases and exotoxins (Boisset *et al.*, 2007).

The production of RNAIII is affected by a second regulatory system – the staphylococcal accessory regulator *sar* (Blevins *et al.*, 2002). Indeed, Blevins *et al.* (2002), Bayer *et al.* (1996) and Dunman *et al.* (2001) all demonstrated that mutating *sarA* resulted in a decrease in the production of RNAIII. Binding of SarA to the P2 and P3 promoters promotes transcription of RNAII and RNAIII, indirectly affecting virulence factor regulation. The *sar* locus also operates independently of *agr*, regulating other virulence factors directly (Dunman *et al.*, 2001).

1.3.5 Molecular studies on the *agr* system

Mutation of the *agr* system has been shown to have a number of effects. The expression of exotoxins such as α, β and δ toxins, serine protease, DNase, fibrinolysin, enterotoxin B and TSS toxin 1 has been shown to decrease, hence reducing virulence. An increase was also seen in the expression of many surface proteins (Recsei *et al.*, 1986).

Mutation of *agr* has also been shown to inhibit proteolytic, lipolytic and haemolytic activity in *S. aureus* (Blevins *et al.*, 2002). Kies *et al.* (2003) demonstrated that production of the antibiotic epidermin in a *S. epidermidis agr* mutant was strongly reduced compared to the wild type, showing that the *agr* system plays an important role in epidermin biosynthesis.

The *agr* system therefore has potential for targeting in an attempt to attenuate virulence in staphylococci. However, there is an alternative argument that inactivating the *agr* system may, in fact, contribute to the conversion of an acute infection to a chronic one (Kong *et al.*, 2006). Some studies have shown that inhibiting the *agr* system enables staphylococci to form biofilms more readily. Vuong *et al.* (2004) demonstrated this in *S. epidermidis* and suggested that this may present problems when using medical implant devices.

1.3.6 Agr in clostridia

During this study, sequence alignments show that homologues of the *agr* genes occur in a number of bacteria besides staphylococci. In particular, they have been found in various clostridial species such as *C. perfringens*, *C. botulinum*, *C. acetobutylicum*, *C. sporogenes* and *C. difficile* (see **Figure 1.6**). *agr* homologues have also been found in *Listeria monocytogenes* (Autret *et al.*, 2003), *Lactobacillus plantarum* (Diep *et al.*, 1994) and *Enterococcus faecalis* (Nakayama *et al.*, 2001). The availability of completed genome sequences and mutagenesis tools have enabled functional genetic analysis of *agr* in clostridia to take place and recently the role of *agr* in *C. perfringens* and *C. botulinum* has been reported (Othani *et al.*, 2009; Cooksley *et al.*, 2010).

The work of Othani *et al.* has shown that in *C. perfringens* strain 13, *agr* plays a role in the regulation of toxin expression. In a *C. perfringens agrBD* mutant strain, θ toxin gene expression was abolished and transcription of both α and β toxin genes was decreased. Toxin expression in the mutant strain was restored by the addition of wild type culture supernatant (Othani *et al.*, 2009).

The *virR/virS* two component system has been shown to regulate toxin gene expression in *C. perfringens* (Shimizu *et al.*, 1994). However the signal detected by VirS and how the signalling system stimulates toxin expression was unknown. Therefore role of the VirR/VirS in *agrBD*-mediated toxin regulation was investigated in *C. perfringens* strain 13. Addition of wild type culture supernatant to

an *agr/virR* mutant strain did not restore toxin production. This indicates that the signal peptide produced and processed by *agrD* and *agrB* respectively, is detected by the ViR/VirS two component system and subsequently transcription of the toxin genes activated (Othani *et al.*, 2009).

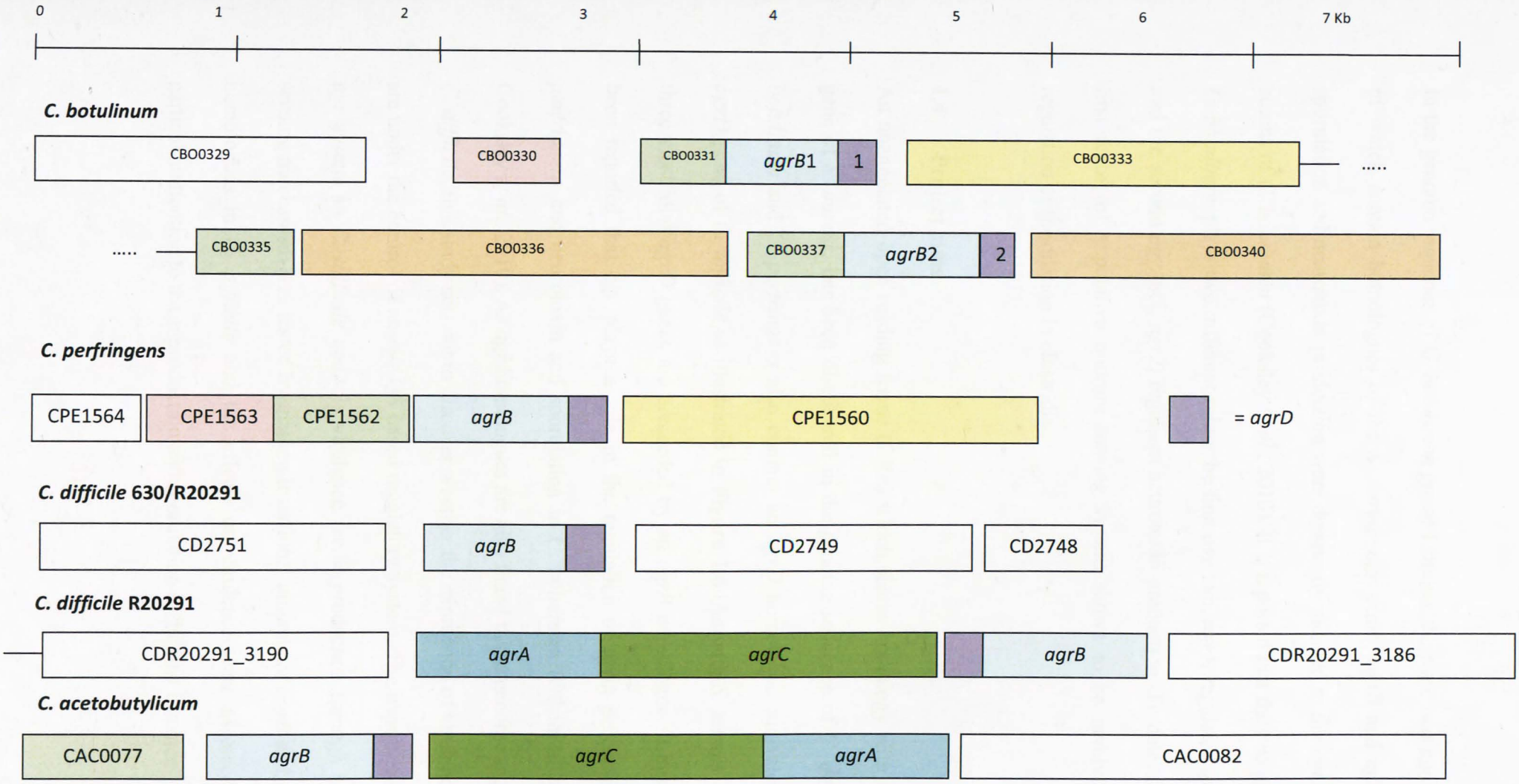


Figure 1.6 – Schematic diagram showing *agr* gene regions in four species of clostridia. The different colours represent homologous genes. White genes do not show any homology.

In the genome sequence of *C. botulinum* group I strains, there are two *agr* loci both of which contain homologues of the *S. aureus agr* genes, *agrB* and *agrD*. Both sporulation and neurotoxin production were drastically reduced in the *agrD* mutant strains of *C. botulinum* (Cooksley *et al.*, 2010). It is reported that the two *agr* loci in *C. botulinum* plays two different roles; the first *agr* loci, *agr1*, regulates sporulation and the second *agr* loci, *agr2*, regulates neurotoxin production. To date, this is the first report of a putative quorum sensing system shown to be involved in the regulation of sporulation in clostridia.

1.4 Project aims

An unannotated open reading frame (ORF) which shares homology with the *agrD* gene of *S. aureus*, has been discovered in the genome sequence of *C. difficile*. *C. botulinum* and *C. perfringens* also contain an *agrD* homologue equivalent to the *agrD* gene of *C. difficile* as illustrated in **Figure 1.6**. As with *S. aureus agrD*, all three clostridial *agrD* genes are preceeded by an *agrB* homologue. It has recently been reported that *agr* plays a role in the regulation of toxin production in *C. perfringens* and neurotoxin and sporulation in *C. botulinum* (Othani *et al.*, 2009; Cooksley *et al.*, 2010). As *agr* homologues are also found in the genome sequence of *C. difficile*, this study sets out to discover whether the expression of virulence factors are under the control of an *agr*-QS based regulatory system. The hypothesis that the *agr* system in *C. difficile* controls virulence factor production through a quorum sensing mechanism was tested by making insertional mutants of *agrB* and other *agr* homologues in *C. difficile* and the effects on virulence were assessed, paying particular attention to toxin production and sporulation in the first instance.

In many pathogenic bacteria quorum sensing controls the regulation of so many processes, including the production of virulence factors in some clostridia species. Therefore, it is a focus for attention as a therapeutic target in the search for alternatives to conventional antibiotic therapy, which is becoming increasingly redundant due to the emergence of resistance.

Characterisation of a putative QS system in *C. difficile* could provide identification of previously unknown factors that participate in the progression of CDAD. Identification of such factors may lead to the generation of much needed novel therapeutic drugs (such as AIP blockers) that interfere with quorum sensing. Targeting the QS signalling pathway or the virulence factors that are discovered to be under its control is a promising therapeutic approach for the treatment of CDAD supportive of primary antibiotic therapy.

Chapter 2

Materials and Methods

2.1 Bacteriological methods

2.1.1 Bacterial strains

The bacterial strains used in this study are listed in Table 2.1. *Clostridium difficile* strains were stored at -80°, as a mixture of spores and vegetative cells resuspended in brain heart infusion (BHIS) broth, supplemented with 20% (v/v) glycerol.

Strain	Relevant properties	Source/reference
Escherichia coli TOP10	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 (StrR) *end*A1 *nup*G	Invitrogen
E. coli CA434	Conjugation donor	Purdy *et al.*, 2002
C. difficile 630Δ*erm*	PCR-ribotype 012 Erythromycin sensitive strain of *C. difficile* 630	Hussain *et al.*, 2005
C. difficile R20291	BI/NAP1/027 (Stoke Mandeville, UK). Isolated in 2004/5	Anaerobe Reference Library, Cardiff, United Kingdom
C. difficile 630Δ*erm agrB*::CT*ermB*	*C. difficile* 630Δ*erm agrB* insertional mutant made using ClosTron	This study
C. difficile 630Δ*erm* CD0160::CT*ermB*	*C. difficile* 630Δ*erm* CD0160 insertional mutant made using the ClosTron	This study
C. difficile 630Δ*erm* CD0611::CT*ermB*	*C. difficile* 630Δ*erm* CD0611 insertional mutant made using the ClosTron	This study
C. difficile 630Δ*erm* CD1743::CT*ermB*	*C. difficile* 630Δ*erm* CD1743 insertional mutant made using the ClosTron	This study
C. difficile 630Δ*erm luxS*::CT*ermB*	*C. difficile* 630Δ*erm luxS* insertional mutant made using the ClosTron	This study
C. difficile R20291 *agrB*1::CT*ermB*	*C. difficile* R20291 *agrB*1 insertional mutant made using the ClosTron	This study
C. difficile R20291 *agrB*2::CT*ermB*	*C. difficile* R20291 *agrB*2 insertional mutant made using the ClosTron	This study
C. difficile R20291 *agrA*::CT*ermB*	*C. difficile* R20291 *agrA* insertional mutant made using the ClosTron	This study
C. difficile R20291 *luxS*::CT*ermB*	*C. difficile* R20291 *luxS* insertional mutant made using the ClosTron	This study

C. difficile R20291 CD2603::CT*ermB*	*C. difficile* R20291 CD2603 insertional mutant made using the ClosTron	This study
C. difficile 630Δ*erm spo0A*::CT*ermB*	*C. difficile* 630Δ*erm spo0A* insertional mutant made using the ClosTron	Heap *et al.*, 2007
C. difficile R20291 *spo0A*::CT*ermB*	*C. difficile* R20291 *spo0A* insertional mutant made using the ClosTron	Heap *et al.*, 2010
C. difficile 630Δ*erm agrB*::CT*ermB* (pMTL84151::*agrBD*1)	*C. difficile* 630Δ*erm agrB* insertional mutant containing *agrBD*1 complementation plasmid	This study
C. difficile 630Δ*erm agrB*::CT*ermB* (pMTL84151)	*C. difficile* 630Δ*erm agrB* insertional mutant containing pMTL84151	This study
C. difficile 630Δ*erm* (pMTL84151)	*C. difficile* 630Δ*erm* containing pMTL84151	Burns *et al.*, 2010
C. difficile R20291 *agrB*1::CT*ermB* (pMTL84151::*agrBD*1)	*C. difficile* R20291 *agrB*1 insertional mutant containing *agrBD*1 complementation plasmid	This study
C. difficile R20291 *agrB*1::CT*ermB* (pMTL84151)	*C. difficile* R20291 *agrB*1 insertional mutant containing pMTL84151	This study
C. difficile R20291 (pMTL84151)	*C. difficile* R20291 containing pMTL84151	Burns *et al.*, 2010
C. difficile R20291 *agrB*2::CT*ermB* (pMTL84151::*agr*2)	*C. difficile* R20291 *agrB*2 insertional mutant containing *agr*2 complementation plasmid	This study
C. difficile R20291 *agrB*2::CT*ermB* (pMTL84151)	*C. difficile* R20291 *agrB*2 insertional mutant containing pMTL84151	This study
C. difficile R20291 *agrA*::CT*ermB* (pMTL84151::*agr*2)	*C. difficile* R20291 *agrA* insertional mutant containing *agr*2 complementation plasmid	This study
C. difficile R20291 *agrA*::CT*ermB* (pMTL84151)	*C. difficile* R20291 *agrA* insertional mutant containing pMTL84151	This study
C. difficile 630 Δ*erm* (pMTL84151::*agr*2)	*C. difficile* 630Δ*erm* containing pMTL84151::*agr*2	This study

Table 2.1. Bacterial strains used in this study.

2.1.2 Plasmids

All plasmids used in this study are listed in Table 2.2.

Plasmid	Relevant properties	Source/reference
pMTL007C-E2	ClosTron plasmid containing *catP* and intron containing *ermB* RAM	Heap *et al.*, 2010
pMTL007C-E2::Cdi-*agrB*-316s	pMTL007C-E2 retargeted to *C. difficile agrB*-316s	This study
pMTL007C-E2::Cdi-CD0160-246s	pMTL007C-E2 retargeted to *C. difficile* 630Δ*erm* CD0160-246s	This study
pMTL007C-E2::Cdi-CD0611-162s	pMTL007C-E2 retargeted to *C. difficile* 630Δ*erm* CD0611-162s	This study
pMTL007C-E2::Cdi-CD1743-390s	pMTL007C-E2 retargeted to *C. difficile* 630Δ*erm* CD1743-390s	This study
pMTL007C-E2::Cdi-*luxS*-48s	pMTL007C-E2 retargeted to *C. difficile luxS*-48s	This study
pMTL007C-E2::Cdi-*luxS*-161a	pMTL007C-E2 retargeted to *C. difficile luxS*-161a	This study
pMTL007C-E2::CdiR20291-*agrB*1-362a	pMTL007C-E2 retargeted to *C. difficile* R20291 *agrB*1-362a	This study
pMTL007C-E2::CdiR20291-*agrB*2-313a	pMTL007C-E2 retargeted to *C. difficile* R20291 *agrB*2-313a	This study
pMTL007C-E2::CdiR20291-*agrA*-306s	pMTL007C-E2 retargeted to *C. difficile* R20291 *agrA*-306s	This study

pMTL84151	*Clostridium* modular plasmid containing *catP*	Heap *et al.*, 2009
pMTL84151::*agrBD*1	pMTL84151 containing 806 bp *agrBD* coding region, a presumed 452 bp upstream promoter region and 152 bp downstream of the *agrBD* locus	This study
pMTL84151::*agr*2	pMTL84151 containing 2,915 bp *agr*2 locus coding region, a presumed 400 bp upstream promoter region and 269 bp downstream of the *agr*2 locus	This study

Table 2.2. List of plasmids used in this study.

2.1.3 Routine culture of aerobic bacterial isolates

Escherichia coli strains were routinely cultured on Luria Bertani (LB) broth or agar as appropriate. Cultures were incubated aerobically with shaking at 200 rpm at 37°C. The media was supplemented with chloramphenicol at 12.5 μg/ml (in broth) or 25 μg/ml (in agar) where appropriate. *E. coli* TOP10 was used throughout as a cloning host and *E. coli* CA434 was used as a donor strain for transfer of plasmid DNA into *C. difficile* by conjugation.

2.1.4 Routine culture of anaerobic bacterial isolates

Unless stated otherwise, strains of *Clostridium difficile* were routinely cultured in BHIS (brain heart infusion supplemented with L-cysteine [0.1%; Sigma, United Kingdom] and yeast extract [5 mg/ml; Oxoid] broth or agar; a medium that has been shown to aid *C. difficile* spore formation (Sorg and Sonenshein, 2008). Cultures were incubated at 37°C in an anaerobic workstation (Don Whitley, United Kingdom) containing an atmosphere of 80% (v/v) nitrogen, 10% (v/v) hydrogen and 10% (v/v)

carbon dioxide. The media was supplemented as appropriate with cycloserine (250 μg/ml), cefoxitin (8 μg/ml), thiamphenicol (15 μg/ml), erythromycin (10 μg/ml) or lincomycin (20 μg/ml). Prior to inoculation, all growth media was pre-reduced overnight.

2.1.5 Preparation of electro-competent *E. coli* cells

A 200ml volume of pre-warmed LB broth was inoculated 1/100 with a fresh overnight culture of *E. coli*. The culture was incubated at 37 °C with 200 rpm shaking until an optical density at 600 nm (OD_{600}) of between 0.5 and 1 was achieved; indicating that the cells were in exponential growth phase. Cells were harvested by aliquoting the culture into 50ml falcon tubes, cooling on ice for 30 min, followed by centrifugation for 15 min at 4000 ×*g* at 4°C. The cell pellets were gently re-suspended in 40 ml of ice-cold, sterile dH_2O before centrifugation as described above. Resuspension of the cell pellets in ice-cold dH_2O was repeated once again. Following centrifugation, the pellets were re-suspended in 4ml of sterile 10% glycerol and centrifuged as before. After discarding the supernatant, pellets were finally resuspended in 600 μl of sterile 10% glycerol and stored at -80 °C in 50 μl aliquots.

2.1.6 Measurement of *C. difficile* growth in BHIS broth

To measure the growth rates of *C. difficile* strains, cultures were set up in BHIS broth using a 1/100 inoculum of a fresh overnight culture and incubated at 37°C in

anaerobic conditions. At different time points, 0.5-1 ml samples were removed from the culture and the OD_{600} was measured. (Biomate 3, Thermo Scientific).

2.1.7 Sporulation assays

2.1.7.1 Preparation of *C. difficile* sporulation cultures in liquid media

Sporulation of *C. difficile* in a nutrient-rich liquid medium was achieved by incubating cultures anaerobically in BHIS broth for five days at 37 °C. To ensure that no spores were present when the sporulation medium was inoculated, a starter culture was prepared in BHIS broth using a 1/100 inoculum of a *C. difficile* culture and incubated until an OD_{600} of between 0.2 and 0.5 was reached. The sporulation medium was then inoculated 1/100 with this exponential starter culture.

2.1.7.2 Heat treatment of sporulation cultures

Sporulating cultures of *C. difficile* were prepared as described above. A 500 µl sample of the culture was removed from the anaerobic chamber and heated at 60 °C for 30 minutes to kill the vegetative cells but not the spores. To control for any effects of oxygen exposure during heat treatment, a non-heated sample was also removed from the anaerobic chamber for 30 minutes. Heat-treated samples were then returned to the anaerobic chamber, serially diluted in PBS, and plated onto BHIS agar supplemented with 0.1% taurocholate (Sigma, United Kingdom). Total cfu/ml (spores and vegetative cells) was also enumerated by serial dilution and plating onto BHIS agar supplemented with 0.1% taurocholate before heat treatment.

After a 24 hour incubation period, bacterial colonies were counted at the appropriate dilution and cfu/ml were calculated by multiplying out the dilution factor. Samples were analysed in the same way every 24 h for five days. *C. difficile spo0A* mutants of both 630Δ*erm* and R20291 were used as sporulation-negative controls (Heap, *et al.*, 2007; Heap, *et al.*, 2010).

2.2 Molecular Biology Methods

2.2.1 Preparation of genomic DNA

Genomic DNA was prepared using the DNeasy Blood & Tissue Kit (Qiagen) according to the instructions provided by the manufacturer.

2.2.2 Preparation of plasmid DNA

Plasmids were prepared using the Plasmid Miniprep Kit (Qiagen) according to the instructions provided by the manufacturer.

2.2.3 Agarose gel electrophoresis

A 1% agarose gel was made by dissolving agarose powder in 1 X TAE buffer and ethidium bromide added to a final concentration of 0.5 μg/ml. Once the gel had been cast, the gel tray was submerged in an electrophoresis tank containing 1 X TAE buffer. DNA samples were mixed 5:1 with a gel loading buffer and 10-15μl of each sample was loaded into individual wells. A DNA ladder (New England Biolabs) was

added to at least one well in the gel to act as a size marker. Gels were electrophoresed at 90V for 20-60 minutes, until DNA bands were suitably resolved. Subsequently, the gel was visualised over a UV trans-illuminator.

2.2.4 Purification of DNA from agarose gels

DNA bands were excised from agarose gels under ultra-violet light, and DNA was purified using the QIAquick Gel Extraction Kit (Qiagen) in accordance with the manufacturer's instructions.

2.2.5 Purification of DNA from enzymatic reactions

DNA fragments were purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions.

2.2.6 Digest of DNA with restriction endonucleases

All DNA restriction enzymes (New England Biolabs) were used in the buffers and under the conditions recommended by the manufacturer.

2.2.7 Dephosphorylation of free DNA ends

To remove 5′ phosphate groups from linearised plasmid DNA fragments and prevent self-ligation, dephosphorylation reactions were carried out using Antarctic Phosphatase (New England Biolabs) in accordance with manufacturer's instructions.

2.2.8 Ligation of linear DNA fragments

Ligations were carried out in 10µl volumes with the appropriate vector : insert ratio, 1µl 10x ligase buffer and 1 U of T4 DNA ligase (Promega). Reactions were incubated at room temperature overnight.

2.2.9 Nitrocellulose membrane dialysis of DNA after ligation

Following ligation reactions, the products were dialysed through an 0.025 µm nitrocellulose membrane (Millipore) over dH_2O for 30 minutes. The reaction mixture was then transformed into *E. coli* as required.

2.2.10 Transformation of electro-competent *E. coli* by electroporation

A 50 µl aliquot of electro-competent *E. coli* cells was thawed on ice. Once thawed, DNA was added to the cells, gently mixed and then transferred into a cold 0.2 cm gap electroporation cuvette (Biorad). A pulse applied across the cuvette using an electroporator with 2.5 kV voltage, 25 µF capacitance and 200 Ω resistance. Immediately after, a 250 µl aliquot of SOC medium (Invitrogen) was added to the

mixture. The transformation mixture was then transferred into a sterile microfuge tube and the cells were allowed to recover for 1 hour under aerobic conditions, with shaking at 200 rpm. The transformation mix was plated onto LB agar supplemented with appropriate antibiotics to select for transformants. Plates were incubated at 37°C for 16 hours, under aerobic conditions. Colonies were screened to identify those harbouring the plasmid vector with the desired insert by restriction mapping and PCR. Cloned inserts were sequenced where necessary.

2.2.11 Spectrophotometric quantification of DNA

The concentration of DNA from preparations was measured using a nanodrop ND-1000 spectrophotometer. The OD_{260} of the preparation was measured and DNA concentration calculated, given that with a 1 cm path, a 50 mg/ml solution of double-stranded DNA has an OD_{260} of 1.

2.2.12 Nucleotide sequencing and analysis of sequence data

DNA sequencing reactions were carried out by Geneservice Ltd, Cambridge and Geneservice Ltd, Nottingham. Sequence data were assembled and analysed using were VectorNTI software (Invitrogen).

2.2.13 Transfer of plasmid DNA into *C. difficile* by conjugation

Conjugative transfer of plasmid DNA into *C. difficile* was carried out as described by Purdy *et al.*, 2002. Briefly, both donor and recipient cells were grown to stationary

phase in overnight cultures. A 1ml volume of donor cells was harvested by centrifugation at 4000 ×*g* for 1 minute. The cell pellet was washed with phosphate-buffered saline (PBS), in order to remove traces of antibiotics, and centrifuged as before. In an anaerobic workstation, the donor cell pellet was re-suspended in a suspension of recipient cells, yielding a suspension containing an approximate 5:1 ratio of donor to recipient cells. The donor-recipient suspension was then plated onto BHIS agar in discrete spots and incubated anaerobically at 37 °C overnight. Cells taken off the plate by gently scraping with a loop and re-suspended into 500 µl PBS and subsequently plated onto media containing cycloserine (250 µg/ml), cefoxitin (8 µg/ml), thiamphenicol (15 µg/ml) and lincomycin (20 µg/ml) as appropriate to counter select against growth of *E. coli* donor strains and to positively select for *C. difficile* transformants. Plates were incubated anaerobically at 37 °C for 24-72 h before colonies were picked and purified by subculture for further analysis.

2.2.14 Amplification of DNA by polymerase chain reaction (PCR)

For cloning, PCR amplifications were carried out using either the Epicentre Failsafe PCR system with Failsafe Premix E (Cambio), or Phusion polymerase (New England Biolabs) according to manufacturer's instructions. For screening PCR, *Taq* DNA polymerase (Promega) was used, in accordance with the manufacturer's instructions. The following amplification conditions were used, unless stated otherwise; 1 cycle at 94°C for 3 minutes, 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 1 minute 30 seconds, and 1 cycle of 72°C for 10 minutes.

Oligonucleotide	Sequence (5'- 3')
Intron retargeting	
EBS Universal	CGAAATTAGAAACTTGCGTTCAGTAAAC
Cdi-agrB-316s-IBS	AAAAAAGCTTATAATTATCCTTAAAATACAAATATGTGCGCCCAGATAGGGTG
Cdi-agrB-316s-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCAAATATAGTAACTTACCTTTCTTTGT
Cdi-agrB-316s-EBS2	TGAACGCAAGTTTCTAATTTCGGTTTATTTCCGATAGAGGAAAGTGTCT
Cdi-CD160-246s-IBS	AAAAAAGCTTATAATTATCCTTAGTAAGCATTATAGTGCGCCCAGATAGGGTG
Cdi-CD160-246s-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCATTATATTTAACTTACCTTTCTTTGT
Cdi-CD160-246s-EBS2	TGAACGCAAGTTTCTAATTTCGATTCTTACTCGATAGAGGAAAGTGTCT
Cdi-CD611-162s-IBS	AAAAAAGCTTATAATTATCCTTAAATTACTACATAGTGCGCCCAGATAGGGTG
Cdi-CD611-162s-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCTACATAGATAACTTACCTTTCTTTGT
Cdi-CD611-162s-EBS2	TGAACGCAAGTTTCTAATTTCGGTTTAATTCCGATAGAGGAAAGTGTCT
Cdi-CD1743-390s-IBS	AAAAAAGCTTATAATTATCCTTAAGTAACTATATCGTGCGCCCAGATAGGGTG
Cdi-CD1743-390s-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCTATATCACTAACTTACCTTTCTTTGT
Cdi-CD1743-390s-EBS2	TGAACGCAAGTTTCTAATTTCGATTTTACTTCGATAGAGGAAAGTGTCT
Cdi-luxS-48s-IBS	AAAAAAGCTTATAATTATCCTTAAAAGTCAAAGCTGTGCGCCCAGATAGGGTG
Cdi-luxS-48s-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCAAAGCTCCTAACTTACCTTTCTTTGT
Cdi-luxS-48s-EBS2	TGAACGCAAGTTTCTAATTTCGGTTACTTTCCGATAGAGGAAAGTGTCT
Cdi-luxS-161a-IBS	AAAAAAGCTTATAATTATCCTTATGCTCCAAACCGGTGCGCCCAGATAGGGTG
Cdi-luxS-161a-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCAAACCGTGTAACTTACCTTTCTTTGT
Cdi-luxS-161a-EBS2	TGAACGCAAGTTTCTAATTTCGGTTGAGCATCGATAGAGGAAAGTGTCT
Cdi027-agrB1-362a-IBS	AAAAAAGCTTATAATTATCCTTACAAATCCCAACCGTGCGCCCAGATAGGGTG
Cdi027-agrB1-362a-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCCAACCCATAACTTACCTTTCTTTGT

Cdi027-agrB1-362a-EBS2	TGAACGCAAGTTTCTAATTTCGATTATTTGTCGATAGAGGAAAGTGTCT
Cdi027-agrB2-313a-IBS	AAAAAAGCTTATAATTATCCTTAAATAACTACTTAGTGCGCCCAGATAGGGTG
Cdi027-agrB2-313a-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCTACTTATATAACTTACCTT TCTTTGT
Cdi027-agrB2-313a-EBS2a	TGAACGCAAGTTTCTAATTTCGGTTTTATTCCGATAGAGGAAAGTGTCT
Cdi027-agrA-306s-IBS	AAAAAAGCTTATAATTATCCTTAAGGAACATAAGAGTGCGCCCAGATAGGGT G
Cdi027-agrA-306s-EBS1d	CAGATTGTACAAAGTGGTGATAACAGATAAGTCATAAGAGATAACTTACCTT TCTTTGT
Cdi027-agrA-306s-EBS2	TGAACGCAAGTTTCTAATTTCGATTTTCCTTCGATAGAGGAAAGTGTCT
ClosTron sequencing	
Spofdx_F1	GATGTAGATAGGATAATAGAATCCATAGAAAATATAGG
pMTL007_R1	AGGGTATCCCCAGTTAGTGTTAAGTCTTGG
Mutant screening	
agrB_126_F	GTTAATATAACCACCATGCTGTTTATAGGTTTTTTATTTGG
agrB_388_R	ATTGGGCACAGAACACAAATTCCAACC
CD0160_19_F	GCGAAGGTAAAAAATCAGAGAGG
CD0160_279_R	TCTCTAAATATCTATAAACTCCTATCTCATAACC
CD0611_124_F	GTCTAGTATACAATCTAGAACACG
CD0611_449_R	GTTAATTTTCTTAGCTCACTTTTAAAGTAGAGG
CD1743_273_F	GATGCTTTTGAAGTAGATGC
CD1743_550_R	TGATTATATAACTACGATGACACC
luxS_95_F	AGTAACTAAATTTGACTTGAGATTTTTACAGCC
luxS_262_R	AACCAATCTTAACTGTTTTGGCATCTACATCTCCCC
agrB1(Cdi027)_238_F	TGTGTTATTGTCCAATAAGACAATTTTCAGG
agrB1(Cdi027)_568_R	CTAACATCACAAAAATCCAAAACATGGCAAATGCGC

agrB2_47_F	TGAGATTGTAGAAAGTGAAGATTTTGAAATATATAGATATGG
agrB2_213_R	TGTTAATGTACACTCTTTATAATTCCTAGCATGATAACCTCC
agrA_234_F	GATTTAGACATATTAATTATGGACATACAAATGAAAACAATAAATGG
agrA_506_R	CTGTTTCTATATAAGTAATAGAATCTATTTTAATTCGG
RT-PCR	
ptsI_F	TAGCTGATGATGTACTTAATATGGGTACTGC
CD2751_R	TTACTCTGCTTAAATCATTTAAATGTACTTTGTTATCTTCC
CD2751_F	AGAGATTTGTTTAGAAGCAGTTAATCAAAGTTATGAAGCGC
agrB_R	ACCTATAAACAGCATGGTGGTTATATTAACTATGAAAGC
agrB_F	TTGTGTTCTGTGTCCAATAGAACATAGGAGTAACCC
agrD_R	TAGCTGACAAAATAGCAGTGCTAAGTGCCAATGAGCTAGC
agrD_F	TAATAGTACCTGTCCTTGGATTATTCATCAGCC
CD2749_R	TGAACAGCTCCACCATTCACATCTAAAACGATATTATATCC
CD2749_F	TCAAGGTCCATGTGTAATAAAGAGAATAACTGGATGTGGAGCTGG
CD2748_R	AATAGTTATTGGTCCTTCATCACCTCTAACTACTGCATTGTACC
CD2748_F	TTGATAATGGGTTCTCCTGGTAAGGTTGTTAGAGAGC
ubiB_R	TCTTCTGGTGGGTCAAATAGTGGTATTTTATAGGC
CDR20291_3192_F	TTGTGGAAAGTGTATAGTTGGGGTTCCTTGTTCGGTAATAAATCCC
CDR20291_3191_R	TTCCTGTATCAATTCAAGTAATTCTTCTGGTAATATATGTATTGC
CDR20291_3191_F	AAGTAGTACGTCTACTCGTGAAGATTTATCAATTAGAAATATGC
CDR20291_3190_R	TACAATACCTAATTACAGGTAACTGGGAGATATATTCCACCTCCC
CDR20291_3190_F	ATGAAATTATGTTAGATATAGGCAAGGAGAAGC
agrA_R	ATTCGATAATGTAGTTCATCATCACATATTCC

agrA_F	TAGTAGAATCAATGAGTGGAAGTACTGTTATAGTAGATGGC
agrC_R	TGATATTCCTATTACCATACATAGAAGAATCTTAATATTTGG
agrC_F	AATGCAATTGAAGCATGTATGGATATAAATAATGAGC
agrD2_R	AACTTGCACTGTTTGCTGATAATACAGCTATCCC
agrD2_F	AGTGCAAGTTCTTGGGTAGCACATCAGGCAAAAGAGCC
agrB2_R	TCCTAGCATGATAACCTCCTGTGAACTGTCTTAAAGTGC
agrB2_F	ACCTCTTAGTGAAAGTGAGAAAAAGCATTATAGAAAGACTGTGC
CDR20291_3186_R	ACCTTAATACTACTCATATTTATGCCCCTCCTAGCCAATCC
CDR20291_3186_F	AAGAAATGAAATATTAGGTGATAGCAAAGCATCTGTAGATGC
CDR20291_3185_R	ATGCAACTACTCCATCTGTTAGGGTTAAATTTATATTATCCAGAGC
CDR20291_3185_F	TTGATATTATTGATTATAGAAGAGTTAAAGATGGTGTGC
CDR20291_3184_R	TTCCTAACCATCCTGCTATAGAATATGTATCCATTATAGG
Complementation	
agrB1_F1(EcoRI)	AAAAAAGAATTCATGTTTAGAAGCAGTAAAGCAAAATTATAGAGC
agrD1_R1(BglII)	AAAAAAGAATTCAGTTTGTCTTGAAGCAGTAAAACAGAATAGAAAAGC
CDR20291_3190_F1 (EcoR1)	AGGTAAATTAAATTATGAAATTATGTTAGATATAGGCAAGGAGAAGC
agrC_R (XbaI)	TCTATCATGAACTTCGTTTATATTTTCATAATTCC
agrC_F (XbaI)	CATCAAGTATAATACTTTTAATAGTAATAGGG
CDR20291_3186_R (BglII)	TATTGGATATATGTGTCCATCGTTGTATGAGATGG

Table 2.3. List of oligonucleotides used in this study. Oligonucleotides were used as described in the text.

2.2.15 Inactivation of *C. difficile* genes

Target genes were insertionally inactivated using the ClosTron system as previously described (Heap, *et al.*, 2007), using the modular ClosTron plasmid pMTL007C-E2, described in Figure 2.1 (Heap, *et al.*, 2010).

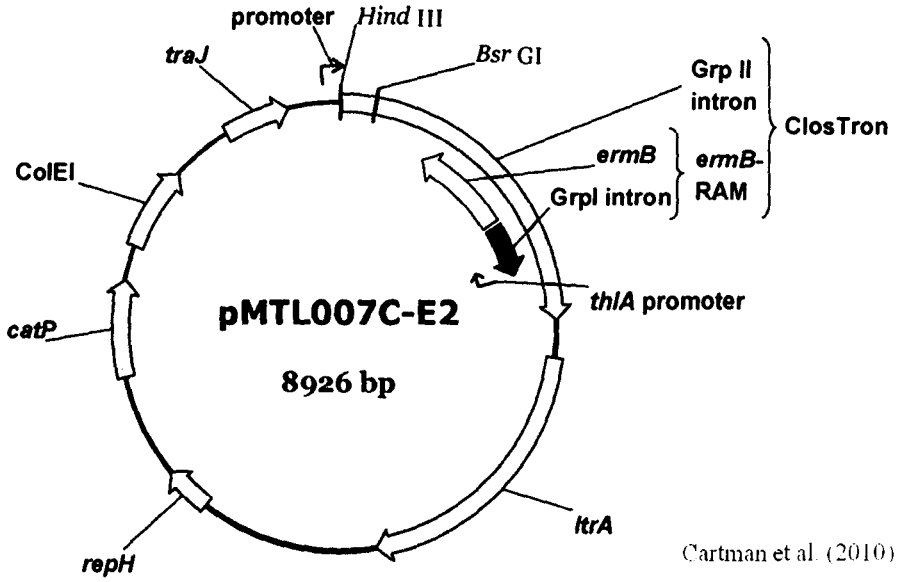


Figure 2.1. The second generation, modular ClosTron plasmid pMTL007C-E2 (Cartman, *et al.*, 2010; Heap, *et al.*, 2010). This plasmid uses the strong *fdx* promoter to drive expression of the ClosTron (Heap, *et al.*, 2007). Intron re-targeting using the HindIII and BsrGI restriction sites replaces a *lacZ'* stuffer fragment with the 5′ exon and the region of the intron conferring target specificity, and allows for the re-targeted pMTL007C-E2 to be analysed by blue/white screening, PCR or restriction analysis.

2.2.16 Construction of re-targeted pMTL007C-E2

Target sites were identified and intron re-targeting PCR primers designed using a computer algorithm (Perutka, *et al.*, 2004), available free-of-charge at http://www.clostron.com. A 353 bp PCR product containing modified IBS, EBS1d

and EBS2 sequences, responsible for target specificity of the intron, were amplified and assembled using SOEing (splicing by overlap extension) PCR. The following cycling conditions were used; 1 cycle at 94°C for 30 seconds, 30 cycles of 94°C for 15 seconds, 55°C for 30 seconds and 72°C for 30seconds, and 1 cycle at 72°C for 2 minutes. The 353bp PCR product was cloned into the *Bsr*GI and *Hind*III sites of plasmid pMTL007C-E2 in order to retarget the group II intron to the gene of interest. Primers Spofdx_F1 and pMTL007_R1 were used to verify the sequence of the insert. Re-targeted introns were named in accordance with the previously published ClosTron nomenclature (Karberg, *et al.*, 2001; Perutka, *et al.*, 2004; Heap, *et al.*, 2007). For example, the intron on plasmid pMTL007C-E2::CdiR20291-*agrA*-306s has been retargeted to insert in the sense orientation after base 306 of the *agrA* open reading frame (ORF) of *C. difficile* R20291. Re-targeted pMTL007C-E2 vectors were initially analysed by blue/white screening, restriction analysis with SacI or BglII and/or PCR using primer pair spofdx_F1/pMTL007_R1. Vectors were then confirmed by sequencing.

2.2.17 Synthesis of re-targeted pMTL007C-E2

The advance of commercial DNA synthesis technology during this study allowed for the construction of re-targeted pMTL007C-E2 plasmids to be outsourced (DNA2.0, USA; Heap, *et al.*, 2010). Target sites and PCR primers were designed as described above, and the supplier synthesised and cloned the subsequent 353 bp fragment into pMTL007C-E2. The re-targeted vector was delivered, sequence-verified, ready for transfer into *C. difficile*.

2.2.18 Isolation and confirmation of *C. difficile* ClosTron mutants

Re-targeted pMTL007C-E2 plasmids were introduced into *C. difficile* by conjugation as described in section 2.2.13. Transconjugants were selected on media supplemented with cycloserine (250 μg/ml), cefoxitin (8 μg/ml) and thiamphenicol (15 μg/ml), and then serially sub-cultured to obtain pure clones. A loop-full of transconjugant cells was re-suspended in 500 μl PBS, serially diluted as appropriate, and plated onto media supplemented with cycloserine (250 μg/ml), cefoxitin (8 μg/ml) and either erythromycin (2.5 μg/ml, *C. difficile* 630Δ*erm*) or lincomycin (20 μg/ml, *C. difficile* R20291), to select for integration of the ClosTron. Genomic DNA was extracted from erythromycin-resistant (630Δ*erm*) or lincomycin-resistant (R20291) clones at random and used in a PCR to amplify the intron-exon junction. One clone of each desired mutant was selected, and the intron site was verified by sequencing using the primers pairs EBS Universal, RAM_R1 and screening primers R1 and F1.

2.2.19 Complementation of a *C. difficile agrB* mutant with parental *agrB*

For complementation studies of *agrB* mutant strains of *C. difficile* 630Δ*erm* and *C. difficile* R20291, a 806 bp fragment encompassing the *agrBD* structural genes and 5′ non-coding region was cloned into the modular plasmid pMTL84151 (Heap, *et al.*, 2009) to generate plasmid pMTL84151::*agrBD1*. The 452 bp 5′ non-coding region likely encompassing the *agrBD* promoter, the 806 bp region containing the *agrBD* structural genes and the 152 bp 3' non-coding region were amplified by PCR using oligonucleotide primer pairs agrB1_F1/agrD1_R1. The primers were designed to

allow subsequent cleavage of the fragment generated with EcoRI/BglII. The cleaved fragment was subsequently ligated with plasmid pMTL84151 cut with EcoRI and BglII, which yielded plasmid pMTL84151::*agrBD1*. As the protein encoded by *agrB* is the same in *C. difficile* 630Δ*erm* and R20291, only one plasmid (pMTL84151::*agrBD1*) was constructed for use with both *agrB* mutants. Both the pMTL84151::*agrBD1* complementation plasmid and a pMTML84151 empty vector control were then transferred into *C. difficile* by conjugation.

2.2.20 Complementation of a *C. difficile* R20291 *agrB2* and *agrA* mutants with the parental *agr2* locus

For complementation studies of *agrB2* and *agrA* mutant strains of *C. difficile* R20291, a 2,915 bp fragment encompassing the *agr2* structural genes and 5′ and 3' non-coding region was cloned into the modular plasmid pMTL841 to generate plasmid pMTL84151::*agr2*. The 400 bp 5′ non-coding region likely encompassing the *agr2* promoter, the 2,915 bp region containing the *agr2* structural genes and the 269 bp 3' non-coding region were independently amplified by PCR using oligonucleotide primer pairs CDR20291_3190_F1/agrC_R and agrC_F/CDR20291_3186_R, respectively. The primers were designed to allow subsequent cleavage of the two fragments generated with EcoRI/XbaI and XbaI/BglII, respectively. The two cleaved fragments were subsequently ligated with plasmid pMTL84151 cut with EcoRI and BglII, which yielded plasmid pMTL84151::*agr2*, in which the two fragments were inserted contiguously. Both the pMTL84151::*agr2* complementation plasmid and a pMTML84151 empty vector control were then transferred into *C. difficile* R20291 by conjugation.

2.2.21 Preparation of total RNA from *Clostridium difficile*

Under anaerobic conditions, a 5ml sample of *C. difficile* liquid culture was added to 10ml RNA protect (Qiagen) and mixed by inverting. The *C. difficile*/RNA protect mixture was incubated at 37°C for 5 minutes and subsequently harvested by centrifugation at 4000 rpm for 10 minutes at room temperature. The supernatant was discarded and the cell pellet was resuspended in 2ml RNA pro solution (MP Biochemicals) in a fume hood. The cell suspension was transferred to a matrix tube and incubated on ice before being lysed in a ribolyser at 5000 rpm for 30 seconds. After the cell lysate had been centrifuged at 13000 rpm for 15 minutes at 4°C, the supernatant was added to a microfuge tube containing 300µl chloroform and mixed by vortexing for 10 seconds. The upper phase was transferred into a fresh microfuge tube containing 500µl 100% ethanol, mixed by inverting and incubated at -20°C overnight to precipitate the RNA.

Following incubation overnight, the sample was centrifuged at 13000 rpm for 15 minutes at 4°C to pellet the RNA. Once the supernatant had been discarded, the RNA pellet was washed with 500µl 70% ethanol and then centrifuged at 13000 rpm for 5 minutes at 4°C. The supernatant was discarded and the RNA pellet air dried at room temperature before being resuspended in 50µl DEPC treated water and subsequently stored at -80°C.

2.2.22 Removal of contaminating genomic DNA from total RNA

Contaminating genomic DNA was removed from RNA samples using TURBO DNase (Ambion). A 50µl aliquot of RNA was mixed with 2µl TURBO DNase, 2µl

RNase inhibitor (Promega),2 µl TUBRO DNase buffer and incubated for 30 minutes at 37°C. Following the incubation period, 2µl TURBO DNase and 2µl RNase inhibitor was added to the RNA sample and incubated for a further 30 minutes at 37°C.

2.2.23 Purification of DNase-treated RNA

DNase treated RNA was purified using the RNeasy MinElute Cleanup Kit (Qiagen) according to manufacturer's instruction. The volume of the RNA sample was adjusted to 100µl using RNase-free water. A 250µl aliquot of buffer RLT was added to the sample and mixed, followed by the addition of 250µl absolute ethanol. The solution was mixed by pipetting before being applied to the spin column and centrifuged at 10000 rpm for 15 seconds. The spin column was placed in a new collection tube and 500µl buffer RPE was added and centrifuged as before. The flow through was discarded and the column washed for a second time by addition of 500µl 80% ethanol before being centrifuged as previously. The column was placed in a new collection tube and centrifuged at 13000 rpm for a further 2 minutes to dry the column. The RNA was eluted from the column by adding 14µl RNase-free water and after leaving the column to stand for 1 minute, the column was centrifuged for 10000 rpm for 1 minute. RNA samples were stored at -80°C prior to use.

2.2.24 One-step Reverse Transcriptase PCR (RT-PCR)

All RNA samples were prepared according to 2.2.21 and DNase-treated and purified as described in 2.2.22 and 2.2.23 respectively. RT-PCR was carried out using One-

Step RT-PCR kit (Qiagen) as described in manufacturer's instructions. The reaction mix was composed of 10µl 5x Qiagen OneStep RT-PCR buffer, 2 µl dNTP mix (containing 10mM of each dNTP), 1 µl of each gene specific primer (10µM), 2µl Qiagen OneStep RT-PCR enzyme mix and 1µg temple RNA. The volume was adjusted to 50µl using RNase-free water. All reactions were set up on ice to inhibit any enzymatic activity. The thermal cycler was pre-heated to 50°C before the samples were placed inside. The RT-PCR reaction was initiated with a reverse transcriptase step, 50°C for 30 minutes, followed by a DNA polymerase activation/reverse transcriptase inactivation step, 95°C for 15 minutes. A total of 40 cycles of the following conditions were used for amplification; denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and elongation at 72°C for 1 minute 30 seconds. The reaction was concluded after a final elongation step of 72°C for 10 minutes. After amplification, RT-PCR products were visualised on an agarose gel as described in section 2.2.3 or stored at -20°C until required.

2.3 *In vitro* methods

2.3.1 Preparation and maintenance of Vero and HT29 cell monolayers

The African green monkey kidney epithelial Vero cell line and the human colon adenocarcinoma grade II HT29 cell line were used for cytotoxicity assays in this study. Complete growth media used for Vero cells was Dulbecco's Modified Eagle Medium (DMEM) (Sigma), supplemented with 10% (v/v) heat inactivated foetal calf-serum (Sigma), 2mM L-glutamine (Sigma) and penicillin-streptomycin (Sigma).

Complete growth media used for HT29 cells was McCoy's 5A medium (Invitrogen), supplemented with 2mM L-glutamine (Sigma) and penicillin-streptomycin (Sigma).

Vero cells and HT29 cells were revived from storage by resuspending them in pre-warmed (37°C) complete growth media to a density of 2 x 10^5 cells/ml. A 20ml volume of this cell suspension was used to seed a 75 cm^2 tissue culture flask (Nunc). Cells were incubated at 37°C in 5% CO_2 and passaged every 2–3 days, once monolayers reached confluence. To passage the cells, growth media was poured off, cells were washed with 5ml PBS twice and incubated with 5ml 1 x trypsin-EDTA (Sigma) at 37°C for 5–10 minutes. After incubation, a 5ml volume of the appropriate medium was added to cells and the cell suspension swirled around the flask to help dislodge any remaining attached cells. The resulting cell suspension was resuspended in fresh growth medium to a final density of 2 x 10^5 cells/ml. A 20ml volume of this cell suspension was used to seed each fresh 75 cm^2 tissue culture flask.

2.3.2 Cytotoxicity assays

A tissue culture flask containing a 70 - 90% confluence monolayer of Vero or HT29 cells was used to seed 96-well plates for cytotoxicity assays. Cells were dislodged from the flask using 1 x trypsin-EDTA as described in section 2.3.1. The cell suspension was transferred into a 20ml tube and centrifuged at 1000 x g for 15 minutes at room temperature. After centrifugation, the cell pellet was resuspended in 10ml of the appropriate medium. The resulting cell suspension was resuspended in fresh growth medium to a final density of 2 x 10^5 cells/ml. A 100µl of the diluted cell suspension

was used to seed the cells into each well of a 96-well plate. The plates were incubated for 48 hours at 37°C in 5% CO_2

For cytotoxicity assays, *C. difficile* strains were grown in Tryptose yeast (TY) media (3% w/v bacto-tryptose, 2% w/v yeast extract, 0.1% w/v sodium thioglycolate, adjusted to pH 7.4) under anaerobic conditions as described in section 2.1.4. A 5ml culture of *C. difficile* was grown overnight and cell densities were standardised before centrifugation and filtration. Supernatants were diluted in a fourfold series and 20µl of the dilutions were added to onto the monolayers of vero cells and HT29 cells pre-incubated in 96-well plates for 48 hours as described above. Cytotoxic effects on the cells were recorded after 24 hours.

2.4 Statistical analysis

All statistical analysis was carried out in GraphPad Prism, using Student's t-test with a p value cut off of $p < 0.05$.

Chapter 3

Identification of a putative quorum sensing system in *Clostridium difficile* 630 and its effect on sporulation and toxin production

3.1 Introduction

3.1.1 Mutational studies in *C. difficile*

The lack of in-depth studies into the genetic basis of *C. difficile* quorum sensing-type gene regulation has been largely attributed to an absence of reliable genetic tools. In the past, targeted inactivation of *C. difficile* genes was limited to single-crossover mutations through homologous recombination of a replication-defective plasmid (O'Connor *et al.*, 2006). However, single cross-over integrants such as these are genetically unstable, meaning they are able to revert back to the genetic and phenotypic status of the parent strain. More recently, the ClosTron, a facile directed mutagenesis system, has recently been developed (Heap, *et al.*, 2007; Heap, *et al.*, 2010) and allows for reverse genetics studies in *C. difficile* through the creation of stable, insertional mutants.

The system is based on a novel way to insertionally inactivate genes using group II intron (Karberg, *et al.*, 2001). The mobile intron can be specifically targeted to a gene of interest by altering a small region of its sequence (350 bp) according to the rules of an algorithm developed by Perutka *et al.* (2004). The only other component required to mobilise the intron is the LtrA protein which is provided *in trans*. Within the group II intron is an antibiotic resistance gene, which is interrupted by a self-splicing group I intron. This particular arrangement ensures that the antibiotic resistance gene will only be restored following integration of the group II intron and self-splicing of the group I intron. Therefore, antibiotic resistance will only be achieved as a consequence of the integration event. The ClosTron system is ideal to inactivate genes encoding homologues of proteins important in quorum sensing in

other gram positive bacteria, and thus presumed to be important in quorum sensing-type gene regulation *C. difficile*.

3.1.2 Previous knowledge of quorum sensing systems in clostridia

The poor availability of tools to manipulate clostridia genetically has held back progress on the investigation of gene regulation in this genus. Prior to the beginning of this study, detailed studies of *C. difficile* quorum sensing systems, in particular *agr* quorum sensing, had not been reported. However, there were reports about the role of a *luxS*-type quorum sensing system in other clostridia. These findings indicate that quorum sensing type systems have a functional role in clostridia.

Ohtani *et al.* (2002) showed that the *luxS* gene is involved in the regulation of toxin production in *C. perfringens* via quorum sensing. *C. perfringens luxS* mutants exhibited reduced levels of α, κ and θ toxins in comparison to the wild type strain. Toxin expression in the *C. perfringens luxS* mutant strains could be complemented with wild type culture supernatant and toxin production was restored.

There have also been reports proposing a role of a LuxS/AI-2 type signalling system in *C. difficile*. Carter *et al.* (2005) demonstrated that the AI-2 signalling molecule is also produced by *C. difficile*. However, they suggested that AI-2 was not involved in the control of toxin production, as down-regulation of the *luxS* gene using antisense RNA did not cause a reduction in toxin production. Lee & Song (2005) also carried out a similar study into *luxS* and quorum sensing in *C. difficile*, and demonstrated an

up-regulation of toxin A mRNA production in response to exposure to AI-2-containing *C. difficile* supernatant. However, they could not demonstrate a concomitant increase in the level of the toxin. Their data do suggest, however, that *luxS* plays some role in the regulation of toxin production in *C. difficile.*

During the course of this study, Othani *et al.*, (2009) and Cooksley *et al.*, (2010) reported a role of *agr* in *C. perfringens* and *C. botulinum* respectively. In *C. perfringens* strain 13, *agr* is involved in the regulation of toxin gene expression. In the *C. perfringens agrBD* mutant strain, θ toxin gene expression was abolished and transcription of both α and β toxin genes was decreased (Othani *et al.*, 2009).

The *virR/virS* two component system has been shown to regulate toxin gene expression in *C. perfringens* (Shimizu *et al.*, 1994). However the signal detected by VirS and how the signalling system stimulates toxin expression was unknown. Therefore role of the *virR/virS* in *agrBD*-mediated toxin regulation was investigated in *C. perfringens* strain 13. Addition of wild type culture supernatant to an *agr/virR* mutant strain did not restore toxin production. This indicates that the signal peptide produced and processed by *agrD* and *agrB* respectively, is detected by the *viR/virS* two component system and subsequently transcription of the toxin genes activated (Othani *et al.*, 2009).

In the genome sequence of *C. botulinum* group I strains, there are two *agr* loci both of which contain homologues of the *S. aureus agr* genes, *agrB* and *agrD*. Both sporulation and neurotoxin production were drastically reduced in the *agrD* mutant strains of *C. botulinum* (Cooksley *et al.*, 2010). It is reported that the two *agr* loci in

C. botulinum plays two different roles; the first *agr* loci, *agr1*, regulates sporulation and the second *agr* loci, *agr2*, regulates neurotoxin production. To date, this is the first report of a putative quorum sensing system shown to be involved in the regulation of sporulation in clostridia. However, to date, there are no reports of the role of an *agr*-QS based system in *C. difficile.*

3.1.3 Aim of this study

As a range of genetic tools are now available for studying *C. difficile*, the way has been opened to understand more thoroughly the role of *agr* in *C. difficile* QS-mediated regulation of virulence factors. In many bacterial pathogens quorum sensing plays pivotal role in the coordinate expression of virulence factors, including the production of virulence factors in some clostridia species. In *S. aureus* expression of virulence factors is regulated by the accessory gene regulator (*agr*) locus of the quorum-sensing (QS) signalling pathway. Homologues of the *S. aureus agrBD* locus have been identified in the genome sequence of *C. difficile* 630. The work described in this chapter set out to characterise the putative *agr* system of *C. difficile* 630. The hypothesis that the *agr* system in *C. difficile* controls virulence factor production through a quorum sensing mechanism was tested by knocking out *agrB* and other putative quorum sensing homologues in *C. difficile* 630 and assessing the effects on virulence, paying particular attention to toxin production and sporulation in the first instance.

3.2 Results

3.2.1 Identification of *agrBD* homologues in *C. difficile* 630

An unannotated open reading frame (ORF) which shares homology with the *agrD* gene of *S. aureus*, was discovered during annotation of the *C. difficile* 630 genome sequence (Sebaihia *et al.*, 2006). *C. botulinum*, *C. acetobutylicum* and *C. perfringens* also contain an *agrD* homologue equivalent to the *agrD* gene of *C. difficile*. As with *S. aureus agrD*, all four clostridial *agrD* genes are preceeded by an *agrB* homologue.

In staphylococci in addition to *agrBD*, the *agr* locus also contains two additional genes; *agrC* and *agrA* encoding a histidine sensor kinase and its cognate response regulator. The two-component regulatory system that is made up of AgrC and AgrA is required for sensing the AIP signal and for activating the response regulator. Homologues of the entire *agr* locus are also found in *C. acetobutylicum*, *Listeria monocytogenes*, *Lactobacillus plantarum* (Sebaihia *et al.*, 2007; Autret *et al.*, 2003; Sturme *et al.*, 2005). However, in *C. difficile* 630 there are no apparent *agrCA* homologues located adjacent to the *agrBD* homologues on the chromosome.

To begin characterising the *agr* system of *C. difficile*, we decided to inactivate *agrB* (responsible for the maturation and export of the post-transcriptionally modified autoinducing peptide-AIP) in the first instance. In addition to inactivating *agrB*, we also decided to inactivate putative *agrA* homologues in *C. difficile* 630 in order to find a response regulator for the *agrBD* locus in the *C. difficile* 630 strain.

3.2.2 Identification of an *agrA* homologue in *C. difficile* strain 630

AgrA from *C. acetobutylicum* was used in a BLASTP search against the *C. difficile* 630 genome. The BLASTP results returned a list of eight orphan response regulators; a gene encoding a response regulator which is not flanked by a gene encoding a kinase. The eight orphan response regulators identified from the BLASTP search (CD3255, CD1089, CD2603, CD0611, CD1743, CD0160, CD0339 and CD2601) all belong to the LtyTR family of response regulators. The LytTR response regulator family is named after the LytT and LytR response regulators from *Bacillus subtilis* and *S. aureus*, respectively. This family of response regulators is characterized by the presence of a novel DNA-binding domain, which shows little similarity to the helix-turn-helix or winged-helix domains that are more prevalent in other response regulators (Nikolskaya and Galperin, 2002). Members of the LytTR response regulator family include AgrA , the master regulator of the *agr* system in *S. aureus* (Morfeldt *et al.*, 1996) and VirR, which controls the synthesis of several toxins in *C. perfringens* (Shimizu *et al.*, 1994).

From the eight orphan response regulators, two (CD3255 and CD1089) had already been investigated by O'Connor and colleagues (2006). Microarray analysis indicated that CD1089 only reduced *agrB* expression by two-fold. We reasoned that if CD1089 was the master response regulator of the *agr* system, *agrB* expression levels would be reduced more substantially in CD1089, to a level of expression similar to that observed by *S. aureus agr* mutants (Abdelnour *et al*, 1993; Gillaspy *et al.*, 1995). Consequently we investigated the remaining six orphan response regulator proteins, identified by the BLASTP search, as part of this work. The four orphan response regulators CD0160, CD0611, CD1743, and CD2603 were chosen to be

investigated in the first instance, as they showed the highest percentage of identity/similarity to AgrA from *C. acetobutylicum* (excluding CD3255 and CD1089).

Shortly after the study began, it was reported that CD2603, named CdtR, positively regulates binary toxin production in *C. difficile* (Carter *et al.*, 2007). Thus, it was decided to omit CD2603 from the list of orphan response regulators chosen to be investigated in this study.

3.2.3 Construction of mutant strains

To investigate the role of *agrB* in *C. difficile* and in order to find a response regulator for the *agrBD* locus in *C. difficile*, the ClosTron system (Heap, *et al.*, 2007; Heap, *et al.*, 2010) was used to create independent insertional mutants of *C. difficile* 630Δ*erm*. The *agrB*, CD0160, CD0611 and CD1743 genes were inactivated, yielding strains *C. difficile* 630Δ*erm agrB*::CT*ermB*, *C. difficile* 630Δ*erm* CD0160::CT*ermB*, *C. difficile* 630Δ*erm* CD0611::CT*ermB* and *C. difficile* 630Δ*erm* CD1743::CT*ermB*, respectively. The group II intron from the ClosTron system was retargeted using a computer algorithm to insert into *agrB*, CD0160, CD0611 and CD1743 in a sense orientation at position 316 bp, 246 bp, 162 bp and 390 bp, respectively, from the start of the ORF.

Verification of correct ClosTron insertion was performed using two independent PCR screens. The gene specific and the intron specific primer revealed insertion of

the RAM into the target gene, and the gene specific primers flanking the insertion site revealed an increase in 1.9 kb in size for the mutants compared to the wildtype (Figure 3.1).

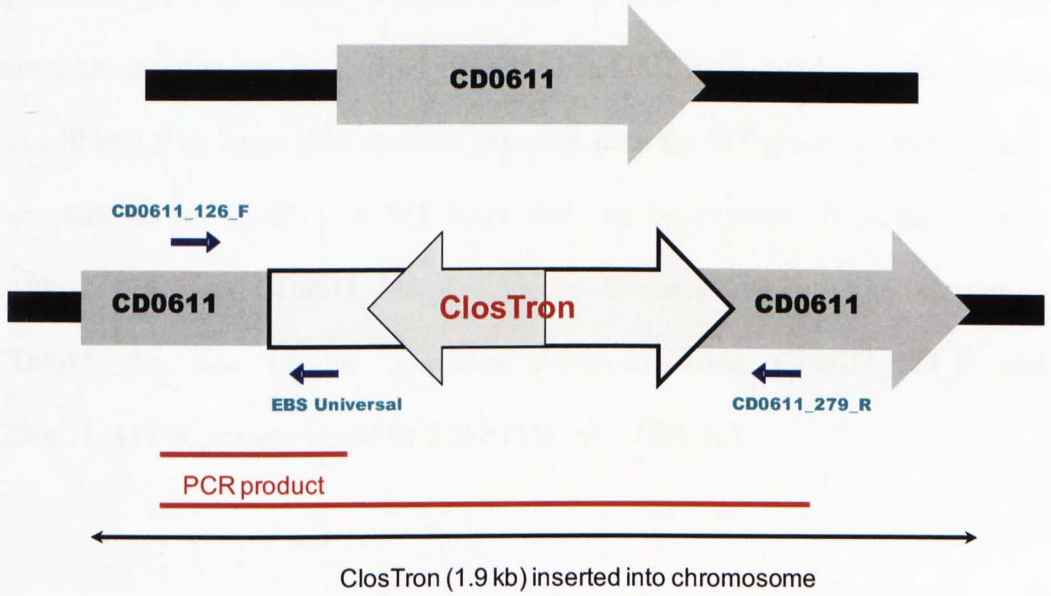


Figure 3.1 Schematic representation of the chromosomal location of the binding sites of the primers used screen putative CD0611 mutants. To confirm correct ClosTron insertion, putative mutant strains were screened by PCR using gene specific_F/R primer (depending on the orientation of the intron insertion) and intron specific primer (EBS Universal), and gene specific primers flanking the insertion site. When the intron is inserted into the gene of interest in a sense orientation a target_F primer is used along with EBS universal to screen putative mutants. Target_F primer binds upstream of the intron insertion site whereas EBS Universal binds within the intron. An amplicon will only be produced from these primers if the intron has inserted into the gene of interest. Putative mutants screened with gene specific primers flanking the insertion site produced an amplicon with a 1.9 kb increase in size compared to the wildtype; indicating intron insertion into the gene.

An example of the screening procedure used is illustrated in Figure 3.2. The example used is for the inactivation of the CD0611 gene in *C. difficile* 630 Δerm. CD0611 putative mutants were screened by PCR using the EBS Universal and

CD0611_124_F primers. Only if the intron has inserted into CD0611, will amplification from these primers occur; a 284 bp band is expected to be seen on the gel. As shown in Figure 3.2, the bands present in Lanes 1-5 indicate that the intron has inserted into CD0611. A second PCR used to screen putative mutants uses gene specific target_F and tagert_R primers with the appropriate PCR controls. If the intron has inserted into the desired gene, the band that is expected to be seen on the gel will be 1.9 kb larger than the band expected from the WT genomic DNA control. For example for CD0611, a WT band that can be expected from the primers CD0611_124_F and CD0611_449_R is 358 bp. Whereas with an intron insertion in CD0611 the size of the amplicon produced using CD0611_124_F and CD0611_449_R primers would be 2.2kb (358 bp + 1900 bp).

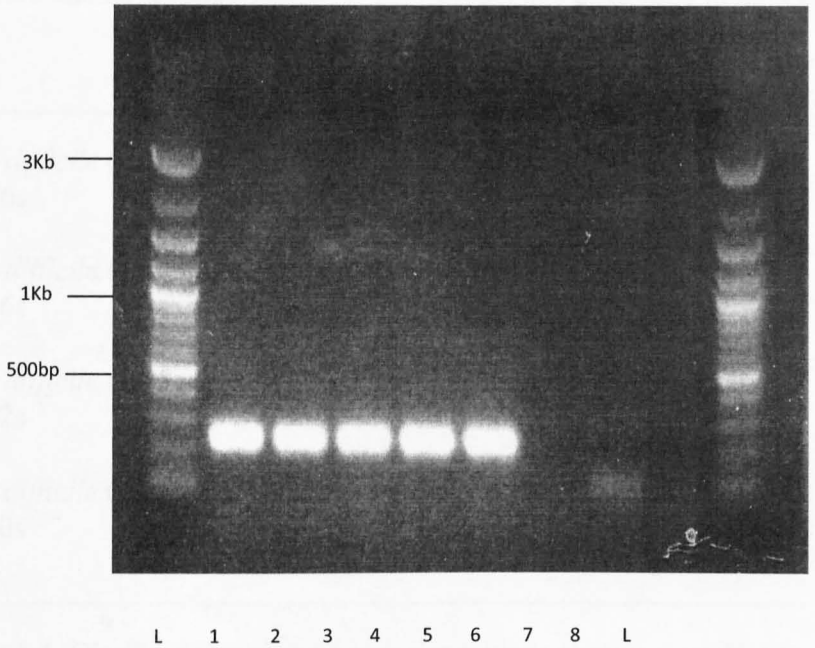


Figure 3.2 An example of PCR screening for putative *C. difficile* 630Δ*erm* CD0611 ClosTron insertion mutants. PCR was used initially to screen for intron insertion using intron-specific EBS Universal primer and the gene-specific primer CD0611_F. Lane L =2-log ladder, 1=KO genomic DNA clone 1, 2 =KO genomic DNA clone 2, 3=KO genomic DNA clone 3, 4 =KO genomic DNA clone 4, 5=KO genomic DNA clone 5, 6=WT genomic DNA control, 7=pMTL007C-E2 plasmid DNA control, 8=water control. The bands (~300bp) shown in Lanes 1-5 indicates that the intron has inserted into CD0611.

One erythromycin-resistant clone each of *C. difficile* 630Δ*erm agrB*::CT*ermB, C. difficile* 630Δ*erm* CD0160::CT*ermB, C. difficile* 630Δ*erm* CD0611::CT*ermB* and *C. difficile* 630Δ*erm* CD1743::CT*ermB* were picked for sequencing at the intron insertion site and the data confirmed the expected sequence (Table 3.1 shows intron insertion sites and the frequencies of the desired mutants obtained).

Strain and target site	Frequency of desired mutant among clones screened	
	%	No. positive/no. screened
C. difficile 630Δ*erm agrB* 316s	80	8/10
C. difficile 630Δ*erm* CD0160 246s	60	3/5
C. difficile 630Δ*erm* CD0611 162s	100	5/5
C. difficile 630Δ*erm* CD1743 390s	100	5/5

Table 3.1. ClosTron insertion frequencies with erythromycin selection. Introns were inserted after the indicated number of bases from the start of the open reading frame in either the sense (s) orientation. Erythromycin-resistant (630Δ*erm*) clones were picked at random and screened by PCR to amplify the intron-exon junction. One clone of each desired mutant was selected, and the intron insertion site verified by sequencing.

An additional experiment that can be performed to further confirm the intron insertion mutants is a southern blot. A southern blot will reveal that a single insertion has been made in the mutant strain following hybridisation of a labelled probe complimentary to the clostron insertion. However, due to time constraints it was not possible to perform southern blots on the insertion mutant strains.

3.2.4 Phenotypic characterisation of mutant strains

C. difficile 630Δ*erm agrB*::CT*ermB*, *C. difficile* 630Δ*erm* CD0160::CT*ermB*, *C. difficile* 630Δ*erm* CD0611::CT*ermB* and *C. difficile* 630Δ*erm* CD1743::CT*ermB*

were phenotypically characterised. To ensure that any observed phenotypes of the *C. difficile* 630Δ*erm* mutants are not a result from growth deficiencies, the change in OD_{600} was used to monitor growth over 24 hours in BHIS broth (Figure 3.3). The growth of all mutants was found to be indistinguishable from the growth of the parental strain, which suggests that there were no obvious growth defects following inactivation of *agrB*, CD0160, CD0611 and CD1743 respectively, in *C. difficile* 630Δ*erm*.

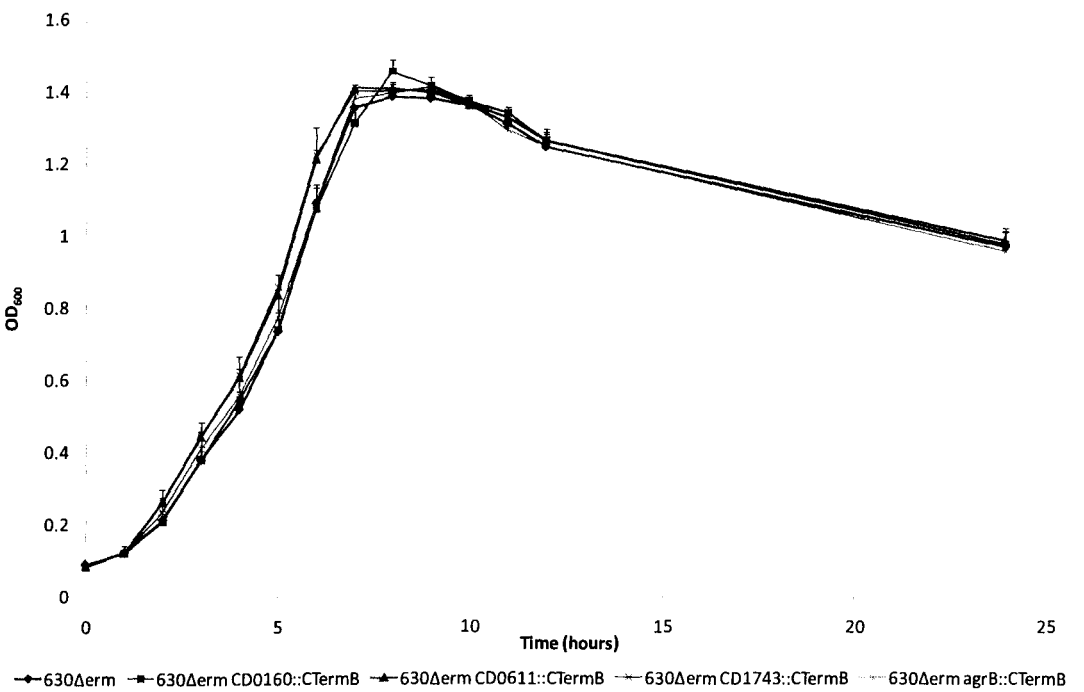


Figure 3.3 The growth of *C. difficile* 630Δ*erm agrB*, CD0160, CD0611 and CD1743 mutant strains in BHIS broth, as indicated by the change in OD_{600}, during a 24 hour period. The symbols represent the averages of three independent experiments, and error bars indicate standard errors of the means.

3.2.4.1 Sporulation in the *C. difficile* 630Δ*erm* mutant strains

Following inactivation of *agrB*, CD0160, CD0611 and CD1743 respectively, in *C. difficile* 630Δ*erm*, these mutant strains were tested to determine their ability to form heat-resistant cfu after a 5-day period on BHIS agar supplemented with the bile salt taurocholate. Total cfu/ml (spores and vegetative cells) and heat-resistant cfu/ml (spores) were enumerated by serial dilution and plating onto BHIS agar supplemented with 0.1% of the bile salt taurocholate before and after heat treatment for 30 mins at 60°C, respectively as described in Chapter 2. After 5 days there was no effect observed on sporulation in the *C. difficile* 630Δ*erm agrB* and the three putative response regulators mutants (Figure 3.4).

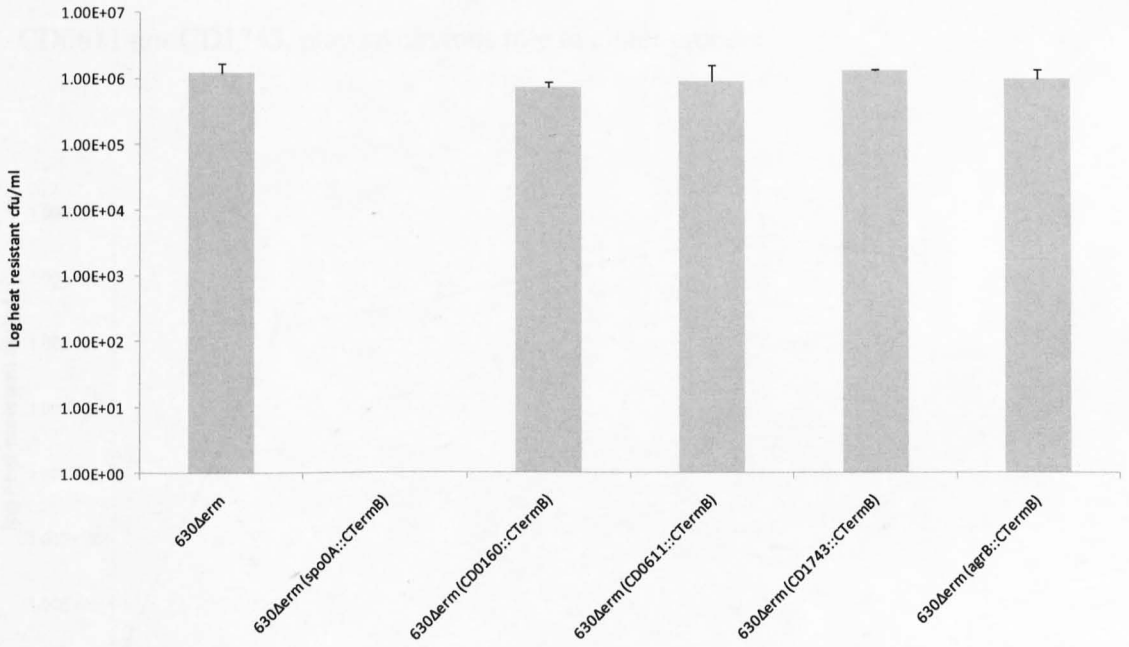


Figure 3.4. Formation of heat resistant cfu of *C. difficile* 630Δ*erm*, *C. difficile* 630Δ*erm spo0A*::CT*ermB*, *C. difficile* 630Δ*erm* CD0160::CT*ermB*, *C. difficile* 630Δ*erm* CD0611::CT*ermB*, *C. difficile* 630Δ*erm* CD1743:CT*ermB* and *C. difficile* 630Δ*erm agrB*::CT*ermB agrB* strains after five days. The symbols represent the averages of three independent experiments, and error bars indicate standard errors of the means. The detection limit for colony counts was 50 cfu/ml.

Therefore to test whether the onset of spore formation is delayed in these mutants and to determine if any effect on sporulation occurs earlier than 5 days, mutant strains were tested to determine their ability to form heat-resistant CFU over a 5-day period on BHIS agar supplemented with the bile salt taurocholate (Figure 3.5 and 3.6).

Inactivation of *agrB*, CD0160, CD0611 and CD1743 appeared to have no effect on sporulation in *C. difficile* 630Δ*erm*. The mutant strains were able to form heat-resistant CFU over the 5-day period at the same rate as the parental strain. This suggests that *agrB* is not required for sporulation and/or taurocholate-induced germination in *C. difficile* 630Δ*erm*. In addition it appears that neither CD0160, CD0611 nor CD1743, play an obvious role in either process.

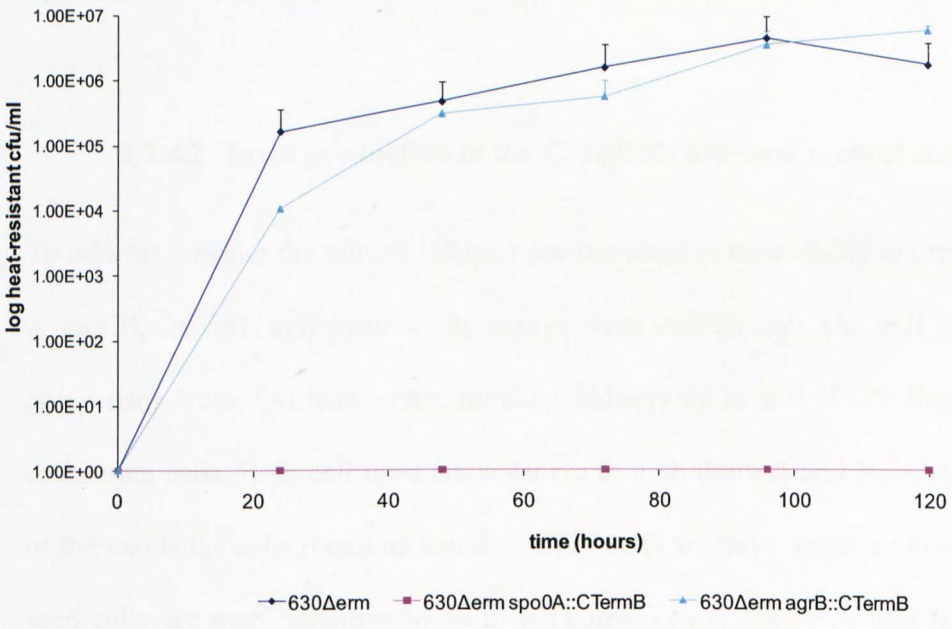


Figure 3.5 The development of heat-resistant cfu of *C. difficile* 630Δ*erm agrB* mutant strain over five days. The symbols represent the averages of three independent experiments, and error bars indicate standard errors of the means. The detection limit for colony counts was 50 cfu/ml.

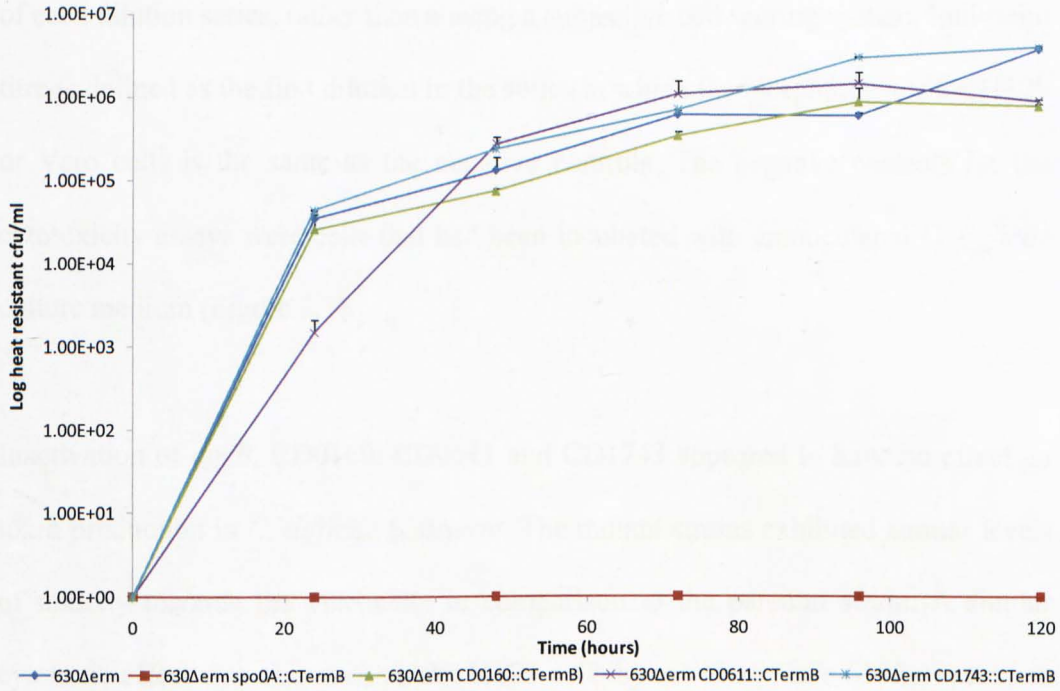


Figure 3.6 The development of heat-resistant CFU of *C. difficile* 630Δ*erm* CD0160, CD0611 and CD1743 mutant strains over five days. The symbols represent the averages of three independent experiments, and error bars indicate standard errors of the means. The detection limit for colony counts was 50 CFU/ml.

3.2.4.2 Toxin production in the *C. difficile* 630Δ*erm* mutant strains

To address whether the mutant strains were impaired in their ability to produce toxin A and B, *in vitro* cell cytotoxicity assays were carried out. The cell cytotoxicity assay uses Vero (African green monkey kidney) cells and HT29 (human colon carcinoma cells. Both cell lines are sensitive to both toxin A and B; in the presence of the toxins the cells round up and die. HT29 cells are more sensitive to toxin A and vero cells are more sensitive to toxin B (Torres *et al.*, 1992). A four-fold dilution series of *C. difficile* culture supernatants were made and added to each cell line separately (i.e. HT29 and Vero cells). After an incubation period of 24 hours,

cytotoxicity was determined as objectively as possible by finding the end point titre of each dilution series, rather than a using a subjective cell scoring system. End-point titre is defined as the first dilution in the series in which the morphology of the HT29 or Vero cells is the same as the negative controls. The negative controls for the cytotoxicity assays were cells that had been incubated with uninoculated *C. difficile* culture medium (Figure 3.7).

Inactivation of *agrB*, CD0160, CD0611 and CD1743 appeared to have no effect on toxin production in *C. difficile* 630Δ*erm*. The mutant strains exhibited similar levels of toxicity towards the vero cells in comparison to the parental strain. A similar cytotoxic effect was also seen on the HT29 cells by the *C. difficile* 630Δ*erm* mutant strains (Figure 3.8a, b and Figure 3.9a, b). This suggests that both the toxins were present in abundance in the supernatant that was collected from the strains prior to being added to the cell monolayer indicating that there toxin gene expression had not been impaired.

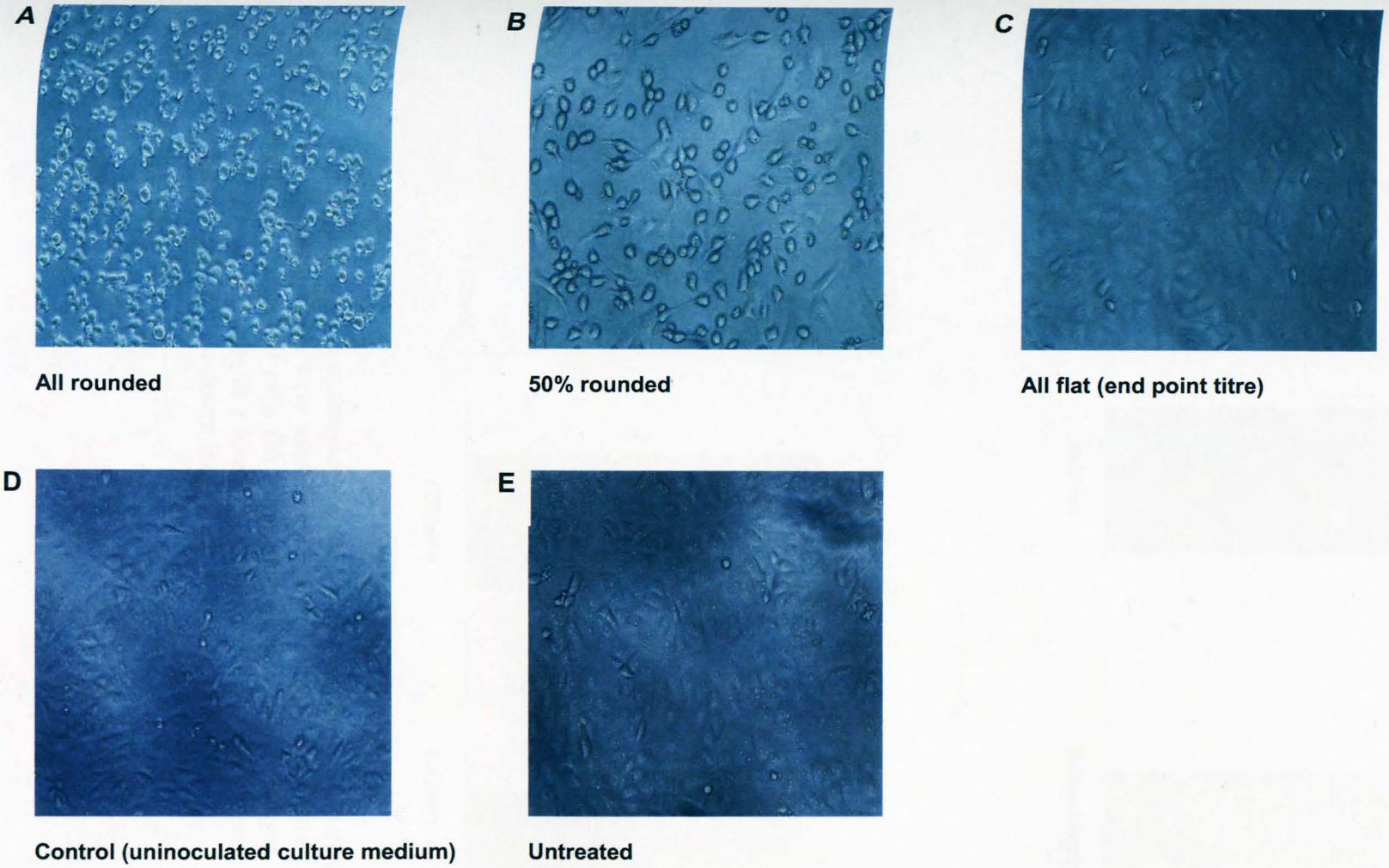


Figure 3.7 The cytotoxic effect of *C. difficile* 630Δ*erm* on Vero cells. Culture supernatant of *C. difficile* 630Δ*erm* was added to a flat monolayer of Vero cells in fourfold dilution series. After a 24 hour incubation, toxin end point titres were determined. **A.** Vero cells inoculated with *C. difficile* 630Δ*erm* supernatant at dilution 1:4 **B.** Vero cells inoculated with *C. difficile* 630Δ*erm* supernatant at dilution 1:4096 **C.** Vero cells inoculated with *C. difficile* 630Δ*erm* supernatant at dilution 1:4194304 **D.** Vero cells incubated with uninoculated culture medium **E.** Untreated Vero cells.

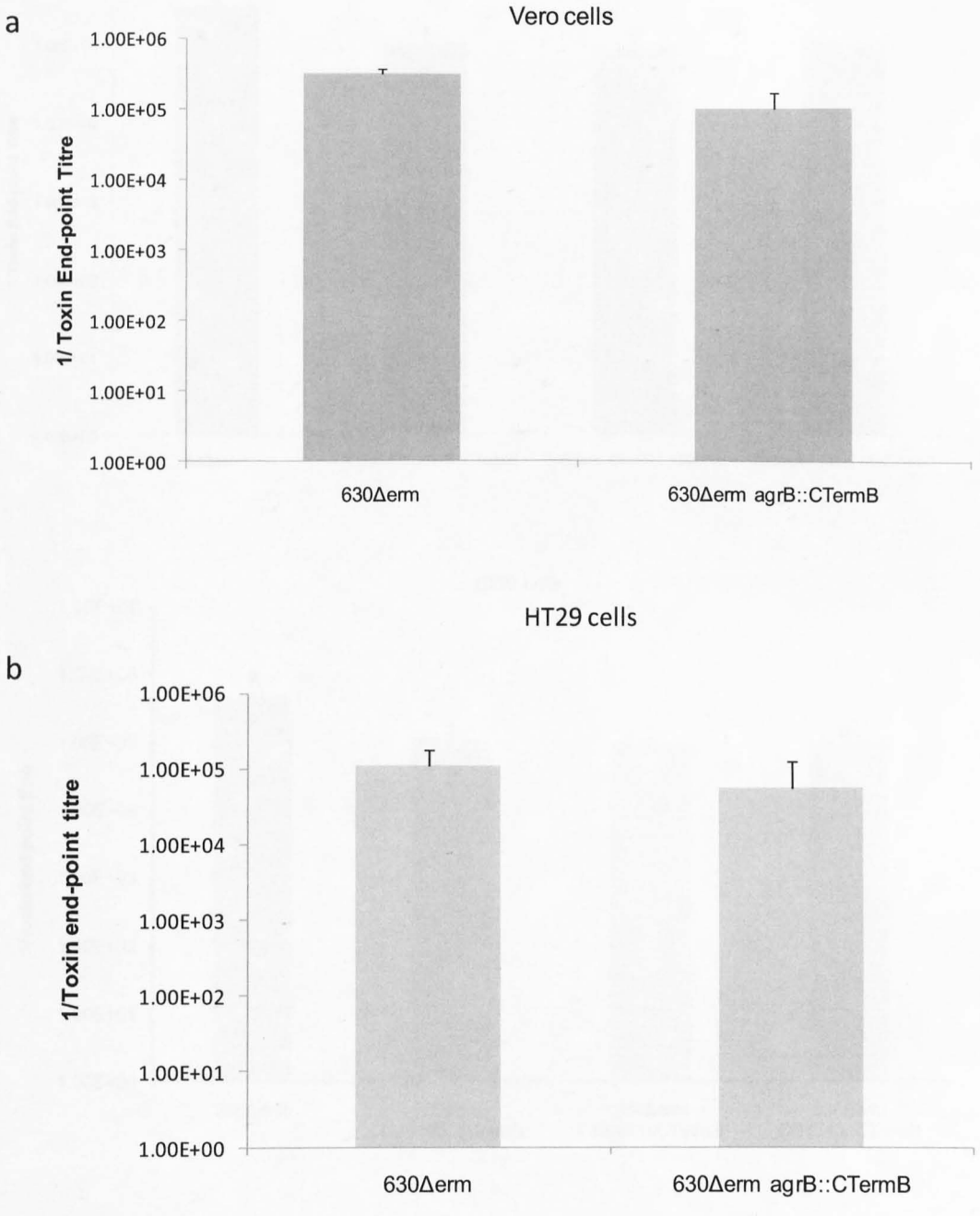


Figure 3.8 *In vitro* cytotoxicity assays. Supernatants of the parental strain and the *agrB* mutant strain were used in cell culture assays to measure cytotoxicity. Vero cells (a) and HT29 cells (b) were cultured to a flat monolayer before adding *C. difficile* supernatants in a fourfold dilution series. After a 24 hour incubation, toxin end point titres were determined

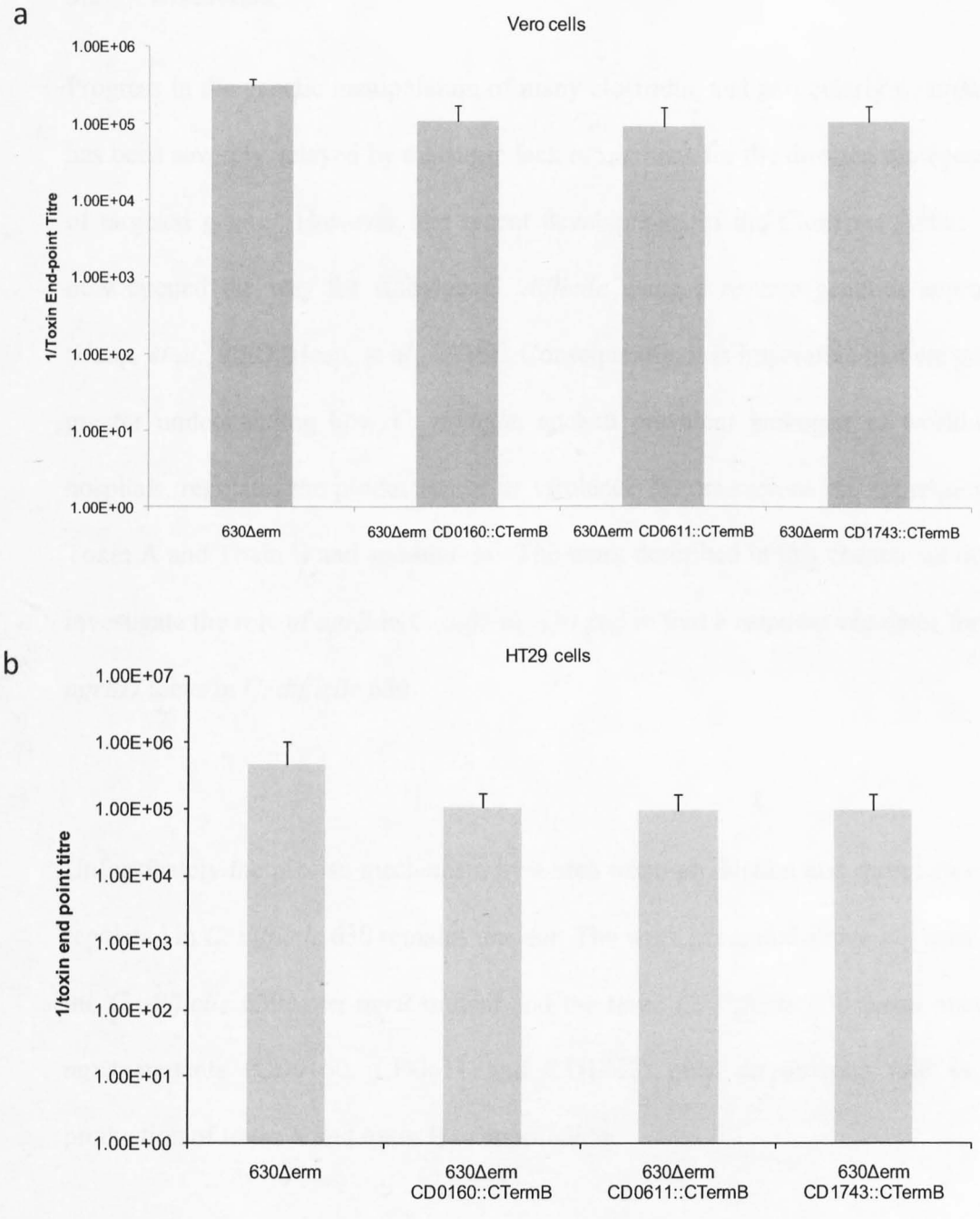


Figure 3.9 Cytotoxicity profiles of the *C. difficile* 630Δ*erm* CD0160, CD0611 and CD1743 mutant strains. See figure legend above for details.

3.2 Discussion

Progress in the genetic manipulation of many clostridia, and particularly *C. difficile*, has been severely delayed by a historic lack of methods for the directed mutagenesis of targeted genes. However, the recent development of the ClosTron system has now opened the way for studying *C. difficile* using a reverse genetics approach (Heap, *et al.*, 2007; Heap, *et al.*, 2010). Consequently, it is imperative that we gain a greater understanding how *C. difficile*, such a prevalent pathogen in worldwide hospitals, regulates the production of its virulence factors such as the expression of Toxin A and Toxin B and sporulation. The work described in this chapter set out to investigate the role of *agrB* in *C. difficile* 630 and to find a response regulator for the *agrBD* locus in *C. difficile* 630.

Unfortunately the precise mechanism by which toxin production and sporulation are regulated in *C. difficile* 630 remains unclear. The work presented above suggests that the *C. difficile* 630Δ*erm agrB* mutant and the three *C. difficile* 630 Δ*erm* putative *agrA* mutants (CD0160, CD0611 and CD1743) play no obvious role in the production of toxin A and toxin B or sporulation.

The mutants were tested in their ability to form heat-resistant cfu after a 5-day period on BHIS agar supplemented with the bile salt taurocholate. After 5 days there was no effect observed on sporulation in the *C. difficile* 630Δ*erm agrB* and the three putative response regulators mutants. However, sporulation in *agr* mutants made in both *C. acetobutylicum* and *C. botulinum* is substantially reduced (Minton lab,

unpublished data; Cooksley *et al.*, 2010). Subsequently we decided to investigate whether the onset of spore formation is delayed in these mutants and to determine if any effect on sporulation occurs earlier than 5 days. The mutant strains were tested to determine their ability to form heat-resistant CFU over a 5-day period on BHIS agar supplemented with the bile salt taurocholate. Our data indicates that the mutant strains were able to form heat-resistant CFU over the 5-day period at the same rate as the parental strain. This suggests that *agrB* is not required for sporulation and/or taurocholate-induced germination in *C. difficile* 630Δ*erm*. In addition it appears that neither CD0160, CD0611 nor CD1743, play an obvious role in either process.

To address whether the mutant strains were impaired in their ability to produce toxin A and B, *in vitro* cell cytotoxicity assays were carried out using Vero cells and HT29 cells. The mutant strains had no effect on toxin production in *C. difficile* 630Δ*erm* as there was no reduction in toxicity towards both the vero and HT29 cells in comparison to the parental strain. This suggests that both the toxins were present in the supernatant that was collected from the strains prior to inoculation onto the cell monolayer, indicating that toxin gene expression had not been impaired. It is unclear using the cell cytotoxicity assays whether the amount of either one of the toxins is more/less prevalent in the mutant strains as both cell lines are sensitive to both toxin A and B. Even though it has been reported that HT29 cells are more sensitive to toxin A and vero cells are more sensitive to toxin B (Torres *et al.*, 1992) this sensitivity was not observed during the cell assays with these mutant strains as a similar degree of toxicity was shown by the mutants strains towards both the vero and HT29 cells. To confirm whether expression of either toxin was impaired in any

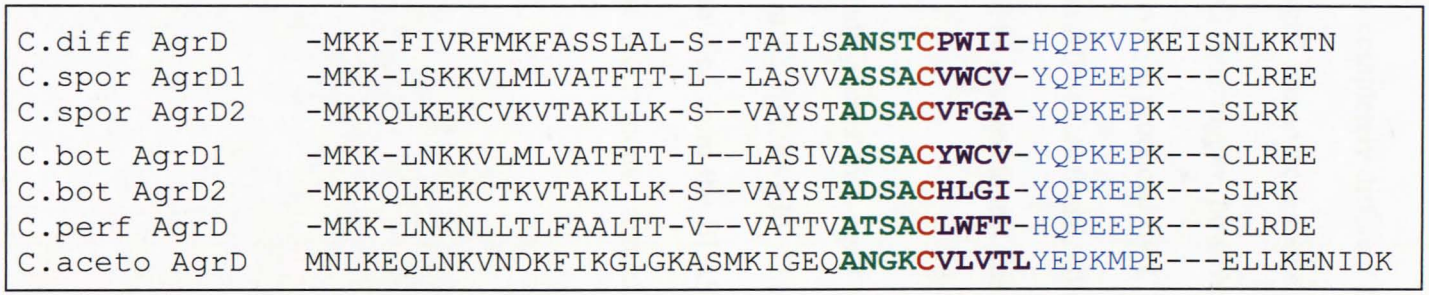
way by inactivation of *agrB* and the response regulator mutants, a toxin neutralisation assay could be performed. The culture supernatant of the mutant strains would be incubated with toxin A or toxin B specific antibodies before being inoculated on the vero or HT29 cells. The culture supernatants are diluted so that only toxin A is detected using the HT29 cells and only toxin B is detected using the vero cells. This assay would allow you to distinguish whether expression of toxin A and toxin B had been impaired by inactivation of *agrB* and the response regulators. If a positive result was observed using the neutralisation assay, quantitative reverse transcriptase PCR analysis could be used to confirm whether toxin expression had been affected in any of the mutant strains.

Further checks on the stability of the insertion mutants could be performed to check that the ClosTron is still located in the same place after growth curves, sporulation assays and toxin assays. This could be done by using the PCR screening method that was initially used to confirm the location of the ClosTron as well as performing a Southern Blot to confirm there is still a single ClosTron insertion in the mutant strains after the functional assays.

Even though sporulation in *agr* mutants made in both *C. acetobutylicum* and *C. botulinum* and neurotoxin production in *agr* mutant strains of *C. botulinum* is substantially reduced (Minton lab, unpublished data; Cooksley *et al.*, 2010), our data shows that *C. difficile* 630 does not regulate sporulation and toxin production in a similar manner to other members of the clostridia species. It remains unclear what

role the *agr* system has in *C. difficile* 630 or even if *agr* has a functional role in regulation of virulence in *C. difficile* 630. With no apparent two-component system (to detect the auto inducing peptide and induce gene expression) present within the vicinity of the *agrBD* locus in genome sequence of *C. difficile* 630, it may be possible that *agr* does not have a functional role in regulation in *C. difficile* 630. This could be determined by investigating whether *agrD* produces an autoinducing peptide (AIP) by trying to isolate it from culture supernatant using mass spectrometry.

A



B

Figure 3.10 AgrD protein alignments. A Alignment of putative clostridial AgrD proteins with those of the four *S. aureus* AIP groups. Bold residues indicate the sequence of the mature AIP. B Alignment of putative AgrD proteins from five clostridial species. C. spor, *C. sporogenes*; C. bot, *C. botulinum*; C. perf, *C. perfringens*; C. diff, *C. difficile*; S. a, *S. aureus*; C. aceto, *C. acetobutylicum*. Residues coloured red are completely conserved. Residues coloured blue are partially conserved. Bold residues indicate the possible AIP sequence, with purple residues forming the thiolactone ring and green residues forming the tail. The length of the tail may, however, be shorter than indicated.

The amino acid sequences of the AIP's in *S. aureus* vary greatly, however a central cysteine is conserved in all of them. The cysteine is important for the formation of a thiolactone ring with the C-terminal amino acid (Muir, 2003). The amino acid sequence of *C. difficile* AgrD is completely different from the amino acid sequence of other AIP's including the AgrD amino acid sequences of *C. acetobutylicum*, *C. botulinum*, *C. sporogenes* and *C. perfringens*, however the same central cysteine is conserved (Figure 3.10). If an AIP is produced by *C. difficile* it would be of great interest to determine whether the AIP is functional, although it would be beneficial if there was a phenotype that could be assayed to test this.

The work presented in this study shows that *agrB* plays no obvious role in the regulation of virulence factors in *C. difficile* 630. The data obtained from the three orphan response regulators, identified from the BLASTP search, suggest that these response regulators may not be the response regulator of the *agrBD* locus in *C. difficile* 630.

Chapter 4

Identification and analysis of a putative quorum sensing system in *Clostridium difficile* R20291

4.1 Introduction

4.1.1 Emergence of 'hypervirulent' strains of *C. difficile*

The recent increase in the number CDAD cases has been linked to the rapid emergence of *C. difficile* strains belonging to restriction endonuclease type BI, North American pulsed-field type 1 (NAP1) and PCR-ribotype 027 (BI/NAP1/027) (Pépin, *et al.*, 2004; Kuijper, *et al.*, 2007). In the UK, these highly virulent and epidemic strains of *C. difficile* were rarely isolated from patients suffering from CDAD prior to 2005, but have recently become highly represented among such clinical isolates. PCR ribotype 027 strains cause >33% of the 50,000 cases of CDAD reported in the UK (Brazier *et al.*, 2008). The BI/NAP1/027 type is now the most commonly isolated *C. difficile* type in England (Figure 4.1). Across mainland Europe, cases of CDAD associated with the BI/NAP1/027 type have been reported in 16 countries (Kuijper, *et al.*, 2008) In North America, these strains have largely been associated with the over-use of quinolone antibiotics and are responsible for an increase in the incidence of nosocomial CDAD, more severe disease, higher relapse rates, increased mortality, and greater resistance to fluoroquinolone antibiotics (Kuijper, *et al.*, 2006). Furthermore, the BI/NAP1/027 type has recently been linked with the emergence of community-associated CDAD, no longer limiting *C. difficile* to the healthcare environment (Rupnik, *et al.*, 2009).

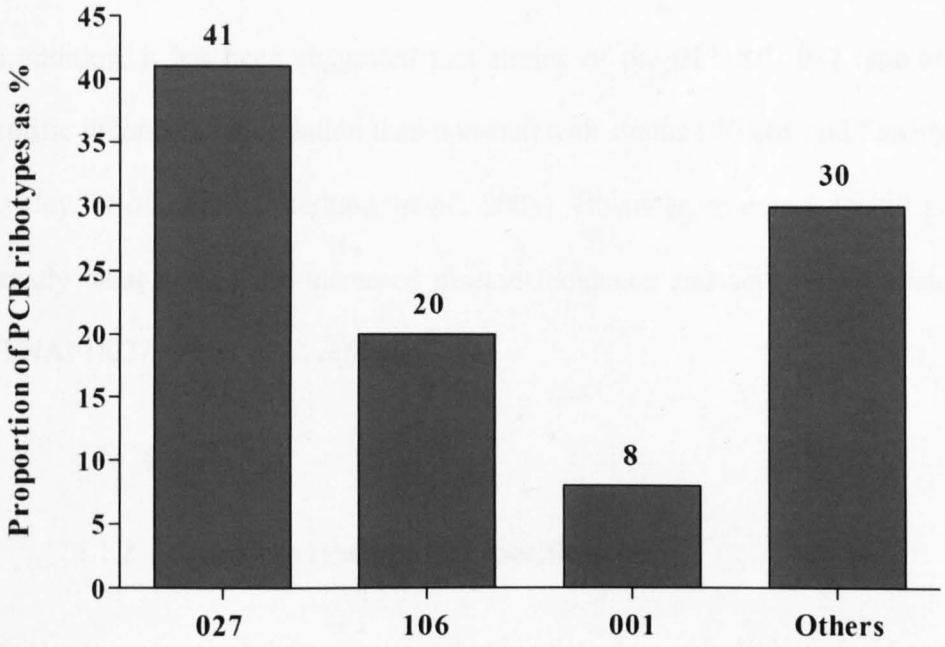


Figure 4.1 Distribution of *C. difficile* PCR ribotypes in England in 2007-8, adapted from Brazier *et al.* (2008). The data represent the national distribution of PCR ribotypes identified from a study of 677 individual isolates. The incidence of cases due to type 027 strains increased by 15.4% compared to a study in 2005, and the percentage of type 001 and type 106 cases fell by 17.3% and 6%, respectively (Brazier, *et al.*, 2008).

There are a number of studies showing that patients infected with the PCR-ribotype 027 strains have more severe diarrhoea, a higher mortality rate and a higher level of reoccurrence (Loo *et al.*, 2008; Goorhuis *et al.*, 2007; Redelings *et al.*, 2007; Pépin *et al.*, 2004). This has been exemplified by the PCR ribotype strain R20291 which was responsible for the outbreak at the Stoke Mandeville hospital in the UK in 2004/2005 (Stabler *et al.*, 2009). Unsurprisingly, there is a widespread interest in the field to understand why strains such as those of the BI/NAP1/027 type can cause a more severe disease than other types, and why incidence and relapse rates appear higher where the BI/NAP1/027 type is isolated. Studies have shown that a number of BI/NAP1/027 strains produce higher levels of toxin in the laboratory, although the

mechanisms for this increased toxin production remain unclear (Warny, *et al.*, 2005). In addition, it has been suggested that strains of the BI/NAP1/027 type are more prolific in terms of sporulation than non-outbreak strains (Wilcox and Fawley, 2000; Fawley, *et al.*, 2007; Akerlund, *et al.*, 2008). However, to-date it is still not clear exactly what causes the increased disease incidence and severity associated with BI/NAP1/027 strains of *C. difficile.*

4.1.2 *C. difficile* ribotype 027 specific genes

Studies to understand the recent emergence of the hypervirulent strain of *C. difficile* is gathering momentum. Stabler *et al.* (2009) compared a 'historic' non-epidemic 027 *C. difficile* (CD196) strain, a recent epidemic and hypervirulent 027 (R20291) strain and a PCR-ribotype 012 strain (630). Their findings showed that even though the genomes are highly conserved, the 027 genomes have 234 additional genes compared to 630. They found that the epidemic 027 strain has five unique genetic regions, which are absent from both the non-epidemic 027 and strain 630. These unique regions include a two component regulatory system and transcriptional regulators.

4.1.3 *C. difficile* ribotype 027 specific regulatory genes

A large proportion of the 027-specific genes are comprised of regulatory genes. The work of Stabler *et al.* (2009) described that in 027 strains there are eight two-component regulators and fifteen other transcriptional regulators. One of the most interesting findings in this study is that in PCR ribotype 027 strains there is an additional copy of the complete *agr* locus, termed *agr*2. The additional *agr*2 locus contains homologues of *agrA*, *agrC*, *agrD* and *agrB*. The *agr1* locus found in *C. difficile* 630 is also present in the ribotype 027 strains. Analysis of the R20291 genome sequence showed that downstream of the *agr2* locus three more 027-specific CDS; encoding two putative membrane proteins and an ABC transporter ATP-binding protein.

The occurrence of the *agr2* locus in different *C. difficile* strains was investigated as part of a comparative study of 94 clinical strains, the most common PCR-ribotypes isolated in mainland Europe and the UK, by array comparative genomic hybridisation (Marsden *et al.*, 2010). The study revealed that the *agr2* locus is found in the genomes of 82 of the 94 (86%) strains tested, including two of four non-toxinogenic strains tested.

4.1.4 Aims of this study

There are a number of gene differences between ribotype 027 strains, in particular the presence of an additional complete *agr2* locus. These genes difference may contribute to understanding why modern PCR ribotype 027 strains are associated with a higher case-fatality ratio and persistence. One of the first characteristic of BI/NAP1/027 strains to be associated with hypervirulence was the production of toxins, the major virulence factor of *C. difficile*. As described previously, a number of BI/NAP1/027 strains have been shown to produce higher levels of toxin *in vitro* (Warny, *et al.*, 2005), although the molecular basis of this observation remains unclear. As the PCR ribotype 027 strains carry an extra copy of the complete *agr* locus, we reasoned that toxin production in these strains may be under the regulatory control of the *agr2* locus. The study presented in this chapter sets out to investigate the role of the second *agr* locus in regulation of virulence factors.

4.2 Results

4.2.1 Construction of mutant strains

To investigate the role of *agr* in *C. difficile* R20291, the ClosTron system (Heap, *et al.*, 2007; Heap, *et al.*, 2010) was used to create independent insertional mutants of *C. difficile* R20291 in which the *agrB1, agrB2* and *agrA* genes were inactivated, yielding strains *C. difficile* R20291 *agrB1*::CT*ermB, C. difficile* R20291 *agrB2*::CT*ermB* and *C. difficile* R20291 *agrA*::CT*ermB*, respectively.

The group II intron from the ClosTron system was retargeted using a computer algorithm to insert into *agrB1* and *agrB2* in an antisense orientation at position 362 bp and 313 bp, respectively (from the start of the ORF), and in the sense orientation for *agrA* at position 306 bp.

As described in previous chapters, to confirm correct ClosTron insertion, putative mutant strains were analysed by PCR, and one lincomycin-resistant clone each of *C. difficile* R20291 *agrB1*::CT*ermB, C. difficile* R20291 *agrB2*::CT*ermB* and *C. difficile* R20291 *agrA*::CT*ermB* were picked for sequencing at the intron insertion site and the data confirmed the expected sequence.

4.2.2 Phenotypic characterisation of mutant strains

C. difficile R20291 *agrB1*::CT*ermB, C. difficile* R20291 *agrB2*::CT*ermB* and *C. difficile* R20291 *agrA*::CT*ermB* were phenotypically characterised. To ensure that

any observed phenotypes of the *C. difficile* R20291 *agr* mutants are not a result from growth deficiencies, all *C. difficile* R20291 *agr mutant* strains were first cultivated in BHIS broth for 24 h, and growth was monitored by measuring the change in OD_{600} (Figure 4.2). All strains showed highly similar growth characteristics, which suggests that there were no obvious growth defects following inactivation of *agrB1*, *agrB2* and *agrA* in *C. difficile* R20291. This allows for the exclusion of growth differences when interpreting the subsequent experiments in this chapter.

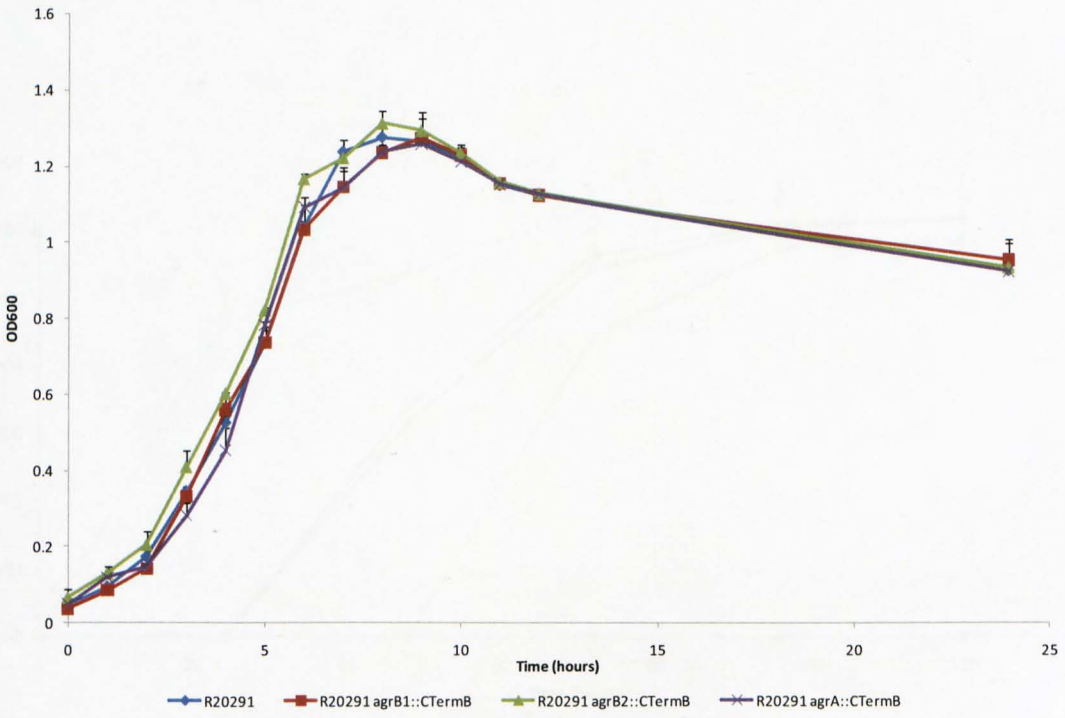


Figure 4.2 Growth of *C. difficile* R20291 *agr* mutants in BHIS broth over five days. Strains were cultivated as described in Chapter Two, and the OD_{600} measured. The symbols represent the averages of three independent experiments, and the error bars represent standard errors of the means.

4.2.2.1 Sporulation in the *C. difficile* R20291 *agr* mutant strains

In order to understand if *agr* is involved in spore formation in *C. difficile* R20291, the development of heat-resistant CFU was measured over 5 days. At 24 h time intervals, samples were heated at 60 °C for 25 min to kill vegetative cells but not spores, and plated onto BHIS agar supplemented with the bile salt taurocholate to recover viable *C. difficile* spores. The observed colony formation appeared to show a degree of variation in the sporulation rates between *C. difficile* R20291 *agr* mutant strains (Figure 4.3)

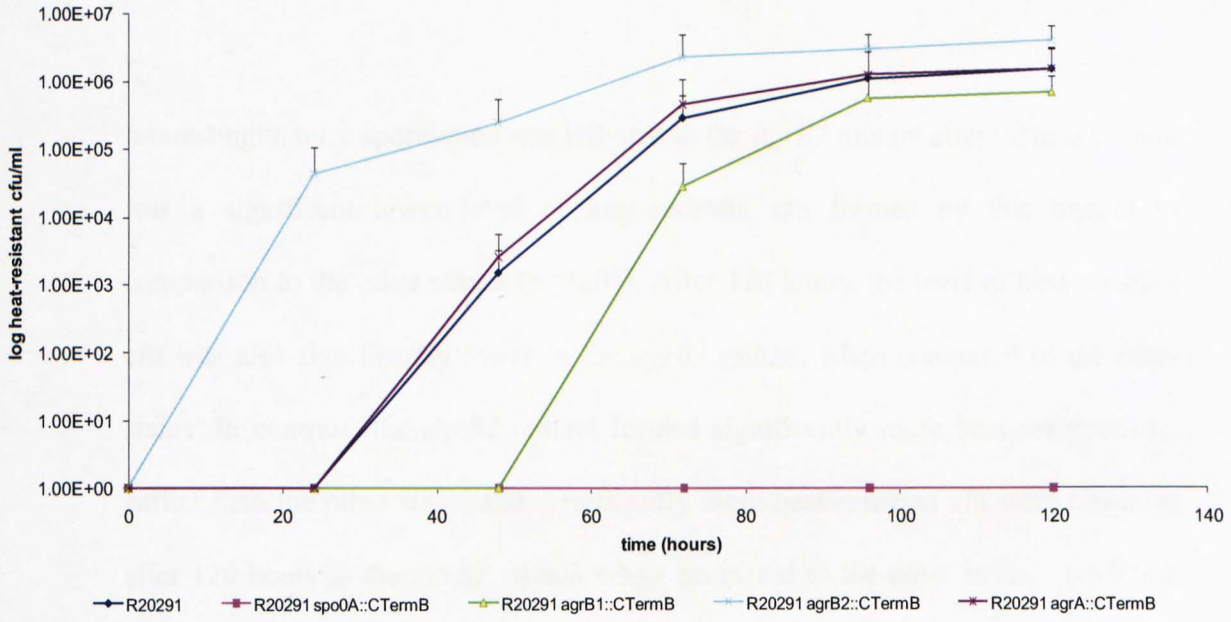


Figure 4.3 Development of heat-resistant CFU of *C. difficile* R20291 *agr* mutants over 5 days on BHIS agar supplemented with 0.1% taurocholate. *C. difficile* strains were cultivated as described in Chapter Two. The symbols indicate the averages of three independent experiments, and the error bars indicate standard errors of the means. The detection limit for the assay was 50 CFU/ml.

There are no obvious differences in the number of heat-resistant cfu or in the rate at which the heat-resistant cfu are formed over 5 days in the *C. difficile* R20291 *agrA* mutant strain in comparison to the parental strain. Surprisingly, the initiation of heat-resistant cfu formation occurs earlier (at 24 hours) in the *C. difficile* R20291 *agrB2* mutant compared to the parental strain and the *C. difficile* R20291 *agrA* mutant. On the other hand, heat-resistant cfu are formed later (at 72 hours) in the *C. difficile* R20291 *agrB1* mutant compared to the parental strain. It would be expected that *agr* mutants of the *agr2* locus (*agrB2* mutant and *agrA* mutant) would behave the same in terms of when they sporulate, however these results show that is not the case here.

Interestingly, once sporulation was initiated in the *agrB1* mutant after 72 hours, there was a significant lower level of heat-resistant cfu formed by this mutant in comparison to the other strains ($p < 0.05$). After 120 hours, the level of heat resistant cfu was also significantly lower in the *agrB1* mutant when compared to the other stains. In contrast, the *agrB2* mutant formed significantly more heat-resistant cfu, earlier than the other stains and significantly more heat-resistant cfu were observed after 120 hours in the *agrB2* mutant when compared to the other strains ($p < 0.05$). This suggests that in a case where a strain initiates sporulation particularly early, the strain also forms a higher total of spores than a strain which initiates sporulation at a later stage.

4.2.2.2 Toxin production in the *C. difficile* R20291 *agr* mutant strains

In order to understand if *agr* is involved in toxin A and B production in *C. difficile* R20291, *in vitro* cell cytotoxicity assays were carried out. Cells were incubated for 24 hours with a fourfold dilution series of *C. difficile* culture supernatants. Cytotoxicity of the *C. difficile* strains towards the vero cells and HT29 were to a similar degree; there was no obvious difference in cytotoxicity between the cell lines. The *agrB1* mutant showed a similar degree in toxicity towards both the vero cells and HT29 as the parental strain. Interestingly, the *agrB2* and *agrA* mutants showed significantly reduced toxicity towards the vero cells and HT29 cells ($p < 0.05$) (Figure 4.4). This indicates that the *agr2* locus in *C. difficile* R20291 may be indirectly involved in toxin A and toxin B production.

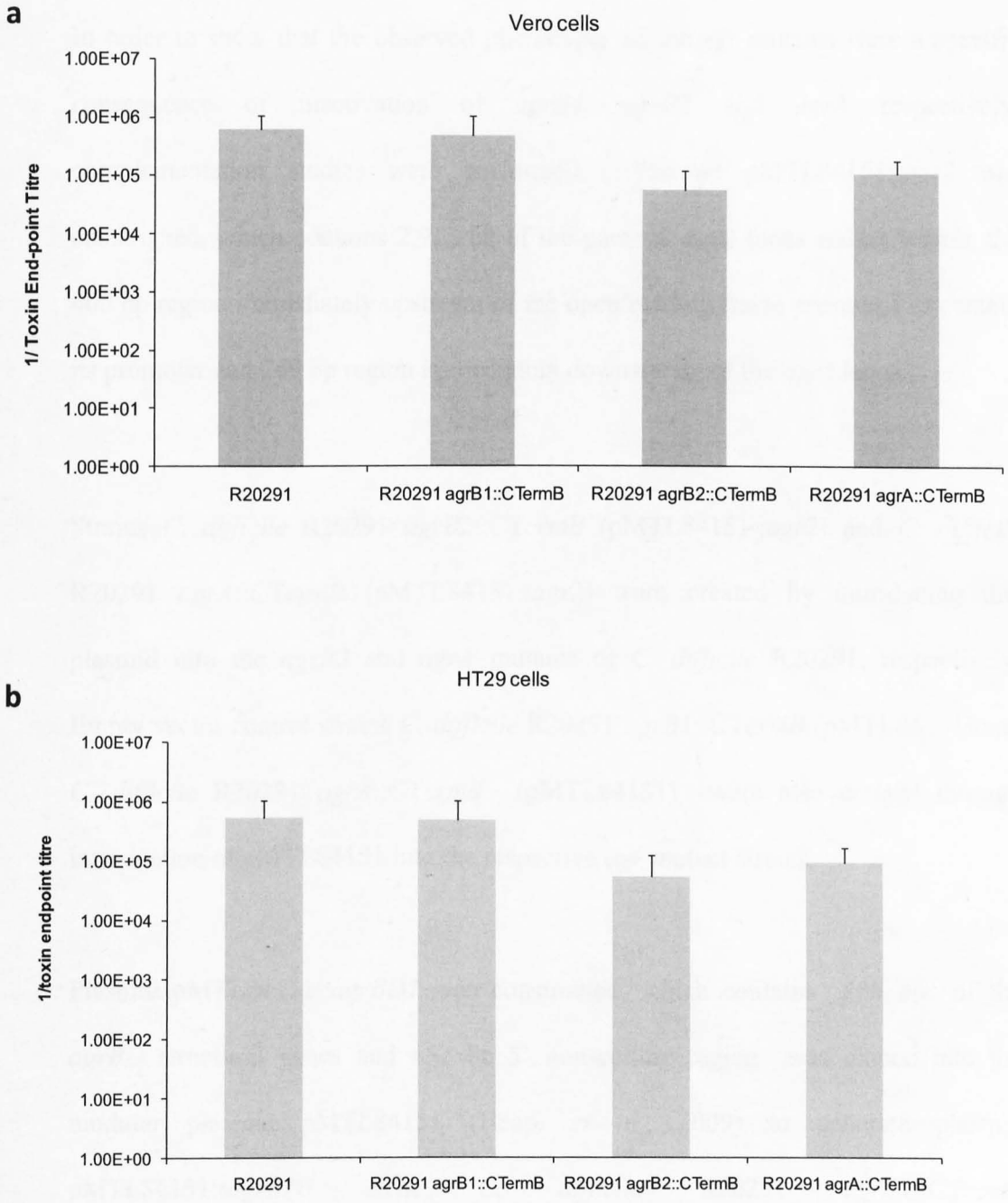


Figure 4.4 *In vitro* cytotoxicity assays. Supernatants of the parental strain and the three *agr* mutant strains were used in cell culture assays to measure cytotoxicity. Vero cells (a) and HT29 cells (b) were cultured to a flat monolayer before adding *C. difficile* supernatants in a fourfold dilution series. After a 24 hour incubation, toxin end point titres were determined.

4.2.3 Complementation of the *agr* mutants with the parental *agr* loci

In order to show that the observed phenotypes of the agr mutants were a specific consequence of inactivation of *agrB1, agrB2* and *agrA* respectively, complementation studies were performed. Plasmid pMTL84151::*agr2* was constructed, which contains 2,915 bp of the parental *agr2* locus coding region, the 400 bp region immediately upstream of the open reading frame presumed to contain its promoter and 269 bp region immediately downstream of the *agr*2 locus.

Strains *C. difficile* R20291 *agrB*2::CT*ermB* (pMTL84151::*agr*2) and *C. difficile* R20291 *agrA*::CT*ermB* (pMTL84151::*agr*2) were created by introducing this plasmid into the *agrB2* and *agrA* mutants of *C. difficile* R20291, respectively. Empty vector control strains *C. difficile* R20291 *agrB*1::CT*ermB* (pMTL84151) and *C. difficile* R20291 *agrA*::CT*ermB* (pMTL84151) were also created through introduction of pMTL84151 into the respective *agr* mutant strains.

Plasmid pMTL84151::*agrBD1* was constructed, which contains 806 bp of the *agrBD* structural genes and 452 bp 5' non-coding region was cloned into the modular plasmid pMTL84151 (Heap, *et al.*, 2009) to generate plasmid pMTL84151::*agrBD1*. The *C. difficile* R20291 *agrB*1::CT*ermB* (pMTL84151::*agr*BD1) strain was created by introducing the plasmid into the mutant strain by conjugation.

Successful plasmid transfer was confirmed by recovery of the plasmid, followed by PCR and/or restriction analysis. In order to control for the thiamphenicol selection

needed to maintain the plasmids in culture, strains *C. difficile* 630Δ*erm* (pMTL84151) and *C. difficile* R20291 (pMTL84151) were used in the assays. The development of heat-resistant CFU was then measured in the same way as previously described, and the data were compared to data obtained previously.

4.2.3.1 Sporulation in the complemented *C. difficile* R20291 *agr* mutant strains

The levels of heat-resistant cfu were fully restored in the *agrB1* mutant strains of *C. difficile* R20291 carrying pMTL84151::*agrBD1* to the levels of the parental strains carrying the control vector pMTL84151 (Figure 4.5). Sporulation in the *agrB1* mutant strain and the *agrB1* mutant strain carrying the empty vector occurred after 72 hours. Whereas the sporulation profile of the *agrB1* mutant carrying pMTL84151::*agrBD1* was the same as the *C. difficile* R20291 parental strain in which sporulation is initiated after 48 hours. The parental strain harbouring the empty vector performed like the plasmid free parental strain, sporulation was initiated after 48 hours in both strains. However, in the case of R20291 there was a slight difference between the level of heat-resistant cfu obtained for the strain with the control plasmid and the level obtained for the plasmid-free parental strain. This difference is presumably as a result of growth under thiamphenicol selection conditions, as the antibiotic selection may affect the sporulation frequency of R20291. The *agrB1* mutant controls containing the empty vector performed like the original mutant, sporulation was initiated after 72 hours. Thus it was possible to complement the defect in the delayed onset of the formation of heat-resistant cfu

observed in the *C. difficile agrB1* mutant, indicating that the observed phenotype was due solely to inactivation of *agrB1*.

There was a slight difference between the level of heat-resistant cfu obtained for the *agrB2* (pMTL84151::*agr2*) strain, the strain with the control plasmid and the level obtained for the plasmid-free parental strain. This difference is presumably as a result of growth under thiamphenicol selection conditions which may affect the sporulation frequency of R20291. The *agrB1* mutant controls containing the empty vector performed like the original mutant, sporulation was initiated after 48 hours.

As expected, there is no difference in the onset of sporulation in the *C. difficile* R20291 *agrA* complemented strains compared to the *agrA* mutant strain (Figure 4.6b). The sporulation profiles of these strains are similar with sporulation being initiated at 48 hours.

The sporulation profile of the *agrB2* mutant strain of *C. difficile* carrying pMTL84151::*agr2* was not the same as the sporulation profile of the parental strain. Sporulation in the *agrB2* mutant is initiated after 24 hours, whereas sporulation in *agrB2* (pMTL84151::*agr2*) strain was also initiated at 24 hours unlike the parental strain which sporulates after 48 hours (Figure 4.6a). The results of the complementation study suggest that *agr2* may not be expressed from the plasmid pMTL84151::*agr2*. This could be tested by modifying the plasmid with a heterologous promoter as it appears that the 5' non-coding region did not result in expression of the *agr2* locus. On the other hand, expression of the *agr2* locus may

not be the cause of the failure to complement the mutant strain using this plasmid pMTL84151::*agr2* and instead this could be due to the ClosTron insertion in these mutant strains having a polar effect on gene expression. There also may be transcriptional terminators present in the DNA sequence within the region of the *agr* locus. It would be useful to search the DNA sequence of the agr region for such transcriptional terminators as this may explain the why it was not possible to complement the mutant strains using plasmid pMTL84151::*agr2*. Unfortunately due to time constraints it was not possible to perform cytotoxicity assays with the complemented *C. difficile agr* mutant strains in this study.

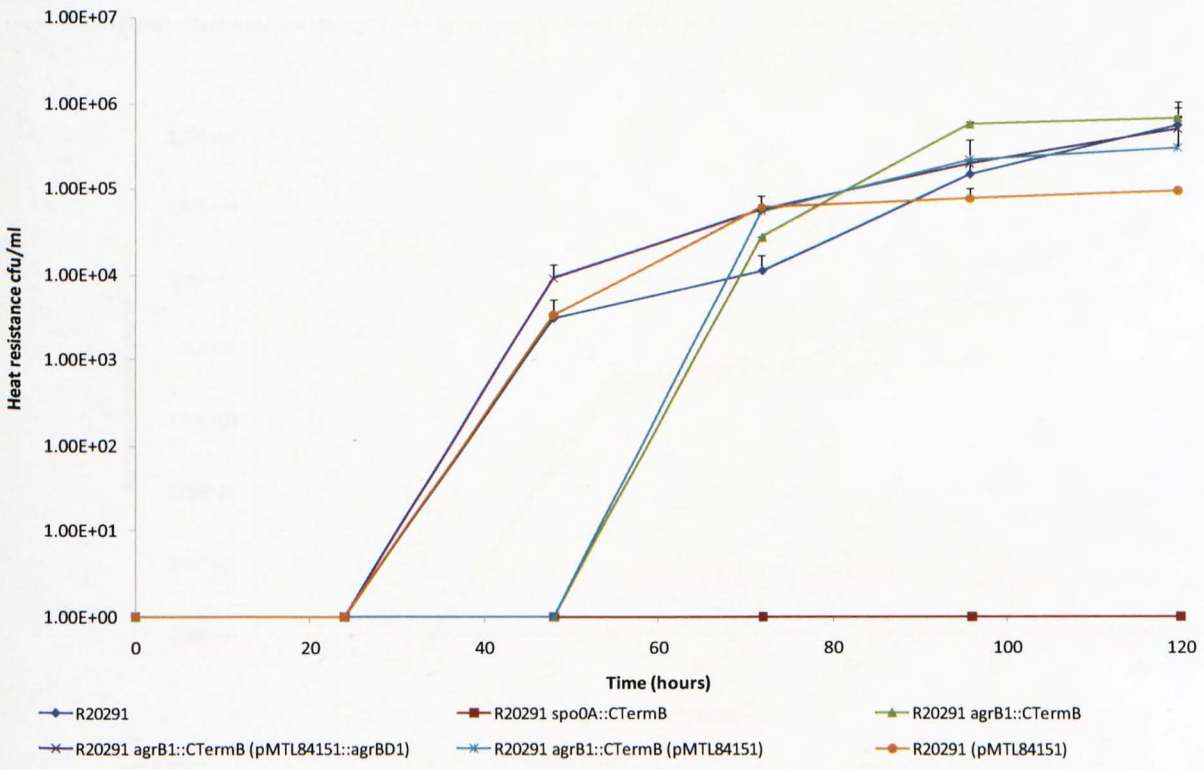
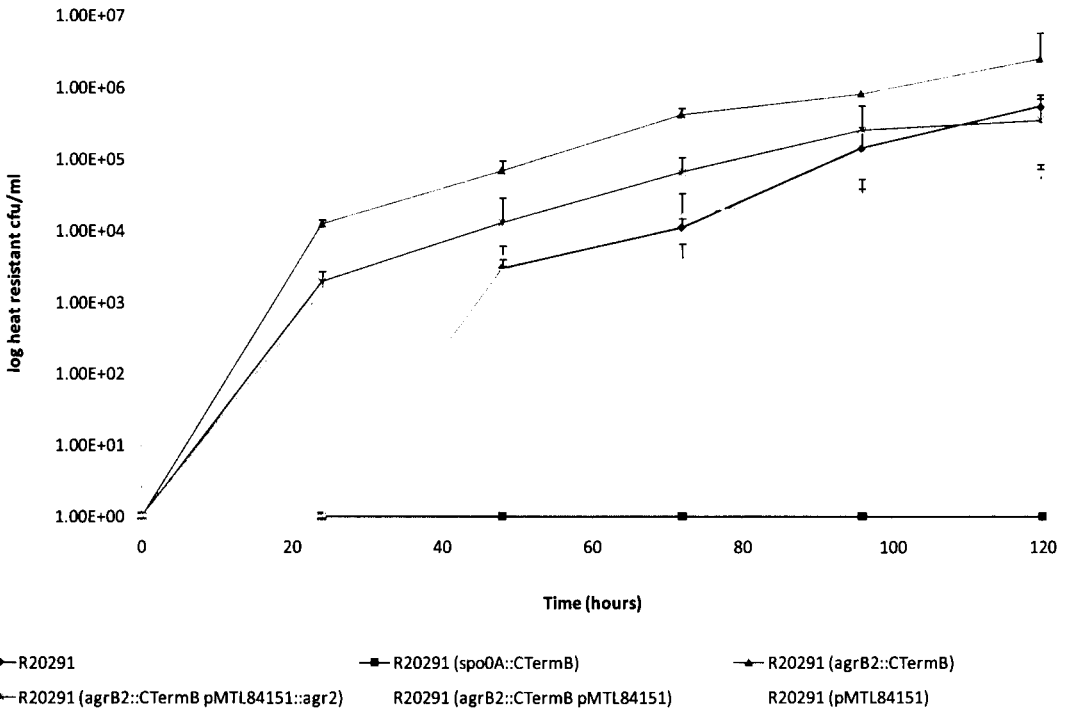


Figure 4.5 Development of heat-resistant CFU of *C. difficile* R20291 *agrB1* complemented mutants over 5 days on BHIS agar supplemented with 0.1% taurocholate. *C. difficile* strains were cultivated as described in Chapter 2. The symbols indicate the averages of 3 independent experiments, and the error bars indicate standard errors of the means. The detection limit for the assay was 50 CFU/ml.

a



b

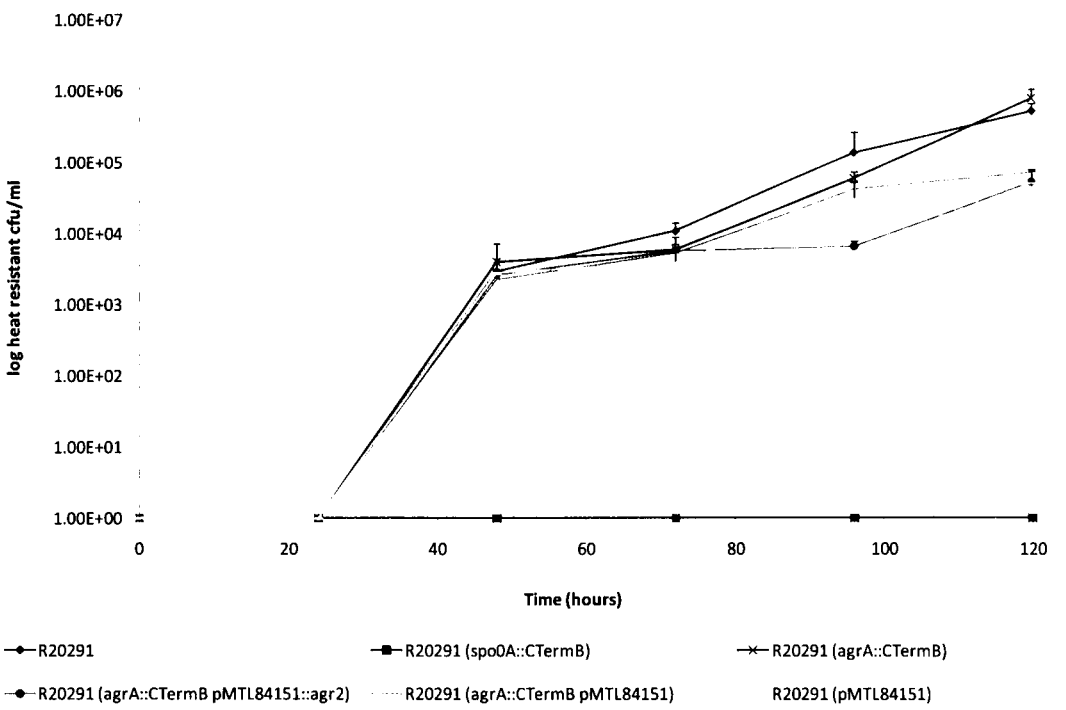


Figure 4.6 Development of heat-resistant CFU of a) *C. difficile* R20291 *agrB2* complemented mutants and b) *C. difficile* R20291 *agrA* complemented mutants over 5 days on BHIS agar supplemented with 0.1% taurocholate. *C. difficile* strains were cultivated as described in Chapter Two. The symbols indicate the averages of three independent experiments, and the error bars indicate standard errors of the means. The detection limit for the assay was 50 CFU/ml.

4.3 Discussion

There are a number of gene differences between PCR ribotype 027 strains, in particular the presence of an additional complete *agr2* locus. These genes differences may contribute to understanding why modern 027 strains are associated with a higher case-fatality ratio and persistence. One of the first characteristic of BI/NAP1/027 strains to be associated with hypervirulence was the production of toxins, the major virulence factor of *C. difficile.* As described previously, a number of BI/NAP1/027 strains have been shown to produce higher levels of toxin *in vitro* (Warny, *et al.*, 2005), although the molecular basis of this observation remains unclear. As the ribotype 027 strains carry an extra copy of the complete *agr* locus, it was hypothesised that toxin production in these strains are under the regulatory control by the *agr2* locus. In this study, the role of the second *agr* locus was analysed to determine whether the *agr* homologues are involved in the regulation of toxin production and sporulation.

The data presented above shows that there is some variation in the sporulation profiles of the *C. difficile* R20291 *agr* mutants. We observed that there are no obvious differences in the rate at which the heat-resistant cfu are formed over 5 days in the *C. difficile* R20291 *agrA* mutant strain in comparison to the parental strain. In contrast to this, the onset of sporulation in the *C. difficile* R20291 *agrB1* mutant is delayed, heat-resistant cfu are formed after 72 hours, in comparison to the parental strain. The delayed onset of sporulation was fully restored in *C. difficile* R20291 *agrB1* mutant strains carrying pMTL84151::*agrBD1* and the complemented *agrB1*

mutant behaved in a similar manner to the *C. difficile* R20291 parental strain (Figure 4.5) indicating that the observed phenotype was due solely to inactivation of *agrB1*.

In the *C. difficile* R20291 *agrB2* mutant heat-resistant cfu formation is initiated after 24 hours, whereas the parental strain sporulates after 48 hours. Unfortunately it was not possible to complement the *agrB2* mutant. Sporulation in the *agrB2* mutant is initiated after 24 hours, whereas sporulation in *agrB2* (pMTL84151::*agr2*) strain was also initiated at 24 hours unlike the parental strain which sporulates after 48 hours (Figure 4.6a). The results of the complementation study suggest that *agr2* may not be expressed from the plasmid pMTL84151::*agr2*. To address this the plasmid could be modified with a heterologous promoter as 5' non-coding region did not result in expression of the *agr2* locus. The stability of the plasmid in the complemented strains should be tested to ensure that the plasmid has not been lost during the sporulation assay. In addition to this it would also be useful to check that plasmid pMTL84151::*agr2* has not recombined with homologous sequences in the genome.

Interestingly, the *agrB1* mutant of *C. difficile* R20291 behaves differently in terms of sporulation when compared to the *agrB* mutant of *C. difficile* 630Δ*erm*. It is not clear why it would be beneficial for R20291, a strain which has two *agr* loci, to regulate the onset of sporulation in this way. Another species of clostridia that carries two copies of the *agr* loci is *C. botulinum*. The work of Cooksley and co-workers (2010) demonstrated that inactivation of *agr1* and *agr2*, respectively, affected sporulation in both of these mutant strains. Even though the *agr1* mutant showed a greater reduction in sporulation than the *agr2* mutant, it is apparent that the two *agr* loci in

C. botulinum are working in tandem. Unfortunately this was not observed in *C. difficile* R20291 thus it is difficult to draw any definite conclusions from these findings but it is clear the sporulation in *C. difficile* is a very complex process.

The *C. difficile* R20291 *agrB1* mutant showed a similar degree in toxicity towards both the vero cells and HT29 cells as the parental strain. However, both *agr2* mutants of *C. difficile* R20291 displayed a reduction in cytotoxicity *in vitro* (Figure 4.4). In light of these findings, it can be concluded that the *agr2* locus may play a secondary role in the regulation of toxin production in *C. difficile*. The exact mechanisms of how this is elucidated remains unclear however it could be possible that the two additional AIP's produced in this strain act as a molecular switch and increase toxin production. In contradiction to our observations, preliminary microarray studies performed with the *C. difficile* R20291 *agrA* mutant has revealed that in the *agrA* mutant strain expression of the PaLoc (pathogenecity locus which encodes the toxin A and B genes) is upregulated during late exponential/stationary growth (R. Stabler, personal communication). The research into regulatory pathways of *C. difficile* is still relatively new. However, with the findings of this study it may help us further our understanding of how the so called hypervirulent strain causes disease.

Chapter 5

Further analysis of the *agr2* locus

5.1 Introduction

C. difficile is one of the most intensively typed pathogens, with a wide range of systems applied in order to understand its epidemiology. The emergence of *C. difficile* strains belonging to restriction endonuclease type BI, North American pulsed-field type 1 (NAP1) and PCR-ribotype 027 (BI/NAP1/027) has contributed to the problem of increased CDAD incidence (Pépin, *et al.*, 2004; Kuijper, *et al.*, 2007). These so-called 'hypervirulent' types of *C. difficile* were in the past isolated infrequently from patients suffering from CDAD, but have recently become highly represented among such clinical isolates.

Outbreaks of CDAD due to the B1/NAP1/027 *C. difficile* strains continue to be reported and these types are now the most clinically relevant type in the United Kingdom (Brazier *et al.*, 2008). In the last decade there has been a 35-fold increase in reported cases of CDAD in the United Kingdom (Stabler *et al.*, 2009). The B1/NAP1/027 strains are associated with more severe disease. In addition, patients infected with these strains often experience more recurrent episodes and higher mortality (Loo *et al.*, 2005). Consequently, the studies of the mechanisms behind the pathogenesis of the B1/NAP1/027 strains are now at the forefront of *C. difficile* research worldwide.

There are a number of genes specific to the PCR ribotype 027 strains, a large proportion of which comprise of regulatory genes. Interestingly, PCR ribotype 027 strains carry an additional copy of the complete *agr* locus which is not found in the *C. difficile* 630 genome.

5.1.1 Aim of this study

The work described in Chapter 4 showed that the second *agr* locus (*agr2*) may play a role in toxin A and toxin B production in *C. difficile* R20291. In light of these findings, the work described in this study sets out to further characterise the *agr2* locus by investigating transcriptional linkage of the *agr* genes. In addition to this, the study also aims to analyse the effect on toxin A and toxin B production in the *C. difficile* 630Δ*erm* strain harbouring a plasmid containing the *agr2* locus.

5.2 Results

5.2.1 Reverse Transcriptase PCR (RT-PCR) analysis to investigate linkage of *agr* gene expression in *C. difficile*

RT-PCR was used to determine whether the genes within and flanking the two *agr* regions of *C. difficile* R20291 and the *agr* region of *C. difficile* 630 are transcriptional linked. RNA was extracted, as described in Chapter 2, during early and late exponential growth, when OD_{600} readings were 0.4 and 1.0 (Figure 5.1). The amount of total RNA temple used was consistent for each RT-PCR reaction, 1μg RNA template per reaction. Nine primer sets for *C. difficile* R20291 and nine primer sets for *C. difficile* 630 were designed; each set spanning two genes within the *agr* regions (Figure 5.2 **1** and **2**). Primer sequences can be found in Chapter 2. Control reactions were carried out without reverse transcriptase to ensure amplification was not due to genomic DNA contamination. In addition, a positive control was also

carried out using a DNA template to ensure that the cycling conditions used were sufficient.

With the exception of the results obtained with the primers spanning *ptsI* and CD2641, the results in Figure 5.2(a) suggests that all of the genes in the *agr* region 1 are transcriptionally linked. It appears that transcription occurs during both early and late exponential growth and there is not an obvious difference in expression levels between the two time points. The negative result obtained with the primers spanning *ptsI* and CD2641 are somewhat surprising as there is no amplification in with the positive control either. Prior to being used in the RT-PCR reactions, the primers that were designed to amplify ptsI/CD2753 were tested on a genomic DNA template using a Phusion Taq polymerase (Promega) to ensure the primers were annealing correctly and there was no non-specific amplification. The results of the test showed that the primers annealed and amplified as expected, data not shown. The lack of amplification in the positive control during the RT-PCR could potentially be due to the HotStar *taq* polymerase (Qiagen), which is used for the RT-PCR, not being able to produce an amplicon greater than 1Kb.

The RT-PCR results shown in Figure 5.2(b) also suggest that in *agr* region 2, all the genes within the region are co-expressed (gene pairs CDR20291_3192/CDR20291_3191, CDR20291_3191/CDR20291_3190, CDR20291_3190/*agrA*, *agrA*/*agrC*, *agrC*/*agrD2*, *agrD2*/*agrB2*. *agrB2*/CDR20291_3186, CDR20291_3186/CDR20291_3185 and

CDR20291_3185/CDR20291_3184). Once again, all gene pairs which are co-expressed show expression throughout early and late exponential growth and there is no obvious difference in the levels of expression at the two time points analysed.

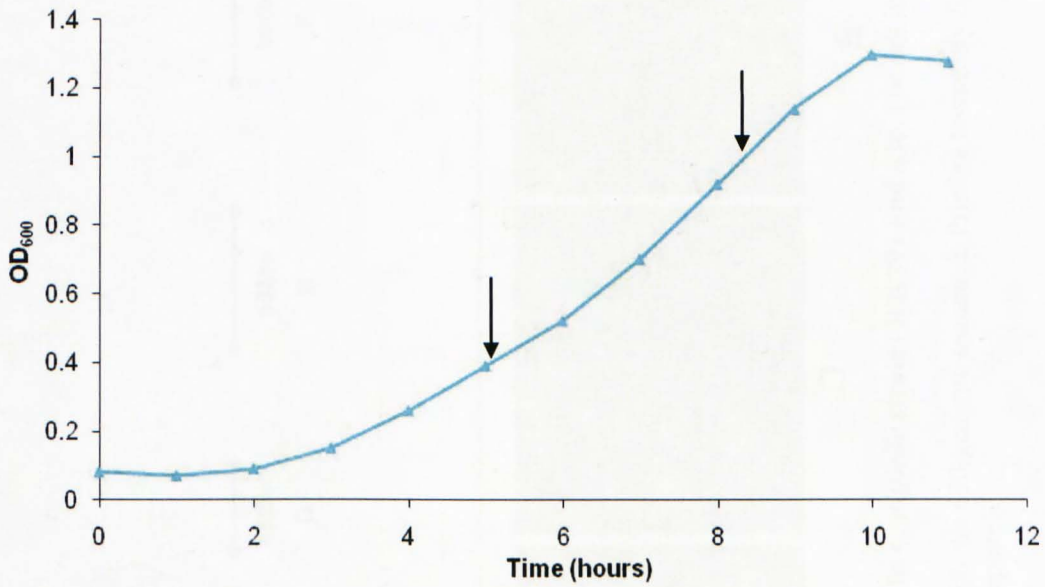
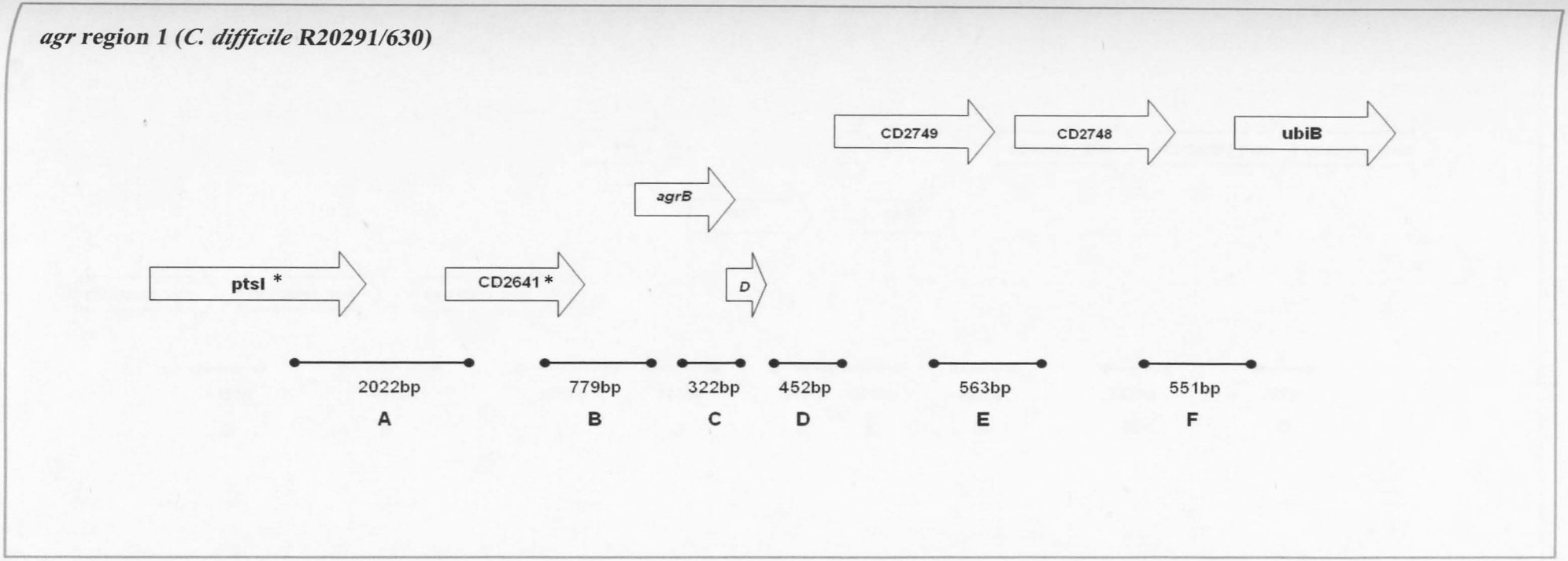


Figure 5.1 Growth of *C. difficile* R20291 in TY broth for RNA extractions. The arrows show when samples were taken for RNA extraction. Strains were cultivated as described in Chapter 2, and the OD_{600} measured.



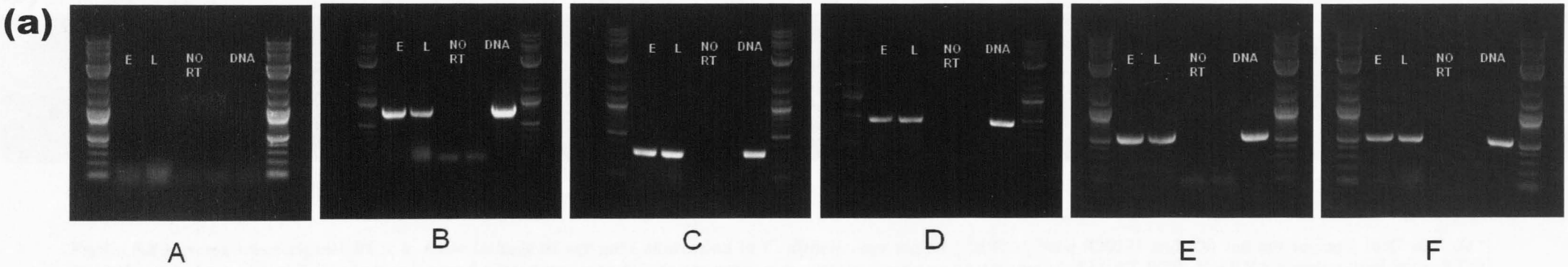


Figure 5.2 Reverse transcriptase PCR to show linkage of agr gene expression in *C. difficile* strains R20291 and 630. For full lengend see next page.

* *ptsI* and CD2641 are annotated as CD2753 and CD2751 respectively in the *C. difficile* 630 genome sequence. Primers to amflify the integenic region between the two genes were the same as those used with *C. difficile* R20291 RNA/DNA template.

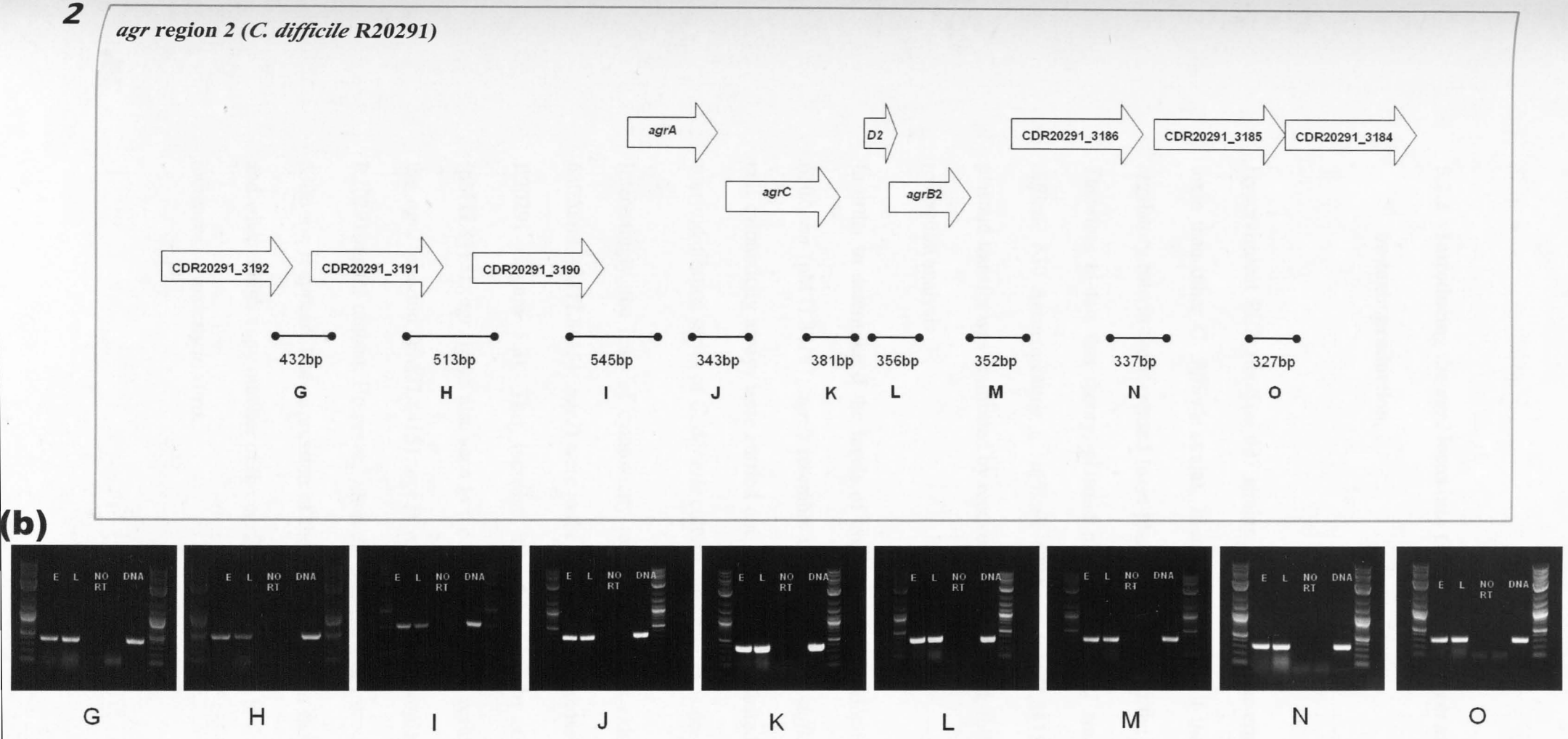


Figure 5.2 Reverse transcriptase PCR to show linkage of agr gene expression in *C. difficile*. *agr* region 1 in *C. difficile* R20291 and 630 and *agr* region 2 in *C. difficile* R20291 are shown by 1 and 2. The bars spanning the intergenic regions between genes represent the fragments (A-O) amplified by RT-PCR. The RNA template used in the RT-PCR reactions were extracted during early (E) and late (L) exponential growth, OD600 0.4 and 1.2 respectively. Negative control reactions were carried out on early and late RNA template without reverse transcriptase (no RT) to ensure amplification was not due to DNA contamination. Positive control reactions were carried out using a DNA template (DNA) in place of the RNA template to ensure the cycling conditions were sufficient.

5.2.2 Introducing the *agr2* locus into *C. difficile* 630Δ*erm* and its effect on toxin production.

Hypervirulent PCR ribotype 027 strains have been characterised to produce more toxin than other *C. difficile* strains. It can speculated that the *agr2* locus plays a regulatory role in the increased toxin phenotype displayed the ribotype 027 strains. Therefore to test this theory, plasmid pMTL84151::*agr2* was introduced into *C. difficile* 630 Δ*erm* creating *C. difficile* 630Δ*erm* (pMTL84151::*agr2*). Successful plasmid transfer was confirmed by recovery of the plasmid, followed by PCR and/or restriction analysis.

In order to determine if the levels of toxin A and B production in the *C. difficile* 630Δ*erm* (pMTL84151::*agr2*) resemble those seen in *C. difficile* R20291, *in vitro* cell cytotoxicity assays were carried out. Cells were incubated for 24 hours with a fourfold dilution series of *C. difficile* culture supernatants as described previously.

Interestingly, the level of cytotoxicity towards vero cells exhibited by *C. difficile* 630Δ*erm* (pMTL84151::*agr2*) were to the same degree as those seen with *C. difficile* R20291 (Figure 5.3). This increase in cytotoxicity by *C. difficile* 630Δ*erm* (pMTL84151::*agr2*) was also seen in the HT29 cells. The results were surprising as the *agr2* plasmid (pMTL84151::*agr2*) was unable to complement the *C. difficile* R20291 *agrB2* mutant. However, when the plasmid was introduced into *C. difficile* 630Δ*erm* it appears that expression of the *agr2* locus from the plasmid is sufficient and when a high copy number of the *agr2* locus is present in *C. difficile* 630Δ*erm*, it increases its toxicity *in vitro*.

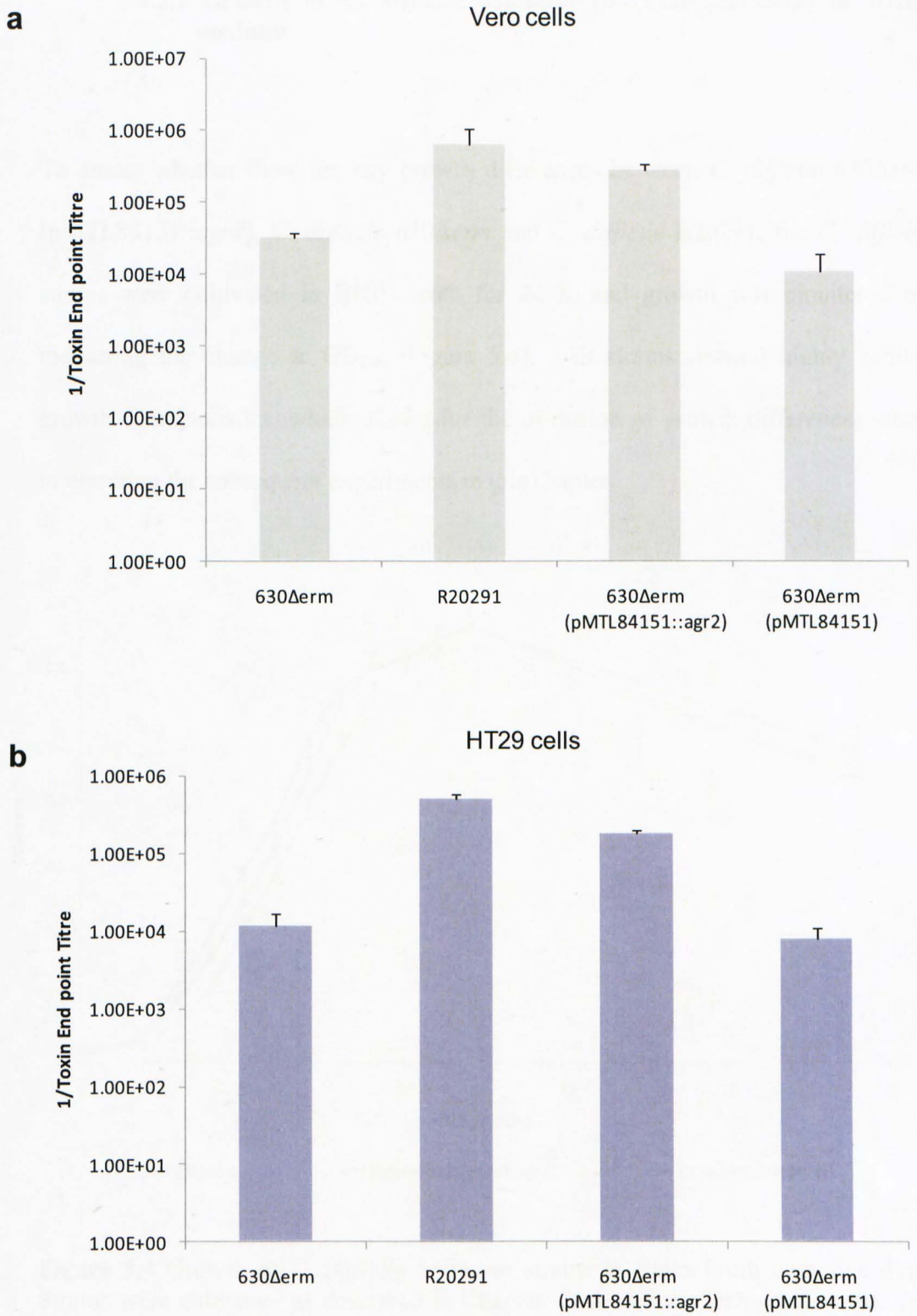


Figure 5.3 *In vitro* cytotoxicity assays. Supernatants of the *C. difficile* 630Δ*erm* strains were used in cell culture assays to measure cytotoxicity. Vero cells (a) and HT29 cells (b) were cultured to a flat monolayer before adding *C. difficile* supernatants in a fourfold dilution series. After 24 hour incubation, toxin end point titres were determined.

5.2.2.1 Growth of *C. difficile 630Δerm* (pMTL84151::*agr2*) in BHIS medium

To assess whether there are any growth differences between *C. difficile* 630Δ*erm* (pMTL84151::*agr2*), *C. difficile* 630Δ*erm* and *C. difficile* R20291, the *C. difficile* strains were cultivated in BHIS broth for 24 h, and growth was monitored by measuring the change in OD_{600} (Figure 5.4). All strains showed highly similar growth characteristics, which allows for the exclusion of growth differences when interpreting the subsequent experiments in this chapter.

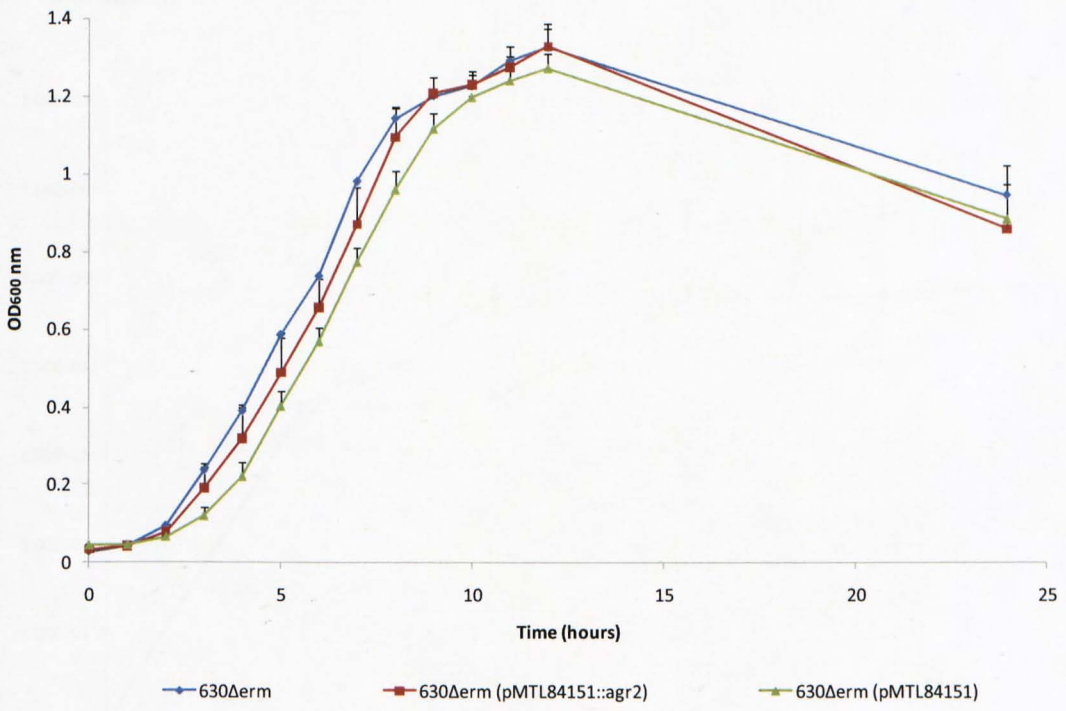


Figure 5.4 Growth of *C. difficile* 630Δ*erm* strains in BHIS broth over five days. Strains were cultivated as described in Chapter Two, and the OD_{600} measured. The symbols represent the averages of three independent experiments, and the error bars represent standard errors of the means.

5.2.2.2 Sporulation in *C. difficile 630Δerm* carrying pMTL84151::*agr2*

Sporulation assays were used to address whether presence of the *agr2* locus changes the sporulation profile of *C. difficile 630Δerm* and its ability to form heat-resistant cfu on BHIS agar supplemented with taurocholate over a five day period (Figure 5.6). *C. difficile 630Δerm* harbouring pMTL84151::*agr2* appeared to have no effect on colony formation after heat treatment. Sporulation in all the strains was initiated at 24 hours. The level of heat resistant CFU formed by *C. difficile* carrying the empty plasmid was slightly lower than the *C. difficile* 630Δ*erm*. These results indicate that *agr2* in *C. difficile* 630Δ*erm* background does not alter the onset of sporulation in this strain.

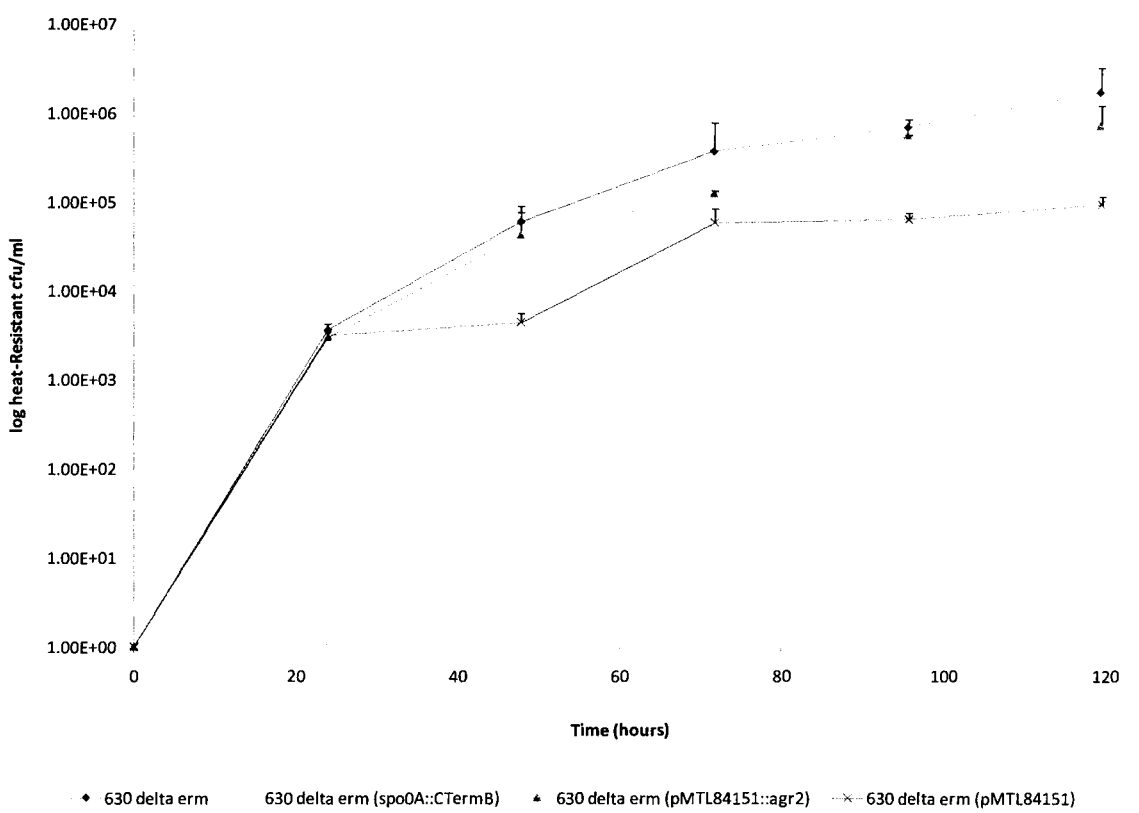


Figure 5.5 Development of heat-resistant CFU of *C. difficile* 630Δ*erm* harbouring pMTL84151::*agr2* over 5 days on BHIS agar supplemented with 0.1% taurocholate. *C. difficile* strains were cultivated as described in Chapter Two. The symbols indicate the averages of three independent experiments, and the error bars indicate standard errors of the means. The detection limit for the assay was 50 CFU/ml.

5.3 Discussion

The work described in Chapter 4 showed that the second *agr2* locus may play a role in toxin A and toxin B production in *C. difficile* R20291. Therefore in light of these findings, the work described in this study set out to further characterise the *agr2* locus by (i) investigating transcriptional linkage of the *agr* genes and (ii) investigating the effect on toxin A and toxin B production in the *C. difficile* 630Δ*erm* strain harbouring a plasmid containing the *agr2* locus.

The RT-PCR results revealed that all the genes within *agr* region 1 and *agr* region 2 are co-expressed. A positive RT-PCR result was obtained for all gene pairs tested with the exception of gene pairs *ptsI* and CD2641. However, there was no amplification from the positive control in this reaction either. The primer pairs were tested prior to being used in the RT-PCR reactions. The primers that were designed to amplify ptsI/CD2753 were tested on a genomic DNA template using a Phusion Taq polymerase to ensure the primers were annealing correctly and there was no non-specific amplification. The results of the test showed that the primers annealed and amplified as expected, data not shown.

All gene pairs which are co-expressed show expression throughout early and late exponential growth and there is no obvious difference in the levels of expression at the two time points analysed. It should be noted that analysis of transcriptional linkage using RT-PCR is not necessarily a quantitative method and therefore, we were unable to predict the size of the mRNA transcript using these results. A

Northern blot is a more quantitative method which would enable us to determine the actual size of the mRNA transcript.

The *agr* regions in other clostridia species, such as *C. perfringens* and *C. botulinum*, have been shown to form an operon and be transcribed from a single RNA transcript (Ohtani *et al.*, 2009; Cooksley *et al.*, 2010). These findings are consistent with what has been shown in *S. aureus* and other gram positive bacteria that carrying an *agr* operon. However, this does not seem to be apparent for *C. difficile* strains 630 and R20291. Our data suggests that maybe the *agr* genes in *C. difficile* 630 and *C. difficile* R20291 are not transcribed as a single operon.

We demonstrated that introduction of the *agr2* locus into the *C. difficile* 630Δ*erm* strain on a plasmid, increases the cytotoxicity of this strain *in vitro*. Interestingly, the cytotoxicity levels of this recombinant strain mirrored the cytotoxicity shown by *C. difficile* R20291. These findings further support the work described in the previous chapter, and suggests that the second *agr* locus in *C. difficile* R20291 may be a contributing factor in increased toxin production phenotype that is observed in PCR ribotype 027 strains. It would be interesting to analyse the effect of adding culture supernatant taken from the *C. difficile* R20291 agr2 mutant strains to *C. difficile* 630Δ*erm* cells to see whether the increase in cytotoxicity occurs in the absence of AIP1 and AIP2 peptides. Further work needs to be undertaken to fully elucidate the role of *agr2* in this strain.

Surprisingly, *C. difficile 630Δerm* harbouring pMTL84151::*agr2* appeared to have no effect on colony formation after heat treatment. Sporulation in all the strains was initiated at 24 hours. The level of heat resistant CFU formed by *C. difficile* carrying the empty plasmid was slightly lower than the *C. difficile* 630Δ*erm*. These results indicate that *agr2* in *C. difficile* 630Δ*erm* background does not alter the onset of sporulation in this strain. We observed that sporulation in the *C. difficile* R20291 occurs after 48 hours, whereas sporulation in *C. difficile* 630Δ*erm* harbouring pMTL84151::*agr2* occurs after 24 hours consistent with its parental strain. Even though it is difficult to draw any definite conclusions from these observations, it is clear sporulation in *C. difficile* is regulated by many different factors and regulation by the *agr* locus may only be one part of this very complex process.

Key outcomes:

1. All the genes within the *agr* 1 region , from *C. difficile* 630 and *C. difficile* R20291, and all the genes within *agr* region 2 of *C. difficile* R20291 are co-expressed. It was not possible to determine whether the agr genes are transcribed on a single mRNA transcript using this method.

2. Introducing the *agr2* locus into a *C. difficile* 630Δ*erm* background increases the cytotoxicity levels of this strain to a similar level of cytotoxicity that is displayed by *C. difficile* R20291. An increased cytotoxicity level means more toxin A and toxin B is being produced in this recombinant strain.

Chapter 6

Analysis of a *luxS*-type signalling system in *Clostridium difficile*

6.1 Introduction

luxS homologues can be found in a variety of Gram-positive and Gram-negative bacteria (Winzer *et al.*, 2003). The quorum sensing molecule auto inducing-2 (AI-2) is synthesised by the *luxS* gene. LuxS is a metabolic side product produced during the activated methyl cycle (a recycling pathway involved in the metabolism of methionine) (Winzer *et al.*, 2003). The AI-2 molecule is produced by many different Gram-negative and Gram-positive species (Bassler *et al.*, 1997) and AI-2 signalling systems are involved in the regulation of virulence factors such as type II secretion in enterohaemorrhagic *E. coli* (Sperandio *et al.*, 1999) and haemolysin and cysteine protease in *Streptococcus pyogenes* (Lyon *et al.*, 2001). As *luxS* homologues have been found in many different bacteria, it has recently been proposed that AI-2 could serve as a 'universal signal' for interspecies cross-talk (Xavier & Bassler, 2003). However, this is yet to be proven in species other than *Vibrio*.

6.1.2 The role of LuxS in clostridia

Several clostridia, including *C. difficile*, have been found to possess LuxS homologues. In *C. botulinum* the *luxS* gene shows 50.3% sequence identity with that of *V. harveyi*, but limited literature is available on its mechanism of control of cellular functions. To date, there is only one documented report on the function of *C. botulinum luxS* (Zhao *et al.*, 2006). This group demonstrated that AI-2 was present in *C. botulinum* culture supernatant and that this supernatant promoted the germination and outgrowth of spores. However, there is no evidence to suggest that this promotion was directly due to the effect of AI-2.

LuxS has also been investigated in other clostridial species and indeed, Ohtani *et al.* (2002) showed that the *luxS* gene is involved in the regulation of toxin production in *C. perfringens* via quorum sensing. This group also demonstrated that *luxS* mutants produced much less α, κ and θ toxins than the wild type, and that these mutants could be complemented with wild type culture supernatant to restore toxin production.

6.1.3 Previous knowledge of LuxS in *C. difficile*

The study of quorum sensing in *C. difficile* is also gathering momentum, due to the emerging incidence of *C. difficile*-associated disease (CDAD) in hospitals. However it can be fair to say that our previous knowledge about the role of *luxS* in *C. difficile* remains limited. Carter *et al.* (2005) demonstrated that AI-2 is produced by this *C. difficile*. In the region upstream of *luxS*, genes encoding a putative two component signal transduction system were identified, and the system was found to be involved in the regulation of AI-2 production. However, they suggested that AI-2 was not involved in the control of toxin production, as down-regulation of the *luxS* gene using antisense RNA did not cause a reduction in toxin production.

In contrast to these findings Lee & Song (2005) also carried out a similar study into *luxS* and quorum sensing in *C. difficile*. They demonstrated an up-regulation of toxin A mRNA production in response to exposure to AI-2-containing *C. difficile* supernatant. However, they could not demonstrate a concomitant increase in the level of the toxin. Their data do suggest, however, that *luxS* plays some role in the regulation of toxin production in *C. difficile*.

Homologues of *luxS* have been identified in the genome sequence of both *C. difficile* 630 and *C. difficile* BI/NAPI/027 strain, R20291. We reasoned that the AI-2/LuxS signalling system plays a role in the regulation of virulence factors in *C. difficile*. Therefore it was decided to target *luxS* in both *C. difficile* 630Δ*erm* and *C. difficile* R20291.

6.1.4 Aims of this study

With a range of genetic tools now at our disposal, the way has been opened to understand more thoroughly the role of *luxS* in *C. difficile* QS-mediated regulation of virulence factors. The study presented in this chapter, therefore, aimed to utilise the ClosTron system to inactivate *luxS* homologues in both *C. difficile* 630Δ*erm* and R20291, and analyse the role played by these genes in the regulation of virulence factors, paying particular attention to sporulation and toxin production in the first instance.

6.2 Results

6.2.1 Construction of mutant strains

To investigate the role of *luxS* in *C. difficile*, the ClosTron system (Heap *et al.*, 2007; Heap *et al.*, 2009; Heap *et al.*, 2010) was used, to create independent insertional mutants of *C. difficile* 630Δ*erm* and *C. difficile* R20291 in which the *luxS* gene was inactivated, yielding strains *C. difficile* 630Δ*erm luxS*::CT*ermB* and *C. difficile* R20291 *luxS*::CT*ermB*, respectively. The group II intron from the ClosTron system was retargeted using a computer algorithm to insert into *luxS* in a sense orientation at position 48 bp, from the start of the ORF, and in an antisense orientation at position 161 bp.

As described in previous chapters, to confirm correct ClosTron insertion, putative mutant strains were analysed by PCR, and one erythromycin/lincomycin-resistant clone each of *C. difficile* 630Δ*erm luxS*::CT*ermB* and *C. difficile* R20291 *luxS*::CT*ermB* were picked for sequencing at the intron insertion site and the data confirmed the expected sequence (Table 6.1 shows intron insertion sites and the frequencies of the desired mutants obtained).

Construction of a *luxS* insertional mutant was first attempted in *C. difficile* 630Δ*erm* using the 48s target site. However, it was not possible to isolate the desired mutant using this target site. Screening of a pool of genomic DNA from >50 erythromycin-resistant clones indicated that no desired mutants were present. The intron or target site was therefore judged to be inefficient (as can occasionally occur when using

group II intron technology), so another target site (base 161 with the antisense orientation) was chosen for *luxS*. This target site was successful in targeting the intron and subsequently after PCR screening a *luxS* insertional mutant in both *C. difficile* strains was obtained.

Strain and target site	Frequency of desired mutant among clones screened	
	%	No. positive/no. screened
C. difficile 630Δ*erm luxS* 48s	0	0/50+
C. difficile 630Δ*erm luxS* 161a	100	5/5
C. difficile R20291 *luxS* 161a	100	5/5

Table 6.1. ClosTron insertion frequencies with erythromycin selection. Introns were inserted after the indicated number of bases from the start of the open reading frame in either the sense (s) or antisense (a) orientation. Erythromycin-resistant (630Δ*erm* or R20291) clones were picked at random and screened by PCR to amplify the intron-exon junction. One clone of each desired mutant was selected, and the intron insertion site verified by sequencing.

6.2.2 Phenotypic characterisation of the *C. difficile luxS* mutant strains

C. difficile 630Δ*erm luxS*::CT*ermB and C. difficile* R20291 *luxS*::CT*ermB* were phenotypically characterised. To ensure that any observed phenotypes of the *C. difficile luxS* mutants are not a result from growth deficiencies, the change in OD_{600} was used to monitor growth over 24 hours in BHIS broth (Figure 6.1 and 6.2). The growth of the mutants in both *C. difficile* strains was found to be indistinguishable

from the growth of the parental strain, which suggests that there were no obvious growth defects following inactivation of *luxS*, in *C. difficile* 630Δ*erm* and R20291.

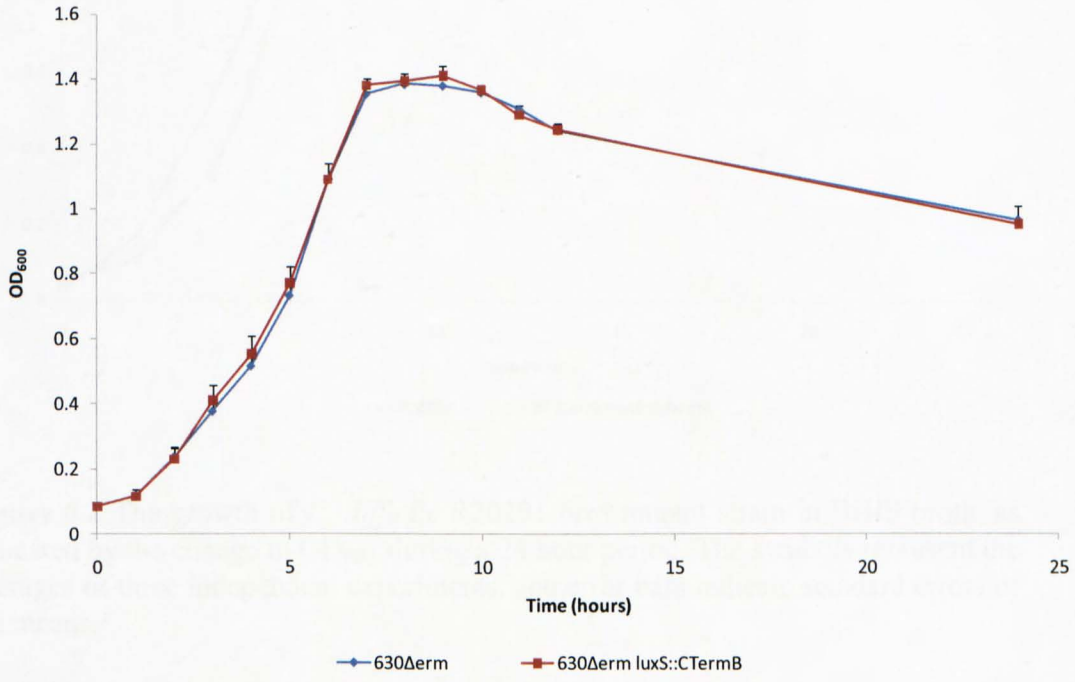


Figure 6.1 The growth of *C. difficile* 630Δ*erm luxS* mutant strain in BHIS broth, as indicated by the change in OD_{600}, during a 24 hour period. The symbols represent the averages of three independent experiments, and error bars indicate standard errors of the means.

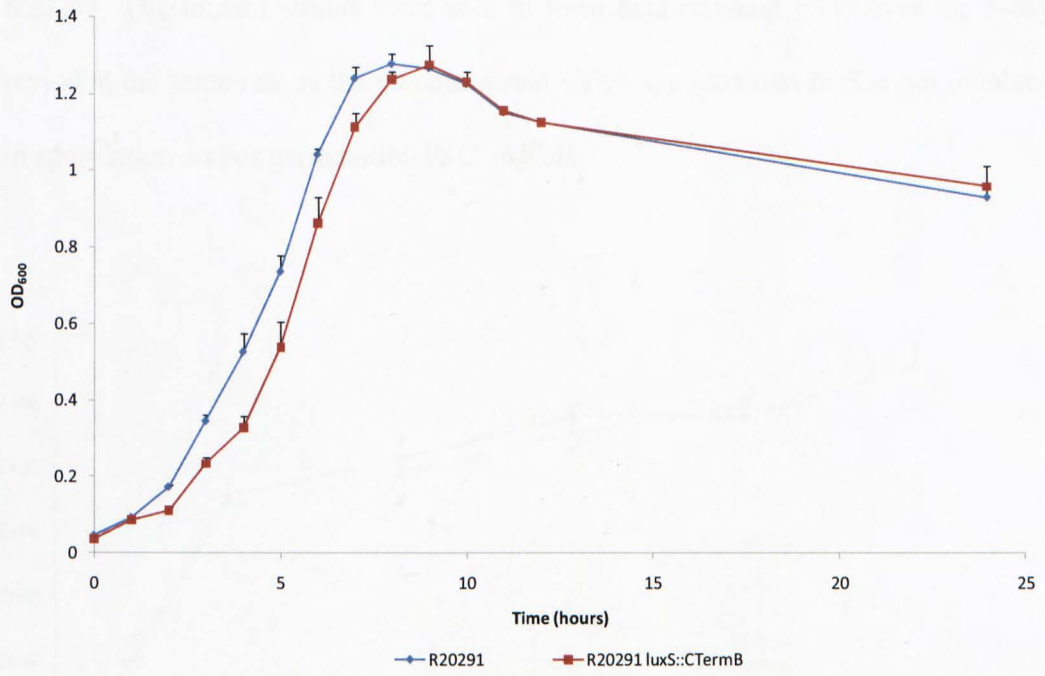


Figure 6.2 The growth of *C. difficile* R20291 *luxS* mutant strain in BHIS broth, as indicated by the change in OD_{600}, during a 24 hour period. The symbols represent the averages of three independent experiments, and error bars indicate standard errors of the means.

6.2.2.1 Sporulation in the *C. difficile luxS* mutant strains

Following inactivation of *luxS* in both *C. difficile* 630Δ*erm* and R20291, the *luxS* mutant strains were tested to determine their ability to form heat-resistant CFU over a 5-day period on BHIS agar supplemented with the bile salt taurocholate (Figure 6.3 and 6.4). Heat-resistant cfu/ml (spores) were enumerated by serial dilution and plating onto BHIS agar supplemented with 0.1% of the bile salt taurocholate after heat treatment for 30 mins at 60°C, respectively as described in Chapter 2. It appears that over 5 days there is no effect on sporulation in the *C. difficile* 630Δ*erm luxS* mutant strain. Inactivation of *luxS* had no effect on sporulation in *C. difficile*

R20291. The mutant strains were able to form heat-resistant CFU over the 5-day period at the same rate as the parental strain. This suggests that *luxS* is not involved in sporulation and/or germination in *C. difficile*.

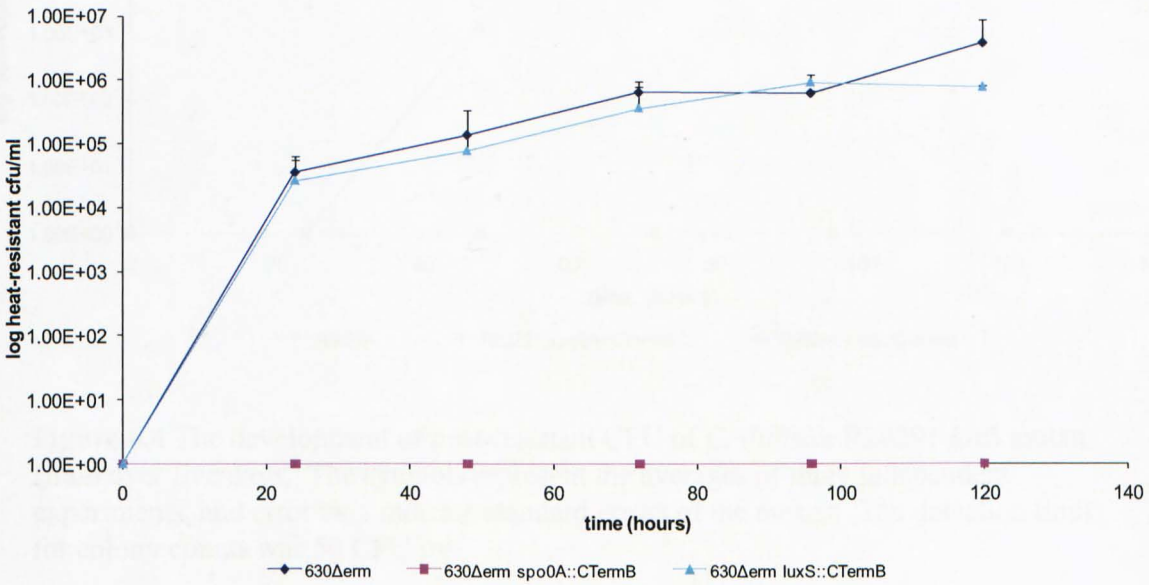


Figure 6.3 The development of heat-resistant CFU of *C. difficile* 630Δ*erm luxS* mutant strain over five days. The symbols represent the averages of three independent experiments, and error bars indicate standard errors of the means. The detection limit for colony counts was 50 CFU/ml.

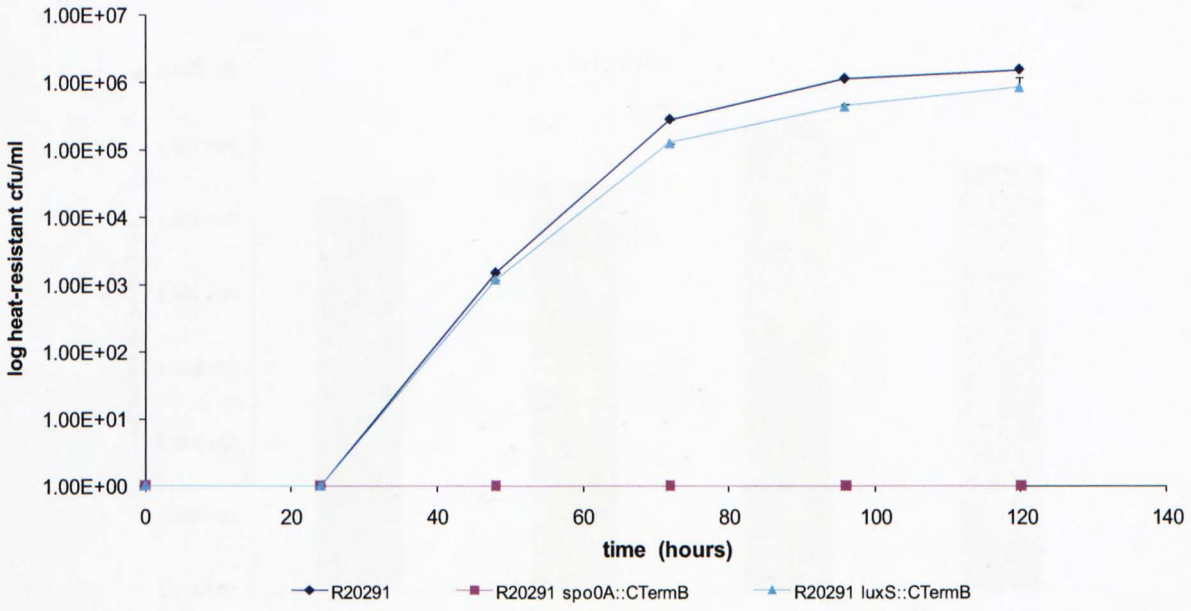
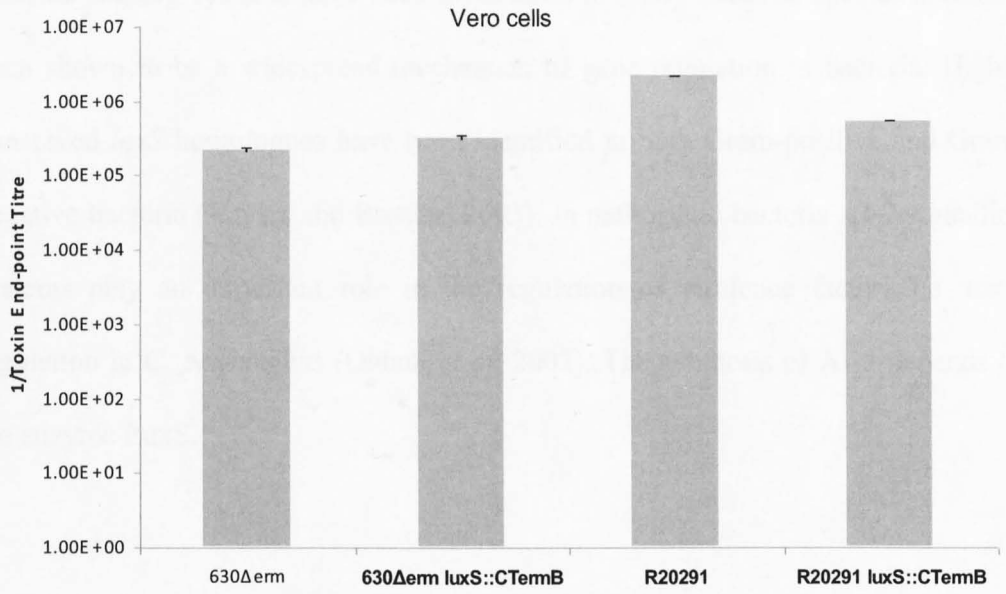


Figure 6.4 The development of heat-resistant CFU of *C. difficile* R20291 *luxS* mutant strain over five days. The symbols represent the averages of three independent experiments, and error bars indicate standard errors of the means. The detection limit for colony counts was 50 CFU/ml.

6.2.2.2 Toxin production in the *C. difficile luxS* mutant strains

In order to understand if *luxS* is involved in the regulation of toxin A and B production in *C. difficile*, *in vitro* cell cytotoxicity assays were carried out. Cells were incubated for 24 hours with a fourfold dilution series of *C. difficile* culture supernatants. The *C. difficile* 630Δ*erm luxS* mutant showed a similar degree in toxicity towards both the vero cells and HT29 as the parental strain. Also there was no obvious difference in cytotoxicity between the *C. difficile* R20291 *luxS* mutant and the parental strain (Figure 6.5a, b). The cytotoxicity results indicate that the *luxS* is not involved in the regulation of toxin A and toxin B production in *C. difficile* R20291.

a



b

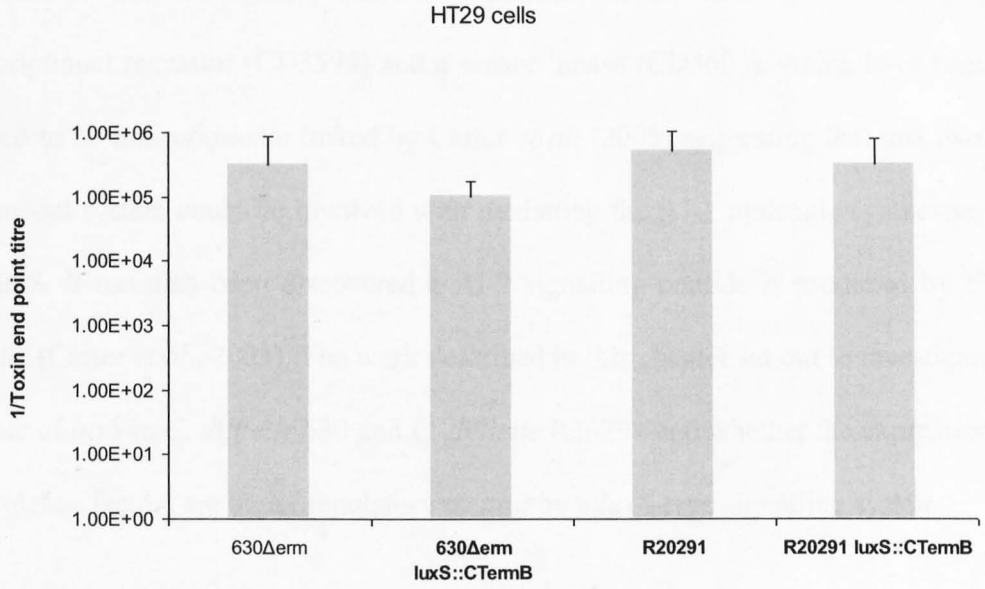


Figure 6.5 *In vitro* cytotoxicity assays. Supernatants of the parental strain and the two *luxS* mutant strains were used in cell culture assays to measure cytotoxicity. Vero cells (a) and HT29 cells (b) were cultured to a flat monolayer before adding *C. difficile* supernatants in a fourfold dilution series. After a 24 hour incubation, toxin end point titres were determined.

6.3 Discussion

Quorum sensing systems have been discovered in many bacterial species and have been shown to be a widespread mechanism of gene regulation in bacteria. Highly conserved *luxS* homologues have been identified in both Gram-positive and Gram-negative bacteria (Xavier and Bassler, 2003). In pathogenic bacteria AI-2 signalling systems play an important role in the regulation of virulence factors i.e. toxin regulation in *C. perfringens* (Ohtani *et al*, 2002). The synthesis of AI-2 depends on the enzyme LuxS.

Homologues of *luxS* have been identified in the genome sequences of both *C. difficile* 630 and *C. difficile* R20291. Upstream of the *luxS* gene there is a transcriptional regulator (CD3599) and a sensor kinase (CD3600), which have been showed to be transcriptional linked by Carter *et al.* (2005) suggesting that this two-component system could be involved with mediating the AI-2 molecule synthesised by LuxS. It has also been discovered a AI-2 signalling peptide is produced by *C. difficile* (Carter *et al.*, 2005). The work described in this chapter set out to investigate the role of *luxS* in *C. difficile* 630 and *C. difficile* R20291 and whether the expression of virulence factors are under regulatory control by a *luxS*-type signalling system.

Inactivation of *luxS* appeared to have no effect on sporulation in *C. difficile* R20291. The mutant strain was able to form heat-resistant CFU over a 5-day period at the same rate as the parental strain. The same result was seen with the *C. difficile*

630Δ*erm* *luxS* mutant. This suggests that *luxS* is not involved in sporulation and/or germination in *C. difficile*.

Cytotoxicity assays performed with *C. difficile* luxS mutants showed a similar degree of cytotoxicity in vitro in comparison to their parental strains. The cytotoxicity results indicate that the *luxS* is not involved in the regulation of toxin A and toxin B production in *C. difficile*. Despite toxin production in *C. perfringens* being regulated by a *luxS*/AI-2 signalling system, it appears that in *C. difficile* toxin production is not under regulatory control of such cell signalling system.

Unfortunately the precise mechanism by which toxin production and sporulation are regulated in *C. difficile* still remains unclear. The work presented above suggests that the production of the toxins and sporulation is not under the regulatory control of a *luxS*-type signalling system in *C. difficile*. It appears that the regulation of virulence factors in *C. difficile* is a very unique and complicated process. It may be possible that they are not under the regulatory control of a simple quorum sensing system and many extracellular and intracellular factors are involved in the regulation of sporulation and toxin production. Despite this, it remains clear that a further comprehensive analysis of regulation is required, for example DNA microarrays, to fully understand how toxin production and sporulation are regulated in *C. difficile*.

Chapter Seven

General Discussion

7.1 Key findings in this study

7.1.1 *agr* in *C. difficile* 630

The work presented in Chapter Three described the initial characterisation of an *agrB* homologue and a number of putative response regulators for their role in sporulation and toxin production in *C. difficile* 630. This study showed that *agrB* plays no obvious role in the regulation of virulence factors in *C. difficile* 630. The data obtained from the three orphan response regulators, identified from the BLASTP search, suggest that these response regulators may not be the response regulator of the *agrBD* locus in *C. difficile* 630. Our data indicates that sporulation and toxin production are not under the regulatory control of an *agr*-QS based system in *C. difficile* 630.

As with *C. perfringens* and *C. botulinum*, *C. difficile* 630 only has *agrBD* homologues and no apparent *agrC/A* homologues located in close proximately to the *agrBD* locus. A role of *agr* has been established in both of these clostridial strains; in both strains *agr* regulates toxin production. The genomic context of the *C. botulinum* and *C. perfringens agr* regions are similar. This feature is not seen in C. *difficile.* Even though it is unknown whether *agr* mutants in C. *perfringens* are impaired in sporulation, *agr* mutants in *C. botulinum* are. This indicates that these two clostridial species are more similar in terms of *agr* control than C. *difficile.*

It remains unclear what role the *agr* system has in *C. difficile* 630 or even if *agr* has a functional role in regulation of virulence in *C. difficile* 630. With no apparent two-component system (to detect the auto inducing peptide and induce gene expression)

present within the vicinity of the *agrBD* locus in genome sequence of *C. difficile* 630, it may be possible that *agr* does not have a functional role in regulation in *C. difficile* 630. This could be determined by investigating whether *agrD* produces an autoinducing peptide (AIP) by trying to isolate it from culture supernatant using mass spectrometry. Further characterisation of the *C. difficile* 630 *agrBD* locus is needed in order to understand whether it is involved in *C. difficile* pathogenesis.

7.1.2 *agr* in *C. difficile* R20291

The recent increase in the number CDAD cases has been attributed to the rapid emergence of *C. difficile* strains belonging to restriction endonuclease type BI, North American pulsed-field type 1 (NAP1) and PCR-ribotype 027 (BI/NAP1/027) (Pépin, *et al.*, 2004; Kuijper, *et al.*, 2007). Patients infected with the PCR-ribotype 027 strains have more severe diarrhoea, a higher mortality rate and a higher level of reoccurrence (Loo *et al.*, 2008; Goorhuis *et al.*, 2007; Redelings *et al.*, 2007; Pépin *et al.*, 2004).

Studies have shown that a number of BI/NAP1/027 strains produce higher levels of toxin in the laboratory, although the mechanisms for this increased toxin production remain unclear (Warny, *et al.*, 2005). In addition, it has been suggested that strains of the BI/NAP1/027 type are more prolific in terms of sporulation than non-outbreak strains (Wilcox and Fawley, 2000; Fawley, *et al.*, 2007; Akerlund, *et al.*, 2008). However, to-date it is still not clear exactly what causes the increased disease incidence and severity associated with BI/NAP1/027 strains of *C. difficile*. There are

a number of genes specific to PCR-ribotype 027 strains, a large proportion of which comprise of regulatory genes. Interestingly PCR-ribotype 027 strains carry an additional copy of the complete *agr* locus which is not found in the *C. difficile* 630 genome.

The study presented in Chapter Four set out to investigate the role of the second *agr* locus in regulation of virulence factors in *C. difficile* R20291. The *C. difficile* R20291 *agrB1* mutant showed a similar degree in toxicity towards both the vero cells and HT29 cells as the parental strain. However, both *agr2* mutants of *C. difficile* R20291 displayed a reduction in cytotoxicity *in vitro* (Figure 4.4). In light of these findings, it can be concluded that the *agr2* locus may play a secondary role in the regulation of toxin production in *C. difficile*. The exact mechanisms of how this is elucidated remains unclear however it could be possible that the two additional AIP's produced in this strain act as a molecular switch and increase toxin production. We observed that there is some variation in the sporulation profiles of the *C. difficile* R20291 *agr* mutants. There are no obvious differences in the rate at which the heat-resistant cfu are formed over 5 days in the *C. difficile* R20291 *agrA* mutant strain in comparison to the parental strain. In the *C. difficile* R20291 *agrB2* mutant sporulation is initiated after 24 hours, whereas the parental strain sporulates after 48 hours. On the other hand, the onset of sporulation in the *C. difficile* R20291 *agrB1* mutant is delayed, heat-resistant cfu are formed after 72 hours, in comparison to the parental strain. The delayed onset of sporulation was fully restored in *C. difficile* R20291 *agrB1* mutant strains carrying pMTL84151::*agrBD1* and the complemented *agrB1* mutant behaved in a similar manner to the *C. difficile* R20291 parental strain (Figure 4.5).

We observed differences in the sporulation profiles of the *agrB* and *agrB1* mutant strains of *C. difficile* 630Δ*erm* and *C. difficile* R20291, respectively. Sporulation in the *agrB* mutant of *C. difficile* 630Δ*erm* occurs after 24 hours whereas in the *C. difficile* R20291 *agrB1* mutant sporulation is initiated much later, after 72 hours. This suggests that the *agrBD* loci in the two *C. difficile* strains may have different regulatory roles. It is not clear why it would be beneficial for *C. difficile* R20291 to indirectly regulate the initiation of sporulation in this way, as the delay in the onset of sporulation is not seen in the *C. difficile* 630Δ*erm agrB* mutant. It could be that this is a phenomenon that only occurs in *C. difficile* R20291. However, it would be interesting to determine whether this sporulation profile is a characteristic of all PCR-ribotype 027 strains, as well as other *C. difficile* strains that carry the *agr2* locus.

A comparative study of UK and European clinical isolates of PCR-ribotype 027 strains revealed that the *agr2* locus is present in genomes of 86% of the strains tested, two of which were non-toxigenic strains (Marsden *et al.*, 2010). If the *agr2* locus is present in non-toxigenic strains it may be feasible that the increased virulence exhibited by the PCR-ribotype 027 strains is not a consequence of carrying the additional *agr2* locus. On the other hand, it could also be said that the *agr2* locus is not functional in the non-toxigenic *C. difficile* strains.

The characterisation of the *agr2* locus described in Chapter Four and Chapter Five has suggested a possible role of the *agr* genes in sporulation and toxin production in *C. difficile*, although it is obvious that the *agr2* locus must be further analysed to determine the role that it may be playing in regulation of *C. difficile* virulence. In

particular, it is necessary to complement the defect in the initiation of sporulation and the reduction of toxin production that was observed in the *agr* mutants, in order to show that the observed phenotypes was a specific consequence of the inactivation of the *agr2* genes. However, initial attempts to complement the sporulation defect in the *agrB2* mutant were not successful. As described in Chapter Two, plasmid pMTL84151::*agr2* was constructed, carrying the parental *agr2* locus coding region, the 400 bp region immediately upstream of the open reading frame presumed to contain its promoter and 269 bp region immediately downstream of the *agr*2 locus.

This plasmid was introduced into the *agrB2* mutant strain of *C. difficile* R20291, but the initiation of sporulation in this 'complemented' mutant strain occurred after 24 hours, the same as the *agrB2* mutant strain. This suggests that pMTL84151::*agr2* was not suitable for complementing the sporulation defect observed in the *agrB2* mutant strain. There are a number of reasons why this sporulation defect was not restored following expression of pMTL84151::*agr2*. Identification of a putative *agr2* promoter region was based on speculation that the promoter would be located immediately upstream of *agr2* locus. However, it appears that the 400 bp region immediately upstream of the open reading frame may not contain its promoter and it is therefore possible that a suitable promoter lies further upstream of the *agr2* locus. Identification of this promoter could be achieved using techniques such as primer extension but, alternatively, use of a known strong promoter may allow for efficient expression of *agr2* locus when attempting complementation studies in the *agr2* mutant strains.

Other explanations for the failure of pMTL84151::*agr2* expression in the complementation studies of the *C. difficile* R20291 *agr* mutants include the loss of pMTL84151::*agr2* during cultivation in the sporulation medium, altered sporulation frequency of mutant strains carrying pMTL84151::*agr2* (this effect could be analysed by observing spores under phase-contrast microscopy), or indeed the possibility that the observed mutant phenotype is not a consequence of inactivation of *agr2*, instead a result of a potential second ClosTron insertion. This could be clarified by using Southern Blot analysis to identify intron insertions in the *agr2* mutant strain. The observed mutant phenotypes could also be the result of a spontaneous mutation, which cannot be confirmed by any method. In such case, the obvious approach would be to create further identical mutants and then attempt to complement their respective mutant phenotypes.

7.1.3 Transcriptional analysis of the *agr* operon

Transcriptional linkage of the *agr* genes was investigated using the RT-PCR. Our results revealed that all the genes within *agr* region 1 and *agr* region 2 are co-expressed and show expression throughout early and late exponential growth; there is no obvious difference in the levels of expression at the two time points analysed. A positive RT-PCR result was obtained for all gene pairs tested with the exception of gene pairs *ptsI* and CD2641. However, there was no amplification from the positive control in this reaction either. The primer pairs were tested prior to being used in the RT-PCR reactions. The primers that were designed to amplify ptsI/CD2753 were tested on a genomic DNA template using a Phusion Taq polymerase to ensure the primers were annealing correctly and there was no non-

specific amplification. The results of the test showed that the primers annealed and amplified as expected, data not shown.

The *agr* regions in other clostridia species, such as *C. perfringens* and *C. botulinum*, have been shown to form an operon and be transcribed from a single RNA transcript (Ohtani *et al.*, 2009; Cooksley *et al.*, 2010). These findings are consistent with what has been shown in *S. aureus* and other gram positive bacteria that carrying an *agr* operon. However, this does not seem to be apparent for *C. difficile* strains 630 and R20291. Our data suggests that maybe the *agr* genes in *C. difficile* 630 and *C. difficile* R20291 are transcribed on a polycistronic mRNA and not as a single operon. Further studies need to be undertaken to determine this. Analysis of transcriptional linkage using RT-PCR is not necessarily a quantitative method and therefore, we were unable to predict the size of the mRNA transcript using these results. A Northern blot is a more quantitative method which would enable us to determine the actual size of the mRNA transcript.

7.1.4 *agr2* and *C. difficile* 630Δ*erm*

We demonstrated that introduction of the *agr2* locus into the *C. difficile* 630Δ*erm* strain on a plasmid, increases the cytotoxicity of this strain *in vitro*. Interestingly, the cytotoxicity levels of this recombinant strain mirrored the cytotoxicity shown by *C. difficile* R20291. These findings further support the work described in the previous chapter, and suggests that the second *agr* locus in *C. difficile* R20291 may be a contributing factor in increased toxin production phenotype that is observed in PCR

ribotype 027 strains. It would be interesting to analyse the effect of adding culture supernatant taken from the *C. difficile* R20291 *agr2* mutant strains to *C. difficile* 630Δ*erm* cells to see whether the increase in cytotoxicity occurs in the absence of AIP1 and AIP2 peptides (assumed to be processed by AgrB1 and AgrB2, respectively). Further work needs to be undertaken to fully elucidate the role of *agr2* in this strain.

Surprisingly, *C. difficile 630Δerm* harbouring pMTL84151::*agr2* appeared to have no effect on heat resistant colony formation after heat treatment. Sporulation in all the strains was initiated at 24 hours. The level of heat resistant cfu formed by *C. difficile* carrying the empty plasmid was slightly lower than the *C. difficile* 630Δ*erm*. These results indicate that *agr2* in *C. difficile* 630Δ*erm* background does not alter the onset of sporulation in this strain. We observed that sporulation in the *C. difficile* R20291 occurs after 48 hours, whereas sporulation in *C. difficile 630Δerm* harbouring pMTL84151::*agr2* occurs after 24 hours consistent with its parental strain.

Sporulation in organisms such as *B. subtilis* has been extensively studied (Sonenshein, 2000). On the other hand, the conditions which lead to sporulation initiation in *C. difficile* are not clear and our understanding of this complex process in *C. difficile* remains poor. Even though it is difficult to draw any definite conclusions from our observations, it is clear sporulation in *C. difficile* is regulated by many different factors and regulation by the *agr* locus may only be one factor of this very complex process.

7.1.5 LuxS/AI-2 signalling in *C. difficile*

Inactivation of *luxS* homologues in *C. difficile* 630Δ*erm* and *C. difficile* R20291 had no obvious effect on sporulation and toxin production, suggesting that *C. difficile* does not use the AI-2 autoinducer to regulate these processes. It has been reported that *luxS* is transcribed at higher levels during late exponential growth when AI-1 production is maximal (Carter *et al.*, 2005). Toxin production in *C. difficile* is maximal during stationary growth phase. Thus is not obvious how a LuxS/AI-2 QS signalling system could regulate the production of toxins in *C. difficile,* as the toxins are maximally produced after the AI-2 signal molecule is depleted. Although LuxS is involved in toxin production in *C. perfringens*, inactivation of *luxS* in *C. difficile* does not have an effect on virulence. The data presented in this study further supports the theory that LuxS is a metabolic side product produced during the activated methyl cycle and does not have a role in quorum sensing (Winzer *et al.*, 2003). The findings of Doherty *et al.* (2006) demonstrated that the inactivation of *luxS* in *Staphylococcus aureus* had no effect on virulence-associated traits. It is possible that the phenotypic changes observed in some bacteria following inactivation of *luxS*, may be due to the absence of the AI-2 signal molecule, but they could also be attributed to the disruption of the activated methyl cycle (Winzer *et al.*, 2003).

7.2 Future research

7.2.1 Furthering our understanding of *agr* in *C. difficile*

To date it is still not clear what role *agr* plays in the regulation of virulence in *C. difficile*. Our investigation into *agr* regulation of virulence factors has only scratched

the surface. This work presented in this thesis mainly focussed on screening *agr* mutants with two phenotypes, toxin production and sporulation. The main reason for this was that at the time this study began these two assays were developed in our laboratory. Unlike *C. perfringens* and *C. botulinum* in which *agr* system regulates toxin production (and sporulation in *C. botulinum*), *agr* in *C. difficile* may be involved in the regulation of other virulence factors or metabolic pathways. Analysis of the transcriptional profiles of the *agr* mutants will be useful to identify what other virulence factors or metabolic pathways are regulated by the *agr* system in *C. difficile*. Once it has been revealed what processes or pathways are regulated by *agr*, it will be easier to characterise mutants by assaying a phenotype known to be regulated by the *agr* locus.

Preliminary microarray studies with the *C. difficile* R20291 *agrA* mutant were commenced as part of this study however due to time constraints we were unable to present the data in this thesis as the data analysis has not been completed. RNA was extracted from the wildtype and mutant strains during early and late exponential/stationary growth, when OD_{600} readings were 0.4 and 1.2. Preliminary analysis of the microarray data revealed that in the *agrA* mutant strain, expression of the PaLoc (pathogenecity locus which encodes the toxin A and B genes) is upregulated during late exponential/stationary growth (R. Stabler, personal communication). This contradicts our observation that there is a decrease in cytotoxicity in the *agrA* mutant towards the vero and HT29 cells. One way to confirm our observation that there is a reduction in toxin production in the *agrA* mutant would be to perform quantitative PCR with reverse transcription (qRT-PCR) analysis.

A follow-up to this study would be a comprehensive analysis into the regulation of virulence factors in a wider range of *C. difficile* strains, incorporating many more isolates from many more types of *C. difficile*. Only with such a study will it be possible to make a more definitive statement about how sporulation and toxin production is regulated in different *C. difficile* strains and types, and its subsequent role in disease incidence and severity.

7.3 Concluding Remarks

Our knowledge of how *C. difficile* regulates the production of virulence and whether quorum sensing is involved in these processes remains limited. However, the work presented in this study has shown that the *agr* system may be involved in toxin production and sporulation. *agr* mutant strains exhibited a substantial reduction in the production of toxins and the sporulation profiles of these mutant strains vary greatly in comparison to the parental strains. Reports to-date have only scratched the surface in furthering our understanding of the regulation of virulence in *C. difficile*, but with a range of genetic tools now at our disposal (Heap, *et al.*, 2007; Heap, *et al.*, 2009; Heap, *et al.*, 2010), the way is open to understand more thoroughly this important aspect of CDAD and thereby develop effective intervention strategies.

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