Analysis of the spore germination mechanisms of *Clostridium difficile*

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Abstract

Clostridium difficile is the leading cause of hospital-acquired diarrhoea and a major burden to healthcare services worldwide. Endospore production plays a pivotal role in infection and disease transmission, but in order to cause disease these spores must germinate and return to vegetative cell growth. Therefore, knowledge of spore germination is important and may have direct applications in future disease prevention. Germination has been well studied in *Bacillus* and in some clostridia, but the mechanisms of *C. difficile* spore germination remain unclear. Apparent homologues of genes important for germination in other spore formers have been identified in the *C. difficile* genome and ClosTron technology was used to inactivate homologues of *sleC*, *cspA*, *cspB* and *cspC* (*Clostridium perfringens*) and *cwlJ*, *sleB* and *cwlD* (*Bacillus subtilis*) in both *C. difficile* 630Δ*erm* and a BI/NAP1/027 isolate (a 'hypervirulent' type associated with outbreaks of increased disease severity). Using a combination of several different assays to study these mutants in detail, a number of the identified target genes appear to be essential for germination and outgrowth of *C. difficile* spores. This is the first report of using reverse genetics to study the germination of *C. difficile* spores and the first gene characterisation by mutagenesis in a BI/NAP1/027 isolate of *C. difficile*. Furthermore, this study uncovered evidence of significant variation in the sporulation and germination characteristics of different *C. difficile* strains, but this variation did not appear to be type-associated.

Publications relating to these studies

Heap, JT, Cartman, ST, Pennington, OJ, Cooksley, CM, Scott, JC, Blount, B, Burns, DA. and Minton, NP (2008) Development of genetic knock-out systems for clostridia. In: Bruggermann, H, Gottschalk, G. (Eds), Clostridia: Molecular biology in the post-genomic era. Caister Academic Press. Norfolk, UK, pp. 179-198.

Burns, DA, Heap, JT and Minton, NP (2010) SleC is essential for germination of *Clostridium difficile* spores in nutrient-rich medium supplemented with the bile salt taurocholate. *J. Bacteriol.* **192**: 657-664.

Burns, DA, Heap, JT and Minton, NP (2010) *Clostridium difficile* spore germination: an update. *Research in Microbiology* **161**: 730-734.

Burns, DA, Heap, JT and Minton, NP (2010) The diverse sporulation characteristics of *Clostridium difficile* clinical isolates are not associated with type. *Anaerobe* **16**: 618-622.

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Abbreviations

CDAD	*Clostridium difficile*-associated diarrhoea
PG	Peptidoglycan
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
BLAST	Basic Local Alignment Search Tool
NAM	N-acetyl-muramic acid
UV	Ultra-violet
DPA	Dipicolinic acid
SASP	Small acid-soluble spore proteins
NAP1	North American pulsed-field type 1
PCR	Polymerase chain reaction
LB	Luria-Bertani medium
Xgal	Bromo-chloro-indolyl-galactopyranoside
BHIS	Supplemented brain-heart infusion medium
OD	Optical density, at the wavelength specified in the text
g	Gravity
PBS	Phosphate-buffered saline
CFU	Colony forming units
BHI	Brain-heart infusion medium
bp	Base-pair(s)
ORF	Open reading frame
SCLE	Spore cortex lytic enzyme

Chapter One

Introduction

1.1 *Clostridium difficile*

1.1.1 Clostridium difficile: emergence of a significant human pathogen

The genus *Clostridium* is comprised of many species of Gram-positive, obligate anaerobic, rod shaped, endospore-forming bacteria. The vast majority are entirely benign, and have recently become of importance due to their potential beneficial properties, both economical and medical. For example, interest in *Clostridium acetobutylicum* has grown rapidly, due to its role in bio-butanol production (Jones and Woods, 1986; Lee, *et al.*, 2008). Furthermore, a significant role in the treatment of cancer has also been described for *Clostridium sporogenes* and *Clostridium novyi* (Minton, *et al.*, 1995; Theys, *et al.*, 2006). However, the beneficial properties of these species have largely been overshadowed by the actions of a few. *Clostridium perfringens* is a significant cause of gastrointestinal diseases in humans and animals (Collie, *et al.*, 1998), and *Clostridium botulinum*, while widely used in the cosmetics industry and of importance for a number of medical applications, 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). Nevertheless, the most serious headlines in recent times have been reserved for the emergence of *Clostridium difficile*.

C. difficile is capable of residing in the gut of mammals, and is the causative agent of *C. difficile*-associated disease (CDAD). Originally named *Bacillus difficilis*, *C. difficile* was first described in 1935 as part of neonatal intestinal

microflora (Hall and O'Toole, 1935). However, its true potential as a human pathogen was not known until 1978, when the first confirmed case of CDAD was reported (Larson, *et al.*, 1978). In 1999, clindamycin, the antibiotic of choice at the time, was identified as a major risk factor for CDAD following the study of a clindamycin-resistant strain of *C. difficile* at the heart of four outbreaks across the United States of America (Johnson, *et al.*, 1999). In recent years, outbreaks of CDAD have led to patient isolation, ward closures, and in some cases, hospital closure. *C. difficile* has now become recognised as the major cause of hospital-acquired diarrhoea and is a huge burden to healthcare services across Europe, North America and some parts of Asia.

Surveillance of positive *C. difficile* laboratory samples in England and Wales was introduced in 1990, as part of a voluntary monitoring scheme. Over the following 15 years, the incidence of CDAD rose dramatically (Figure 1.1). As a result, a mandatory reporting scheme was introduced in 2004 which required all National Health Service Trusts in England to report cases of CDAD in patients aged 65 and over. Since then, similar mandatory surveillance schemes have begun across the United Kingdom, covering every patient aged 2 or over, in an attempt to accurately assess the incidence of CDAD. In 2008, *C. difficile* was the underlying cause of more than 2500 deaths in England and Wales alone (Figure 1.2), more than 10 times that of methicillin-resistant *Staphylococcus aureus* (from the website for the Office of National Statistics, http://www.statistics.gov.uk).

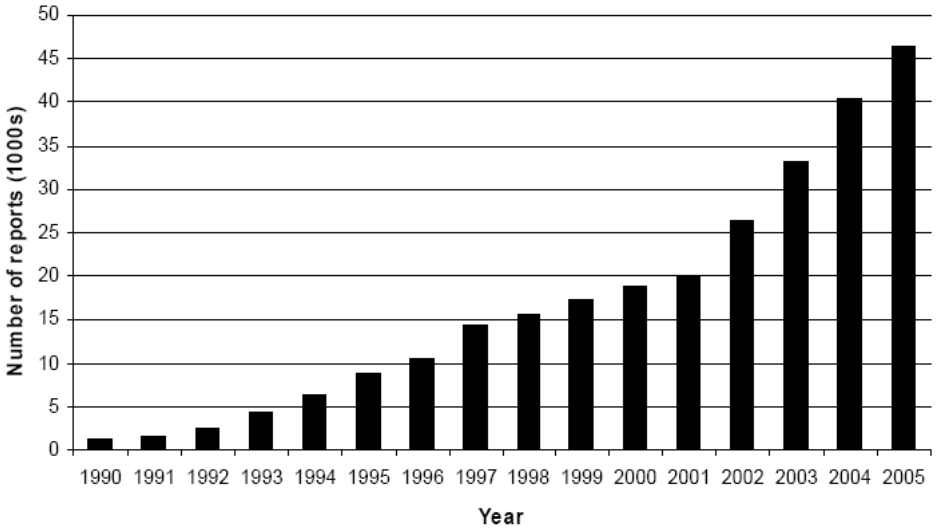

Figure 1.1. Reports of positive *C. difficile* laboratory samples isolated from faecal specimens under the voluntary reporting scheme in England between 1990 and 2005. From the website of the Health Protection Agency (http://www.hpa.org.uk).

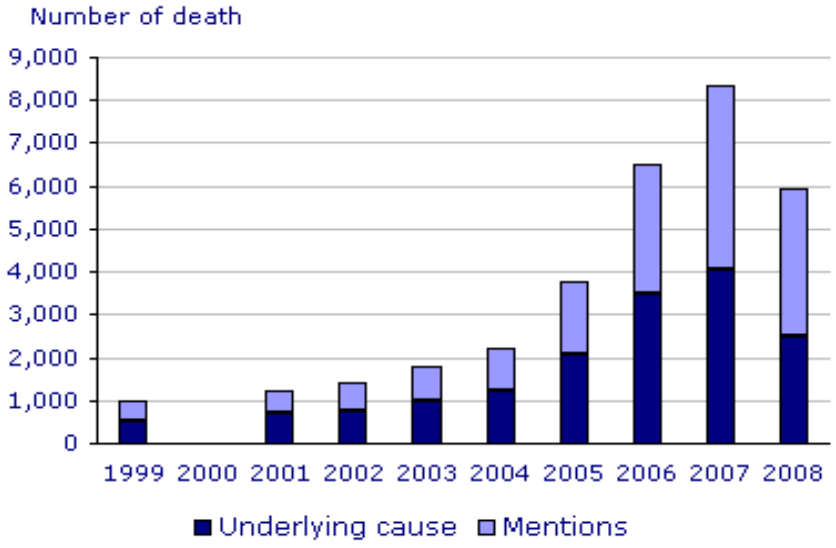

Figure 1.2. Number of death certificates mentioning *C. difficile* in comparison to where *C. difficile* was noted as the underlying cause of death in England and Wales between 1999 and 2008. A mandatory surveillance scheme was introduced between 2004 and 2005, which may partly account for the large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

1.1.2 C. difficile disease

C. difficile is commonly isolated from faecal specimens obtained from neonates and the elderly. In many cases carriage is asymptomatic (as is especially the case in neonates); however, in susceptible individuals *C. difficile* can cause diarrhoeal diseases ranging from asymptomatic carriage to a fulminant, relapsing and potentially fatal pseudomembranous colitis (Poxton, *et al.*, 2001). The major risk groups for CDAD are the elderly and those individuals whose normal intestinal microflora has been disrupted following antibiotic treatments (Johnson, *et al.*, 1999). *C. difficile* subsequently capitalises on this disruption of gut flora to colonise the large intestine, causing disease symptoms through the action of two large toxins TcdA and TcdB, which are commonly referred to as toxin A (enterotoxin) and toxin B (cytotoxin) (Poxton, *et al.*, 2001; Voth and Ballard, 2005).

Treatment of CDAD is far from straightforward as *C. difficile* possesses a large number of mobile elements in the genome carrying antibiotic resistance genes (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). Currently, the only available antibiotics for treating CDAD are metronidazole and vancomycin and these antibiotics, when combined with patient isolation procedures, remain the principal therapeutic option.

1.1.3 C. difficile virulence factors

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. This causes disruption of the actin cytoskeleton and tight junctions, ultimately resulting 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). 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).

Initially, it was generally accepted that toxin A was the principal virulence factor responsible for CDAD. However, a recent study concluded that toxin B is required for disease in Golden Syrian hamsters (Lyras, *et al.*, 2009), and this hypothesis is supported by evidence that a significant number of clinically relevant *C. difficile* strains do not produce toxin A, yet remain highly virulent. Further research into this area is clearly needed, as data from our laboratory would appear to suggest that both toxin A and toxin B alone are sufficient for disease in the hamster model (Kuehne, *et al.*, 2010).

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), although data from our laboratory suggest that this may not be the case (Soza Baban, Sarah Kuehne and Nigel Minton, unpublished results).

1.2 Endospores of *C. difficile*

Many bacterial species are able to use specialised differentiated cell types as a means of surviving in conditions otherwise detrimental to their vegetative cell form. The primary purpose of endospore production is to ensure survival of the organism, but as a consequence the endospore form is now widely recognised as a route of infection for transmissible diseases such as CDAD. While toxins are the principal virulence factors of *C. difficile*, the ability of the organism to produce endospores is necessary for disease transmission. Clostridial 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). Thus, 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 presumably germinate to form the characteristic toxin-producing vegetative cells and, in susceptible individuals, diarrhoeal disease.

1.2.1 Sporulation

Sporulation is the adaptive process by which a bacterial species forms metabolically dormant, highly stress resistant endospores. The most widely examined spore-forming organism is *Bacillus subtilis*, and the mechanisms by which it (i) senses a suitable environment for sporulation; and (ii) initiates and completes the process have become a useful paradigm for such systems in other organisms.

The mechanisms of sporulation initiation in *B. subtilis* are now understood to an exquisite level of detail, beyond the scope of this introduction. The process is driven by a temporally and spatially controlled program of gene expression (Sonenshein, 2000; Paredes, *et al.*, 2005), the commencement of which relies on the vegetative cell reaching a certain stage of its growth cycle (Grossman, 1995; Parker, *et al.*, 1996). Briefly, the main stimulus for sporulation in *B. subtilis* is nutrient-starvation (Schaeffer, *et al.*, 1965; Sonenshein, 2000; Driks, 2002). This environmental signal can drive phosphorylation of the master

regulator of sporulation, Spo0A, which then functions to repress *abrB*, a repressor of numerous stationary phase genes, a number of which are required for sporulation (Weir, *et al.*, 1991). Upon reaching a threshold of Spo0A-P, the sporulation cascade is initiated (Fawcett, *et al.*, 2000; Sonenshein, 2000). There are five principal events which follow commitment to sporulation (Errington, 2003; Paredes, *et al.*, 2005). As described in Figure 1.3, these stages involve (i) an asymmetrically positioned septum divides the cell into the prespore and mother cell compartment; (ii) the prespore is engulfed to form a protoplast within a double membrane; (iii) the spore peptidoglycan (PG) cortex is formed; (iv) an ordered sequence of gene expression results in spore coat formation; and (v) the mature spore is finally released by cell lysis into the surrounding environment.

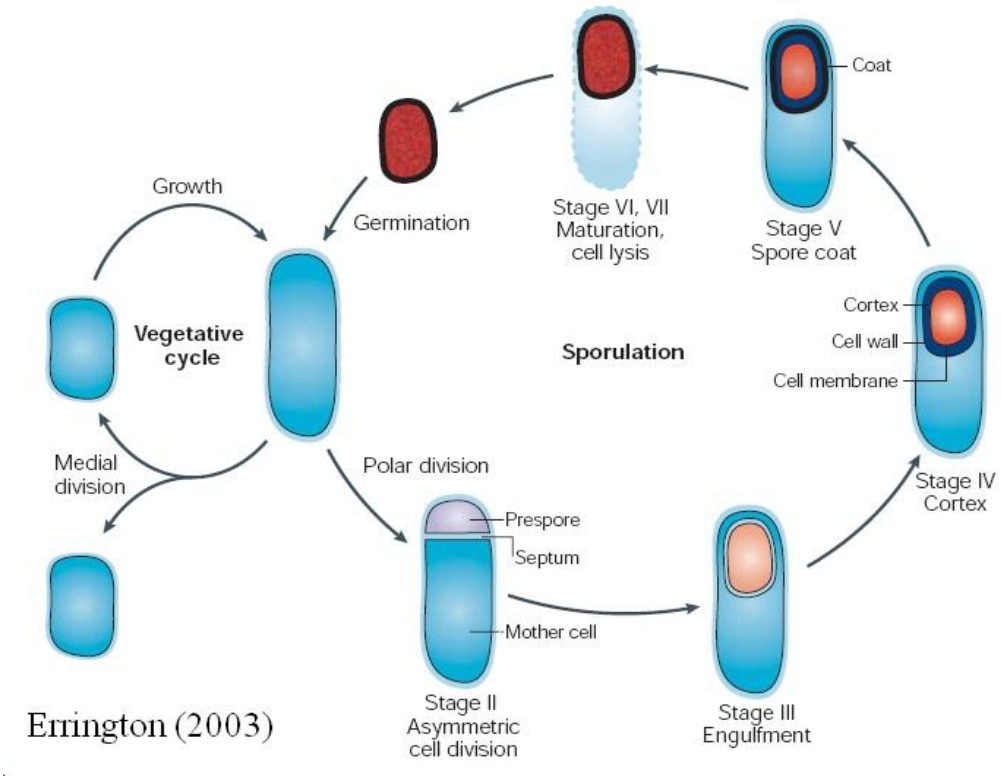

Figure 1.3. A simplified diagram of the *B. subtilis* cell cycle, indicating the principal stages of sporulation (Errington, 2003).

Sporulation in organisms such as *B. subtilis* has been extensively studied. On the other hand, the conditions which lead to sporulation initiation in *C. difficile* are not clear. Figure 1.4 illustrates the genetic basis of the *B. subtilis* sporulation cascade, with appropriate annotations of proteins of interest that have been identified in *C. difficile*. It has been shown that Spo0A is required for spore formation in *C. difficile* (Heap, *et al.*, 2007), and that a putative sporulation-associated kinase, CD2492, may play a role in phosphorylation of Spo0A (Underwood, *et al.*, 2009), but our understanding of this complex process in *C. difficile* remains poor.

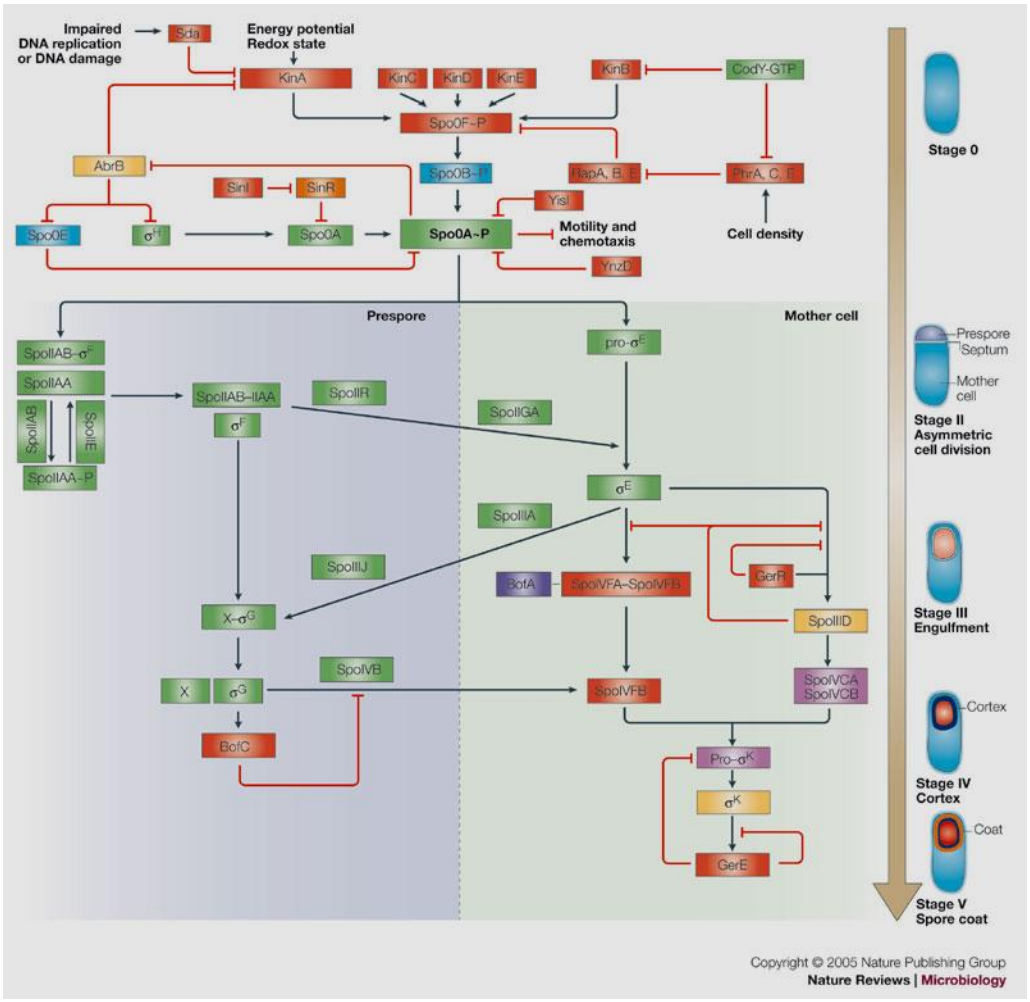

Figure 1.4. The sporulation cascade in *B. subtilis* (Paredes, *et al.*, 2005). Those labelled in green, pink and yellow represent proteins identified in *C. difficile*, and presumed to play a role in sporulation.

1.2.2 The structure of a bacterial spore

In spores of *B. subtilis*, the core containing the spore DNA, RNA, and most enzymes, is enclosed within the spore inner membrane. This membrane has a low permeability to small molecules and perhaps even water. As a result, the spore core is protected from DNA-damaging chemicals and has a very low water content, presumed to play a role in the heat resistance of the spore

(Popham, *et al.*, 1996a; Nicholson, *et al.*, 2000; Cortezzo and Setlow, 2005; Setlow, 2006; Setlow, 2007). Surrounding the spore inner membrane is the germ cell wall, a thin layer of PG identical to that of vegetative cell PG, which becomes the cell wall on the return to vegetative cell growth. The germ cell wall is itself surrounded by the cortex, a thicker layer of spore-specific PG which differs from vegetative cell PG in two major ways (Atrih, *et al.*, 1996; Popham, *et al.*, 1996b; Atrih and Foster, 1999; Popham, 2002). Essentially all *N*-acetylmuramic acid (NAM) residues of germ cell wall and vegetative cell PG carry short peptides, but in spore PG only about 25% of NAM residues carry these peptides. Consequently, spore PG is less highly cross-linked than vegetative cell PG, which may play a role in spore heat resistance (Atrih and Foster, 1999). Furthermore, approximately every second muramic acid residue in spore PG is replaced by muramic-δ-lactam (Warth and Strominger, 1969; Warth and Strominger, 1972), and, as is discussed below, it has been shown that these δ-lactam residues are heavily involved in the germination process.

The cortex is surrounded by an outer membrane, required for sporulation but not known to be a permeability barrier in dormant spores (Setlow, 2007). In many species of *Bacillus*, the outermost layer of the spore is the coat, consisting of an ordered assembly of more than 40 different proteins and functioning mainly to protect the spore by acting as a barrier to harmful molecules (Zheng, *et al.*, 1988; Driks, 1999). In some, but not all, *Bacillus* species, the outermost layer is a "balloon-like" protein layer known as the exosporium (Charlton, *et al.*, 1999; Todd, *et al.*, 2003; Redmond, *et al.*, 2004;

Setlow, 2007). The precise function of the exosporium is not known. As it is not present in spores of the non-pathogenic *B. subtilis*, but is present in spores of the pathogenic *Bacillus anthracis* (Todd, *et al.*, 2003; Redmond, *et al.*, 2004), it is plausible that the exosporium may play a role in the pathogenesis of some spore forming organisms. This hypothesis is supported by the evidence that *C. difficile* spores possess an exosporium layer (Lawley, *et al.*, 2009). However, it is not clear what role the exosporium may play in the disease caused by *C. difficile*.

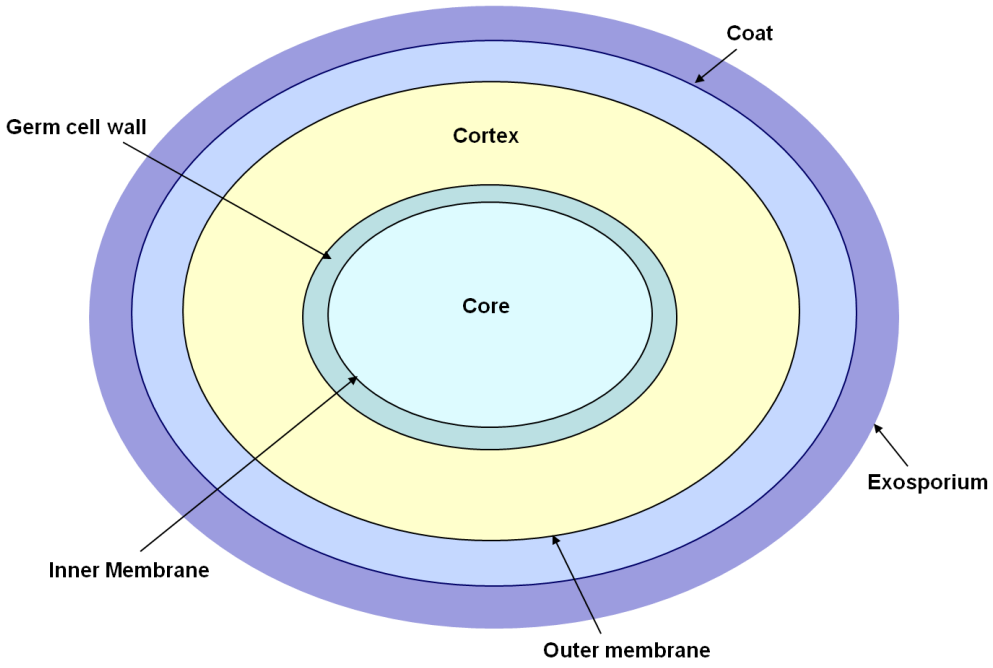

Figure 1.5. Structural diagram of a bacterial spore, adapted from Setlow (2003). The respective layers are not drawn to scale and the large exosporium is not present in all species of *Bacillus*, but has been shown to exist in *C. difficile* spores.

1.2.3 Resistance properties of bacterial spores

Bacterial spores are unified by their ability to resist extreme environments that would otherwise be fatal to vegetative cells. Spores of *Bacillus* and *Clostridium* can survive exposure to stress conditions such as wet and dry heat, UV radiation and many toxic chemicals including ethanol (Nicholson, *et al.*, 2000; Setlow, 2006; Setlow, 2007). As described above, the major role of the coat and spore inner membrane is likely to protect the spore, presumably acting as a barrier against harmful chemicals. The PG cortex has been strongly linked to maintaining spore dormancy and heat resistance (Ellar, 1978; Atrih and Foster, 1999), as spores lacking the coat are heat resistant but cannot grow as a vegetative cell until the cortex is degraded.

Despite many structural factors contributing to spore resistance, the crucial aspect of these properties, and as a result the key to the long-term survival of the spore, lies in the mechanisms by which the DNA in the spore core is protected and repaired (Setlow, 2007). The spores' large stock of dipicolinic acid (DPA), almost certainly existing as a 1:1 chelate with Ca^{2+} (CaDPA), plays a major role in protecting core DNA from damage associated with wet and dry heat, desiccation and chemicals such as hydrogen peroxide (Setlow, *et al.*, 2006; Setlow, 2007). Furthermore, arguably the most important mechanism of spore DNA protection is provided by the α/β-type small, acid-soluble spore proteins (SASPs), which are synthesised during sporulation and saturate spore DNA to prevent potentially fatal DNA-damaging mutations (Setlow, 2007). These α/β-type SASPs have been identified in many *Bacillus*

and *Clostridium* species and are crucial for spore DNA protection, as decreased levels of these SASPs render the spore far more susceptible to DNA-damaging agents (Raju, *et al.*, 2006; Setlow, 2006; Setlow, 2007).

1.3 Spore germination

1.3.1 Model of bacterial spore germination

Spore germination is defined as the irreversible loss of spore-specific characteristics, is required for conversion of dormant spores to vegetative cells and, in pathogenic spore formers, is essential for subsequent disease. Current mechanistic knowledge of spore germination has been principally obtained from studying *B. subtilis* while, in general terms, little is known of the mechanisms responsible for germination in clostridia and, in particular, *C. difficile*.

Germination is initiated when a spore senses specific effectors, termed germinants. Spores of *B. subtilis* can germinate through the binding of germinants, either L-alanine or a mixture of asparagine, glucose, fructose and potassium ions, to specific receptors located in the spore inner membrane (Paidhungat and Setlow, 2000). The spore is then committed to germination and subsequent events include the release of monovalent cations (H^+, Na^+ and K^+) and a large depot (~25% of spore dry weight) of CaDPA (Setlow, 2003), a process for which SpoVA proteins are required (Vepachedu and Setlow, 2004; Vepachedu and Setlow, 2007a; Vepachedu and Setlow, 2007b). The third

major step in germination involves the hydrolysis of the spore PG cortex. It is during this hydrolysis that the previously low water content of the spore is returned to that of a vegetative cell and the core is able to expand, in turn allowing for enzyme activity, metabolism and, ultimately, vegetative cell outgrowth (Moir, 2003; Setlow, 2003; Moir, 2006).

Generally speaking, the germination mechanisms of *Clostridium* spores have been studied in far less detail than *Bacillus* spores. A tricistronic *gerA* operon has been identified in *C. botulinum* and *C. sporogenes*, and recent work in *C. perfringens* has indicated that monocistronic *gerA* and *gerKB*, and a bicistronic *gerKA-gerKC* operon play a role in triggering the germination process (Paredes-Sabja, *et al.*, 2008; Paredes-Sabja, *et al.*, 2009a). However, none of these systems are present in *C. difficile*.

1.4 Spore germination in *C. difficile*

Very little is known of the germination mechanisms in *C. difficile*, and this is due in part to a historical absence of genetic tools. Consequently, studying the genetics of *C. difficile* has been extremely challenging. In the years preceding this thesis, reports of *C. difficile* germination mechanisms have almost exclusively focussed on the interactions of spores with their surrounding environment, and the host factors which may influence the behaviour of *C. difficile* spores *in vivo*.

1.4.1 Role of bile salts in C. difficile spore germination

Bile is produced in the liver and functions to aid digestion and suppress significant bacterial colonisation in the small intestine (Ridlon, *et al.*, 2006). The two primary bile acids synthesised in the liver are cholate and chenodeoxycholate, which are further metabolised via conjugation to glycine or taurine. As bile acids pass through the distal ileum, they are readily reabsorbed and recycled into the liver, although a proportion (~5%) of this bile avoids active transport and escapes into the cecum (Ridlon, *et al.*, 2006). A number of bacterial species, including *C. perfringens*, possess bile-salt hydrolases (Coleman and Hudson, 1995), which contribute to the removal of conjugated amino acids from the bile salt. These deconjugated cholate and chenodeoxycholate derivatives can be further converted, by bacterial species in the large intestine, into the secondary bile salts deoxycholate and lithocholate, respectively (Ridlon, *et al.*, 2006).

The specific conditions by which *C. difficile* spores sense a suitable environment for germination remain unclear. However, recent work has suggested that bile salts play a pivotal role. Thus, following on from the earlier observation that taurocholate improves the recovery of *C. difficile* spores from environmental surfaces (Wilson, *et al.*, 1982), a study by Sorg and Sonenshein demonstrated that specific bile salts and glycine act as cogerminants of *C. difficile* spores, while the secondary bile salt deoxycholate prevented vegetative cell growth (Sorg and Sonenshein, 2008). They hypothesise that, in a healthy host, *C. difficile* spores can survive passage into

the jejunum where they germinate in response to the high concentrations of cholate derivatives and glycine. However, in such individuals, cholate derivatives that escape enterohepatic circulation are metabolised into the secondary bile salt deoxycholate. Thus, outgrowth of *C. difficile* spores is prevented. Conversely, following disruption of intestinal flora, secondary bile salts such as deoxycholate are not readily produced. *C. difficile* vegetative cell growth is therefore not prevented and disease may occur in susceptible individuals.

A further study by Sorg and Sonenshein has described the role of the primary bile salt chenodeoxycholate in the inhibition of *C. difficile* spore germination (Sorg and Sonenshein, 2009). This adds an interesting element to their model of *C. difficile* colonisation. In equal concentrations, both cholate and chenodeoxycholate derivatives may compete for binding to putative *C. difficile* germinant receptors. However, as the rate of absorption by the colon of chenodeoxycholate is 10 times that of cholate (Mekhjian, *et al.*, 1979), spores reaching the large intestine encounter a higher concentration of cholate derivatives. This suggests that germination may be inhibited until *C. difficile* spores reach the anaerobic environment of the large bowel, where conditions are suitable for *C. difficile* vegetative cell growth. This model of colonisation has been largely supported by a recent study which analysed the kinetics of *C. difficile* germination in response to bile salts and glycine, and proposed that taurocholate/glycine receptor complexes are present in *C. difficile* spores (Ramirez, *et al.*, 2010).

A recent study has further expanded our knowledge of how *C. difficile* spores may interact with bile salts (Giel, *et al.*, 2010). The authors described that, using an extract from mouse small intestine, a factor produced *in vivo* stimulated colony formation of *C. difficile* spores. Treatment of the extract with cholestyramine, a bile salt binding resin, reduced the ability of the extract to stimulate colony formation, suggesting that the factor is likely a bile salt. Furthermore, colony formation was stimulated to greater levels in extracts from mice treated with clindamycin, ampicillin or streptomycin, suggesting that disruption of normal intestinal flora plays a role in the germination frequency of *C. difficile* spores *in vivo*. Indeed, a possible role of antibiotics such as fluoroquinolones and clindamycin in inducing *C. difficile* germination has been described, further supporting this hypothesis (Baines, *et al.*, 2009; Saxton, *et al.*, 2009).

These studies now suggest that the interaction of *C. difficile* with bile salts and antibiotics, coupled with disruption of normal intestinal flora, plays an important role in *C. difficile* colonisation and subsequent disease. Consequently, it now becomes important to understand the detailed mechanism of such interactions and this requires knowledge of the genetic basis of *C. difficile* spore germination.

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

1.5.1 BI/NAP1/027 strains of C. difficile

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. Indeed, the BI/NAP1/027 type is now the most commonly isolated *C. difficile* type in England (Figure 1.6). 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).

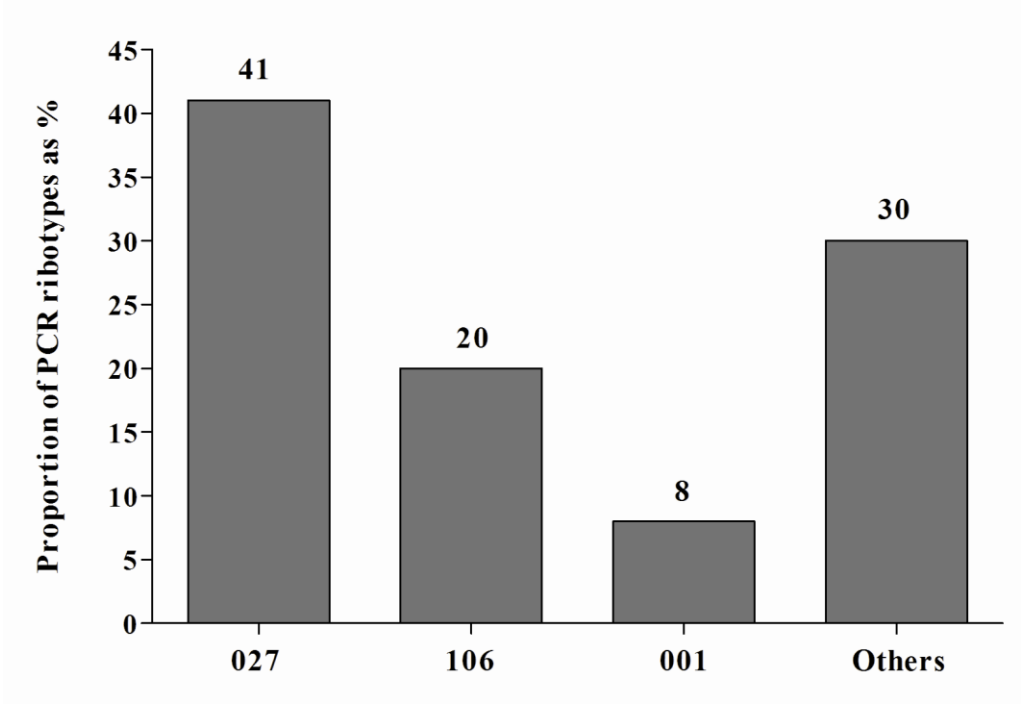

Figure 1.6. Distribution of *C. difficile* PCR ribotypes in England in 2007-8, adapted from Brazier *et al*. (2008). The data represent the national distribution of PCR ribotypes identified from a study of 677 individual isolates. The incidence of cases due to type 027 strains increased by 15.4% compared 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).

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), and one study has also suggested that BI/NAP1/027 strains may differ in their germination characteristics following antibiotic treatment in the patient (Saxton, *et al.*, 2009). 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*.

1.5.2 Other important C. difficile types

Whilst BI/NAP1/027 strains of *C. difficile* have received the most attention, a number of other groups of highly virulent *C. difficile* strains must be considered. Among only a handful of PCR-ribotypes isolated in the UK prior to 2003 (Dawson, *et al.*, 2009), PCR-ribotype 106 strains are the most commonly isolated non-BI/NAP1/027 *C. difficile* type in England (Figure 1.6), although this type is extremely rarely isolated outside of the United Kingdom (Barbut, *et al.*, 2007). Furthermore, of particular interest are those strains belonging to PCR-ribotypes 017 and 078, which have been isolated in parts of Asia and Europe (Drudy, *et al.*, 2007; Kim, *et al.*, 2008) and have also been associated with severe disease. In addition, and along with BI/NAP1/027 strains, these types have also been identified in animals, suggesting a reservoir and indeed a possible role of *C. difficile* as a food-borne pathogen (Songer and Anderson, 2006; Gould and Limbago, 2010).

1.6 Development of genetic tools to study *C. difficile*

The lack of in-depth studies into the genetic basis of *C. difficile* spore germination has been largely attributed to an absence of reliable genetic tools. Historically, 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). Unfortunately, such integrants are unstable and more preferential double-crossover mutants, achieved through allelic exchange, had not been described in *C. difficile* prior to the beginning of this work. However, 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 technology (Karberg, *et al.*, 2001). The mobile intron can be specifically targeted to a gene of interest by modification of intron RNA, and an intron-encoded protein (required for intron mobility) is provided *in trans*, which allows for its subsequent removal following integration and ensures integrant stability. Nested within the group II intron is an antibiotic resistance gene, itself interrupted by a self-splicing group I intron. This 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 is strictly a consequence of the integration event. In terms of studying *C. difficile* germination, the clear application of the

ClosTron is the systematic identification and inactivation of genes encoding homologues of proteins important for germination in other spore formers, and thus presumed to be important for the germination of *C. difficile* spores.

When studying the function of a particular gene mutation, complementation studies are vital to show that any observed mutant phenotype(s) is a specific consequence of target gene inactivation. This is commonly achieved through introduction of an ectopic copy of the gene in question, but such complementation has only been briefly reported in *C. difficile* (O'Connor, *et al.*, 2006; Carter, *et al.*, 2007). A modular system for *Clostridium* shuttle plasmids has now been described, allowing for simple construction and modification of plasmids based on a set of standardised components (Heap, *et al.*, 2009). Complementation of *C. difficile* mutants may, therefore, be easier to undertake in the future with such a system.

1.7 This project

1.7.1 Clinical importance of studying C. difficile spore germination

C. difficile remains the major cause of hospital-acquired diarrhoea and cases of CDAD are now emerging in the community and in animals used for food (Songer and Anderson, 2006). In addition, the emergence of hypervirulent types associated with a more severe disease, higher relapse rates, and increased mortality has compounded the need to understand this prominent pathogen. Vegetative cells of *C. difficile* cannot survive for extended periods of time in

the presence of oxygen. In order to persist outside the anaerobic environment of the large bowel, *C. difficile* must be in its spore form. The production of spores provides an effective means by which *C. difficile* can persist on surfaces in healthcare facilities. The considerable resistance properties of these spores provide continuous challenges in the search for effective cleaning procedures, and the spore form is widely recognised as the principal route of disease transmission (Riggs, *et al.*, 2007; Setlow, 2007; Gerding, *et al.*, 2008). However, the major virulence factors of *C. difficile*, the toxins, are produced exclusively by vegetative cells. Thus, in order to cause disease these spores must germinate and return to vegetative cell growth. Therefore, knowledge of germination is important, with likely practical implications for routine cleaning, outbreak management and potentially in the design of new therapeutics.

1.7.2 Aim of this project

The aim of this project was to gain an understanding of the genetic basis of the mechanisms through which *C. difficile* spores germinate and return to vegetative cell growth. Specifically, target genes in *C. difficile* were to be identified based on their homology to genes important for germination in other spore formers, inactivated using ClosTron technology, and the subsequent phenotypes analysed.

Chapter Two

Materials and Methods

2.1 Materials

Unless indicated otherwise, chemicals and biochemicals were supplied by Sigma-Aldrich, enzymes for molecular biology and their buffers were supplied by New England Biolabs (NEB) or Promega, and bacterial growth media were supplied by Oxoid.

2.2 List of bacterial strains

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	(Hussain, *et al.*, 2005)
C. difficile R20291	Erythromycin sensitive strain of *C. difficile* 630 BI/NAP1/027 (Stoke Mandeville, UK). Isolated in 2004/5	Anaerobe Reference Laboratory, Cardiff
CRG789	*C. difficile* 630Δ*erm spo0A*::intron *ermB*	(Heap, *et al.*, 2007)
CRG878	*C. difficile* 630Δ*erm* CD3563::intron *ermB*	This work
CRG879	*C. difficile* 630Δ*erm* CD0552::intron *ermB*	This work
CRG1115	*C. difficile* 630Δ*erm sleC*::intron *ermB*	This work
CRG1143	*C. difficile* R20291 CD0552::intron *ermB*	This work
CRG1166	*C. difficile* R20291 *sleC*::intron *ermB*	This work
CRG1375	*C. difficile* R20291 *spo0A*::intron *ermB*	(Heap, *et al.*, 2010)
CRG1555	CRG1115 containing pMTL-DB1 (*sleC* complementation plasmid)	This work
CRG1556	CRG1115 containing pMTL84151	This work
CRG1628	CRG1166 containing pMTL84151	This work
CRG1634	CRG1166 containing pMTL-DB1 (*sleC* complementation plasmid)	This work

CRG1651	*C. difficile* 630Δ*erm* containing pMTL84151	This work
CRG1652	*C. difficile* R20291 containing pMTL84151	This work
CRG1718	*C. difficile* 630Δ*erm cspC*::intron *ermB*	This work
CRG1719	*C. difficile* 630Δ*erm cwlD*::intron *ermB*	This work
CRG1720	*C. difficile* 630Δ*erm* CD0065::intron *ermB*	This work
CRG1894	*C. difficile* 630Δ*erm cspBA*::intron *ermB*	This work
CRG1948	*C. difficile* R20291 *cwlD*::intron *ermB*	This work
C. difficile CDC 32	Historical BI/NAP1/027 (USA)	(Killgore, *et al.*, 2008)
C. difficile CDC 38	BI/NAP1/027 (USA)	(Killgore, *et al.*, 2008)
C. difficile M13042	BI/NAP1/027 (Canada)	(Killgore, *et al.*, 2008)
C. difficile DH326	BI/NAP1/027 (Sheffield, Yorkshire and Humberside, UK). Isolated in 2005	Anaerobe Reference Laboratory, Cardiff
C. difficile DH1329	BI/NAP1/027 (Coventry, West Midlands, UK). Isolated in 2007/8	Anaerobe Reference Laboratory, Cardiff
C. difficile R12087	Historical BI/NAP1/027 (European Union)	Michel Popoff, Institut Pasteur, Paris
C. difficile GAI 95601	PCR-ribotype 017 (Japan)	(van den Berg, *et al.*, 2007)
C. difficile 001-3	PCR-ribotype 001	ECDC – Cardiff collection
C. difficile Serosubtype A2	PCR-ribotype 002	ECDC – Cardiff collection
C. difficile Wilcox 078	PCR-ribotype 078	Mark Wilcox
C. difficile R10459	PCR-ribotype 106	ECDC – Cardiff collection
C. difficile VPI 10463	PCR-ribotype 003, toxinotype 0 reference strain	(Sullivan, *et al.*, 1982)

Table 2.1. Bacterial strains used in this study.

2.3 List of plasmids

Strain	Relevant properties	Source/reference
pMTL007C-E2	ClosTron plasmid containing *catP* and intron containing *ermB* RAM	(Heap, *et al.*, 2010)
pMTL007C-E2::Cdi-CD3563-226s	pMTL007C-E2 retargeted to Cdi-CD3563-226s	This work
pMTL007C-E2::Cdi-CD0552-75a	pMTL007C-E2 retargeted to Cdi-CD0552-75a	This work
pMTL007C-E2::Cdi-*sleC*-493s	pMTL007C-E2 retargeted to Cdi-sleC-493s	This work
pMTL007C-E2::Cdi-*sleC*-128a	pMTL007C-E2 retargeted to Cdi-sleC-128a	This work
pMTL007C-E2::Cdi-*cspC*-737a	pMTL007C-E2 retargeted to Cdi-cspC-737a	This work
pMTL007C-E2::Cdi-*cwlD*-198s	pMTL007C-E2 retargeted to Cdi-cwlD-198s	This work
pMTL007C-E2::Cdi-CD0065-440a	pMTL007C-E2 retargeted to Cdi-CD0065-440a	This work
pMTL007C-E2::Cdi-*cspBA*-825s	pMTL007C-E2 retargeted to Cdi-cspBA-825s	This work
pMTL007C-E2::Cdi-*cspBA*-1844a	pMTL007C-E2 retargeted to Cdi-cspBA-1844a	This work
pMTL84151	*Clostridium* modular plasmid containing *catP*	(Heap, *et al.*, 2009)
pMTL-DB1	pMTL84151 containing 1,272 bp SleC coding region and 244 bp upstream promoter region	This work
pMTL-DB2	pMTL84151 containing 1674 bp CspC coding region and a presumed 305 bp upstream promoter region.	This work

Table 2.2. List of plasmids used in this study.

2.4 Bioinformatics methods

2.4.1 Sequence data analysis

Sequence data were routinely handled using GENtle, VectorNTI and DNA Baser.

2.4.2 BLAST

Searches of translated nucleotide databases using the Basic Local Alignment Search Tool (BLAST) were routinely carried out using the tBLASTn algorithm available at http://www.ncbi.nlm.nih.gov.

2.4.3 Plasmid map design and production

Vector maps were designed using Vector NTI or GENtle, produced using Savvy, and edited using Inkscape.

2.4.4 Sequence alignments during cloning studies

GENtle or Vector NTI was used for the alignment of two sequences. For alignments of more than two sequences, DNA Baser and/or Vector NTI were used.

2.4.5 Oligonucleotide design

Oligonucleotides for PCR primers were designed manually using GENtle or DNA Baser, and analysed for predicted hairpin formation, self dimerisation and melting temperatures with Oligo Calc software available at http://www.basic.northwestern.edu.

2.4.6 Data analysis

All data and statistical analysis were carried out in GraphPad Prism, using Student's t-test for individual comparisons, and one-way analysis of variance with Tukey's *post hoc* compensation for multiple comparisons.

2.5 Microbiological materials and methods

2.5.1 Aerobic bacterial strains and growth conditions

All *Escherichia coli* were cultured using Luria-Bertani (LB) broth or agar as appropriate at 37 °C, with 200 rpm shaking for liquid cultures. Media was supplemented as appropriate with chloramphenicol (25 µg/ml in agar or 12.5 µg/ml in broth), 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (Xgal, 40 µg/ml) and kanamycin (50 µg/ml in agar or 25 µg/ml in broth). *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.

2.5.2 Anaerobic bacterial strains and growth conditions

Unless indicated otherwise, all *C. difficile* strains were cultured at 37°C in an anaerobic workstation (Don Whitley, United Kingdom) 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). The medium was supplemented as appropriate with cycloserine (250 μg/ml), cefoxitin (8 μg/ml), thiamphenicol (15 μg/ml in agar or 7.5 μg/ml in broth), erythromycin (2.5 μg/ml or 10 μg/ml) or lincomycin (20 μg/ml). All liquid media were pre-reduced with overnight incubation in the anaerobic chamber, and all solid growth media were pre-reduced for at least 4 h prior to inoculation.

2.5.3 Preparation of electro-competent E. coli cells

A 500 ml conical flask, containing 200 ml of LB broth (pre-warmed to 37 °C), supplemented with antibiotics as required, was inoculated 1/100 with fresh overnight culture. *E. coli* cells were cultured at 37 °C with 200 rpm shaking until an optical density at 600 nm (OD_{600}) of between 0.5 and 1 was reached, indicative of the exponential growth phase. Cells were harvested by pooling into separate 50 ml falcon tubes, cooling on ice for 30 min, and then centrifugation for 15 min at 4000 ×*g*. Pellets were then gently re-suspended in 40 ml of ice-cold, sterile dH_2O before centrifugation as described above. Following centrifugation, the pellets were re-suspended in 500 μl of sterile 10% glycerol, pooled, and stored at -80 °C in 50 μl aliquots.

2.5.4 Transformation of electro-competent E. coli by electroporation

A 50 µl aliquot of electro-competent *E. coli* cells was thawed on ice. DNA was then added and gently mixed with a pipette tip. The mixture was then gently pipetted into a cold 0.2 cm gap electroporation cuvette and a pulse applied across the cuvette using an electroporator with 2.5 kV voltage, 25 µF capacitance and 200 Ω resistance. A 250 µl supplement of Invitrogen SOC medium was then immediately added to the mixture and the entire contents of the electroporation cuvette were then transferred to an eppendorf tube and incubated at 37 °C, at 200 rpm, for 1 h. This incubation step is necessary to allow expression of antibiotic resistance genes. The mixture was then plated onto LB agar at suitable dilutions, supplemented with appropriate antibiotics.

2.5.5 Transfer of plasmid DNA into C. difficile by conjugation

Conjugative transfer of plasmid DNA into *C. difficile* was carried out as described by Purdy and co-workers (Purdy, *et al.*, 2002). Briefly, both donor and recipient cells were grown to stationary phase in overnight cultures and washed with phosphate-buffered saline (PBS) as necessary to remove traces of antibiotics. A pellet of donor cells was prepared by centrifugation for 2 min at 4000 ×*g*, before being moved to the anaerobic chamber and 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 inoculated onto BHIS agar in discrete spots and incubated anaerobically at 37 °C. For conjugation into *C. difficile* 630Δ*erm* (Hussain, *et al.*, 2005),

suspensions were incubated for 4-8 h, and for conjugation into *C. difficile* R20291, suspensions were incubated for a minimum of 10 h as the efficiency of conjugation into R20291 appears lower than the efficiency of conjugation into 630Δ*erm*. Cells were then re-suspended in 750 µl of PBS by gently scraping, and then 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.

2.5.6 Preparation of C. difficile spores using a Trypticase Peptone broth

C. difficile was cultivated in a sporulation medium which contained 90 g of Trypticase Peptone (BBL Microbiology Systems), 5 g of Proteose Peptone no. 3 (Difco), 1 g of $(NH_4)_2SO_4$ and 1.5 g of TrisHCl in 1 litre of dH_2O, adjusted to pH 7.4 with hydrochloric acid, and pre-reduced for at least 24 h prior to inoculation. Briefly, a loop-full of *C. difficile* cells was used to inoculate the sporulation medium, and the mixture was incubated anaerobically, at 37 °C, for 7 days. To measure spore development, 500 µl samples were removed from the anaerobic chamber and either heat treated at 80 °C for 10 min, or incubated in EtOH (100%), Chloroform (10% in dH_2O), NaOH (0.25M) or PBS for 10 min. Following this incubation, samples were washed twice in PBS and then returned to the anaerobic chamber, serially diluted in PBS and

plated onto BHI agar. The plates were incubated for 24 h before colony forming units (CFU) were enumerated.

2.5.7 Preparation of C. difficile spores on solid media

Sporulation of *C. difficile* on solid media was achieved by incubating cultures on BHI agar for 10-14 days. Briefly, *C. difficile* was cultivated in BHI broth and an overnight culture spread onto BHI agar. Plates were incubated anaerobically, at 37 °C, for 10-14 days, or until significant spore titres were observed under phase-contrast microscopy. Following incubation, a spore/vegetative cell mixture was re-suspended in PBS and removed from the anaerobic chamber. This mixture was separated into two identical samples which were incubated in either ethanol (EtOH, 100%) or PBS, for 10 min. Samples were then washed twice in PBS and then returned to the anaerobic chamber, serially diluted in PBS and plated onto BHI agar supplemented with 0.1% taurocholate (Sigma, United Kingdom). Plates were incubated for 24 h before CFU were enumerated.

2.5.8 Preparation of C. difficile spores in a nutrient-rich liquid medium

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.

2.5.9 Purification of C. difficile spores

Sporulation cultures were set up as described in *2.5.8*. To prepare pure *C. difficile* spores following five days of incubation, cultures were repeatedly washed with ice-cold distilled H_2O (dH_2O) until they contained no cell debris or vegetative cells as observed by phase-contrast microscopy. Spores were stored at -20 °C in dH_2O.

2.5.10 Measurement of C. difficile growth in BHIS broth

To measure the growth rates of *C. difficile*, sporulation cultures were set up as described in *2.5.8*. At different time points, 1 ml samples were removed from the sporulation medium and the OD_{600} was measured (Biomate 3, Thermo Scientific).

2.5.11 Measurement of C. difficile colony formation after heat treatment

Sporulating cultures of *C. difficile* were prepared as described in *2.5.8*. At different times, 500 µl samples were removed from the anaerobic chamber and heated at 60 °C for 25 min 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 25 min. 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). *C. difficile spo0A* mutants of both 630Δ*erm* and R20291 were used as sporulation-negative controls (Heap, *et al.*, 2007; Heap, *et al.*, 2010). Plates were incubated for 24 h before CFU were enumerated. Samples were analysed in the same way every 24 h for five days.

2.5.12 Counting C. difficile spores by phase-contrast microscopy

Sporulation cultures were set up as described in *2.5.8*. Following incubation in BHIS broth for five days, spores were enumerated by phase-contrast microscopy using a Bright-Line haemocytometer (Sigma, United Kingdom). To load the haemocytometer, a drop of water was placed on each corner of the coverslip. The coverslip was then placed in the appropriate position, and 10 µl of *C. difficile* spore culture was loaded onto the haemocytometer. *C. difficile* spores were then visualised by phase-contrast microscopy. Spores were counted in 10, 200 µm haemocytometer squares, averaged, and expressed as spores/ml using the appropriate dilution factors.

2.5.13 Decoating C. difficile spores

Sporulation cultures were set up, and spores were purified as described above. Purified spores were decoated as described by Popham and co-workers. Briefly, a spore pellet was re-suspended in 1 ml of 50 mM Tris-HCl (pH 8.0)-8

M urea-1% (wt/vol) sodium dodecyl-sulphate-50 mM dithiothreitol and incubated at 37 °C for 90 min (Popham, *et al.*, 1995). Following this incubation, decoated spores were washed three times in PBS. Samples were then heat treated as described above and plated onto BHIS medium supplemented with 1 μg/ml lysozyme.

2.6 Molecular biological materials and methods

2.6.1 Extraction and purification of C. difficile chromosomal DNA

Chromosomal DNA was extracted and purified from *C. difficile* cultures using previously published methods (Fenicia, *et al.*, 2007). Briefly, a *C. difficile* pellet was re-suspended in 500 μl of 5% chelex 100 (Sigma, United Kingdom). This suspension was vortexed and then incubated at 100 °C for 10 min, before centrifugation at 10,000 ×*g* for 10 min. The supernatant (containing purified chromosomal DNA) was removed and stored at -20 °C.

2.6.2 Extraction and purification of plasmid DNA

Plasmid DNA was extracted from all organisms using the Qiagen QIAprep Spin Miniprep kit in accordance with the manufacturer's instructions.

2.6.3 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 µg/ml solution of double-stranded DNA has an OD_{260} of 1.

2.6.4 Amplification of DNA by PCR

For cloning, DNA was amplified using either the Epicentre Failsafe PCR system and Failsafe Premix E, or NEB Phusion polymerase in accordance with the manufacturer's instructions. For screening PCR, Promega *Taq* DNA polymerase was used, in accordance with the manufacturer's instructions.

2.6.5 Amplification and splicing of DNA using SOEing PCR

Complete or partial genetic elements were spliced together without the use of restriction endonucleases by splicing by overlap extension (SOEing) PCR with appropriate primers (Horton, *et al.*, 1989; Horton, *et al.*, 1990).

Oligonucleotide	Sequence (5′-3′)
Intron retargeting	
Cdi-CD3563-226s-IBS	AAAAAAGCTTATAATTATCCTTAATGAGCGACAGGGTGCGCCCAGATAGGGTG
Cdi-CD3563-226s-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCGACAGGTTTAACTTACCTTTCTTTGT
Cdi-CD3563-226s-EBS2	TGAACGCAAGTTTCTAATTTCGGTTCTCATCCGATAGAGGAAAGTGTCT
Cdi-CD0552-75a-IBS	AAAAAAGCTTATAATTATCCTTATTCTCCACAATAGTGCGCCCAGATAGGGTG
Cdi-CD0552-75a-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCACAATATCTAACTTACCTTTCTTTGT
Cdi-CD0552-75a-EBS2	TGAACGCAAGTTTCTAATTTCGGTTGAGAATCGATAGAGGAAAGTGTCT
Cdi-*sleC*-493s-IBS	AAAAAAGCTTATAATTATCCTTAGTAGTCCCTGAAGTGCGCCCAGATAGGGTG
Cdi-*sleC*-493s-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCCTGAATTTAACTTACCTTTCTTTGT
Cdi-*sleC*-493s-EBS2	TGAACGCAAGTTTCTAATTTCGATTACTACTCGATAGAGGAAAGTGTCT
Cdi-*sleC*-128a-IBS	AAAAAAGCTTATAATTATCCTTACATTACTTCTTAGTGCGCCCAGATAGGGTG
Cdi-*sleC*-128a-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCTTCTTAGGTAACTTACCTTTCTTTGT
Cdi-*sleC*-128a-EBS2	TGAACGCAAGTTTCTAATTTCGGTTTAATGTCGATAGAGGAAAGTGTCT
EBS Universal	CGAAATTAGAAACTTGCGTTCAGTAAAC
ClosTron sequencing	
Spofdx_F1	GATGTAGATAGGATAATAGAATCCATAGAAAATATAGG
pMTL007_R1	AGGGTATCCCCAGTTAGTGTTAAGTCTTGG
Mutant screening	
CD3563_F1	CTTTTAGAACTGTTAATCCACCTAATCCCG
CD3563_R1	CTTTACATTTTTGTGTTTAACAACAACTTATTTATCGC

CD0552_F1	ATGAATTTAGTTCCCTTATATTTCAAGATATGAATATAGC
CD0552-R1	CTTGTGTGTCATTTATTATACTTTAATTATTTATGTCTTTCC
*sleC*_F1	GGGAACTAAATTCATTTAAAGAAAGGGTG
*sleC*_R1 (630Δ*erm*)	GGCTGTTATGAACTAATATATACCATAAGTATTAC
*sleC*_R1 (R20291)	GTATTTTTACTTATAAGTATTGTAGTCTTAACAGCC
*cspC*_F1	CAAGGTGATATAGAATCTGCCCTACAAG
*cspC*_R1	CCCATATCCCTGCGATACATTTGGAT
*cspBA*_F1	CATGGGACACATGTTGCAGGTATTTGT
*cspBA*_R1	CTATATCCCATATCCTCTGTTTCTAAGTCTTG
*cspBA*_F2	GATTCGGGAATAGATTATACTCTACCTG
*cspBA*_R2	GTGAGAGAATACGGTGCTATAGGG
*cspBA*_F3	GCAAGTGATGCCAGAATAATAGTGGTTA
*cspBA*_R3	CCTACTGTTATAACTCTACTTGCTGTTC
*cspBA*_F4	CTACTCAAGAGCTTACTGTAACAGTTCCT
*cspBA*_R4	GCGATAGCTCGATTTATATCTTCTCGTG
*cwlD*_F1 (630Δ*erm*)	GTTAGATAAACATAGCGAATTAAATAGGGGCAG
*cwlD*_F1 (R20291)	GTTAGATAAACATAGTGAATTAAATAGGGGCAG
*cwlD*_R1	CCTATGTAGATTGCCCATGCTATTTTTTCTTG
CD0065_F1	GCAACAAAAGGTATTGGATTAGCATCAGC
CD0065_R1	GCTATATCATCTGGTTCCCCTATACG
RAM_R1	ACGCGTGCGACTCATAGAATTATTTCCTCCCG
R20291 specific primers	
CDR20291_F1	GCTATTATTATGCCAGGATACTTTTATACACC
CDR20291_R1	GACTCACTAATTTCTATTCCATATATTGATGC

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

2.6.6 Restriction endonuclease digestion of DNA

DNA was cleaved at specific points using appropriate NEB restriction endonucleases in accordance with the manufacturer's instructions.

2.6.7 De-phosphorylation of DNA

To remove 5′ phosphate groups from linearised plasmid DNA fragments and prevent self-ligation, NEB Antarctic Phosphatase was used in accordance with manufacturer's instructions.

2.6.8 Agarose gel electrophoresis

PCR products, restriction fragments and plasmid DNA were separated by electrophoresis at 90 V for 20 – 60 min through 1% agarose gels in TAE containing 0.5 μg/ml ethidium bromide. DNA was subsequently visualised under ultra-violet light.

2.6.9 Extraction of DNA from agarose gels and reaction mixtures

Agarose gel pieces containing DNA were excised under ultra-violet light, and DNA was extracted using the Qiagen QIAquick Gel Extraction Kit in accordance with the manufacturer's instructions. DNA was extracted from reaction mixtures using the Qiagen QIAquick PCR Purification Kit in accordance with the manufacturer's instructions.

2.6.10 Ligation of DNA

Digested plasmid DNA fragments were ligated to small DNA insert fragments with compatible ends using NEB T4 DNA ligase, in accordance with the manufacturer's instructions.

2.6.11 Nitrocellulose membrane dialysis of DNA during ligations

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

2.6.12 Nucleotide sequencing

DNA sequencing was performed by Geneservice Ltd, Cambridge and Geneservice Ltd, Nottingham.

2.6.13 Distinguishing between C. difficile 630Δerm and C. difficile R20291

During construction of mutant strains, it was occasionally necessary to confirm the presence of *C. difficile* 630Δ*erm* and *C. difficile* R20291 DNA. Accordingly, genomic DNA was extracted from putative mutant strains and amplified by PCR using the primer pair CDR20291_F1/CDR20291_R1. This primer pair amplifies CDR20291_0242, which encodes a flagella glycosyl

transferase not found in *C. difficile* 630Δ*erm* (Logan, 2006; Sebaihia, *et al.*, 2006; Stabler, *et al.*, 2009) When analysed by agarose gel electrophoresis, the presence of R20291 DNA is indicated by a single 294 bp band.

2.7 Strain construction

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).

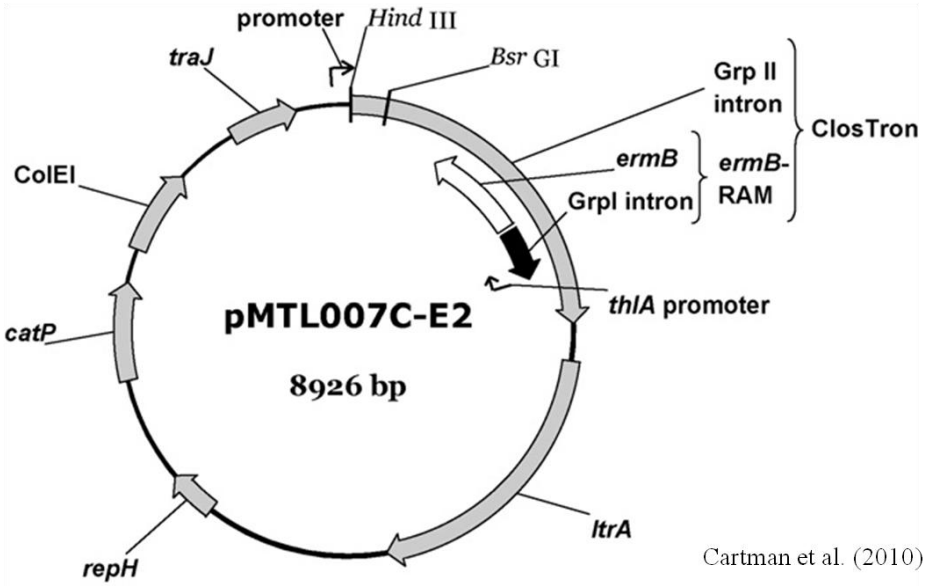

Figure 2.1. The second generation, modular ClosTron plasmid pMTL007C-E2 (Cartman, *et al.*, 2010; Heap, *et al.*, 2010). This plasmid uses the strong *fdx* promoter to drive expression of the ClosTron (Heap, *et al.*, 2007). Intron re-targeting using the HindIII and BsrGI restriction sites replaces a *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.

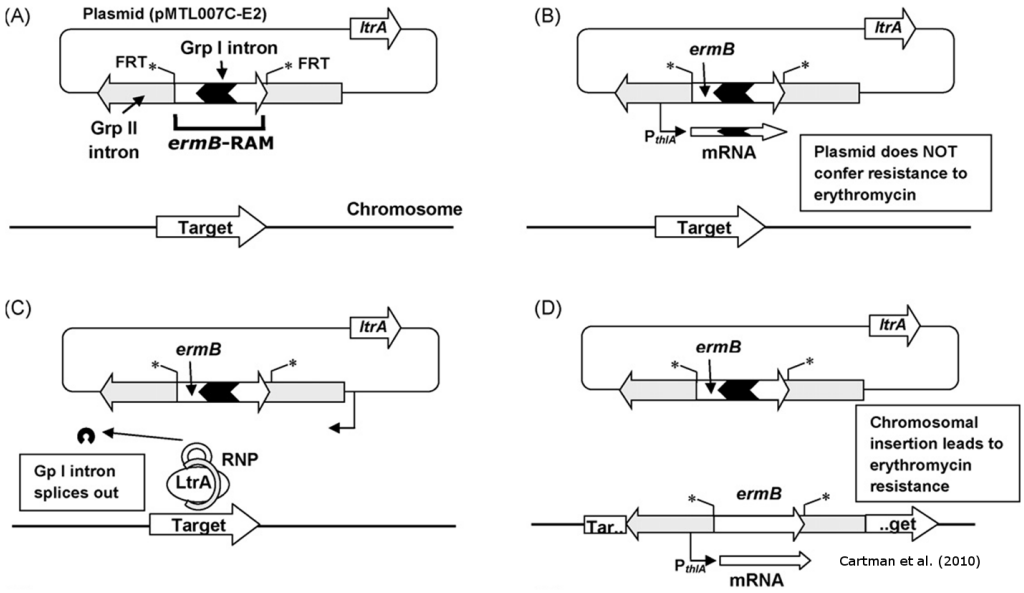

Figure 2.1A. ClosTron insertion using pMTL007C-E2, adapted from Cartman et al., 2010. (A) pMTL007C-E2 is transferred into *C. difficile* by conjugation. (B) As the group I intron is transcribed in the incorrect orientation to splice out, expression from the *C. acetobutylicum* thiolase promoter does not confer erythromycin resistance. (C) Expression of the group II intron yields a ribonuclear protein complex (RNP) upon binding of LtrA to the intron transcript. This results in the group I intron being transcribed in the correct orientation, allowing it to splice out. (D) The RNP locates the target DNA, the group II intron RNA is inserted, and LtrA reverse transcribes the cDNA strand. As the group I intron has spliced out, erythromycin resistance is conferred in successful integrants. The pMTL007C-E2 plasmid is then lost due to its instability.

2.7.1 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. The 353 bp PCR products containing modified IBS, EBS1d and EBS2 sequences, responsible for target specificity of the intron,

were amplified and assembled using SOEing PCR. This 353 bp fragment was cloned as a HindIII/BsrGI fragment into pMTL007C-E2, and the re-targeted intron verified by sequencing, using oligonucleotide primer pairs Spofdx_F1/pMTL007_R1. 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::cdi-*sleC*-128a has been retargeted to insert in the antisense orientation after base 128 of the *sleC* open reading frame (ORF) of *C. difficile*. 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.

2.7.2 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*.

2.7.3 Isolation and confirmation of C. difficile ClosTron mutants

Re-targeted pMTL007C-E2 plasmids were transferred into *C. difficile* by conjugation as described above. 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.

2.7.4 Complementation of a C. difficile sleC mutant with parental SleC

For complementation studies of *sleC* mutant strains of *C. difficile* 630Δ*erm* and *C. difficile* R20291, a 1,516 bp fragment encompassing the *sleC* structural gene and 5′ non-coding region was cloned into the modular plasmid pMTL84151 (Heap, *et al.*, 2009) to generate plasmid pMTLDB1 (Figure 2.2). The 244 bp 5′ non-coding region likely encompassing the *sleC* promoter and the 1,272 bp region containing the *sleC* structural gene were independently amplified by PCR using oligonucleotide primer pairs pSleC_F1/pSleC_R1 and SleC_F2/SleC_R2, respectively. The primers were designed to allow subsequent cleavage of the two fragments generated with NotI/NdeI and NdeI/XhoI, respectively, where the ATG of the NdeI site was synonymous with the translational start codon of *sleC*. The two cleaved fragments were

subsequently ligated with plasmid pMTL84151 cut with NotI and XhoI, which yielded plasmid pMTL-DB1, in which the two fragments were inserted contiguously. The strategy of separating the two regions into two independent fragments was adopted to allow the plasmid to be easily modified for subsequent use of a heterologous promoter if the presence of the 5′ non-coding region did not result in expression of *sleC*. As the protein encoded by *sleC* is the same in *C. difficile* 630Δ*erm* and R20291, only one plasmid (pMTL-DB1) was constructed for use with both *sleC* mutants. Both the pMTL-DB1 complementation plasmid and a pMTL84151 empty vector control were then transferred into *C. difficile* by conjugation, using additional lincomycin (20 μg/ml) selection in *sleC* mutant strains.

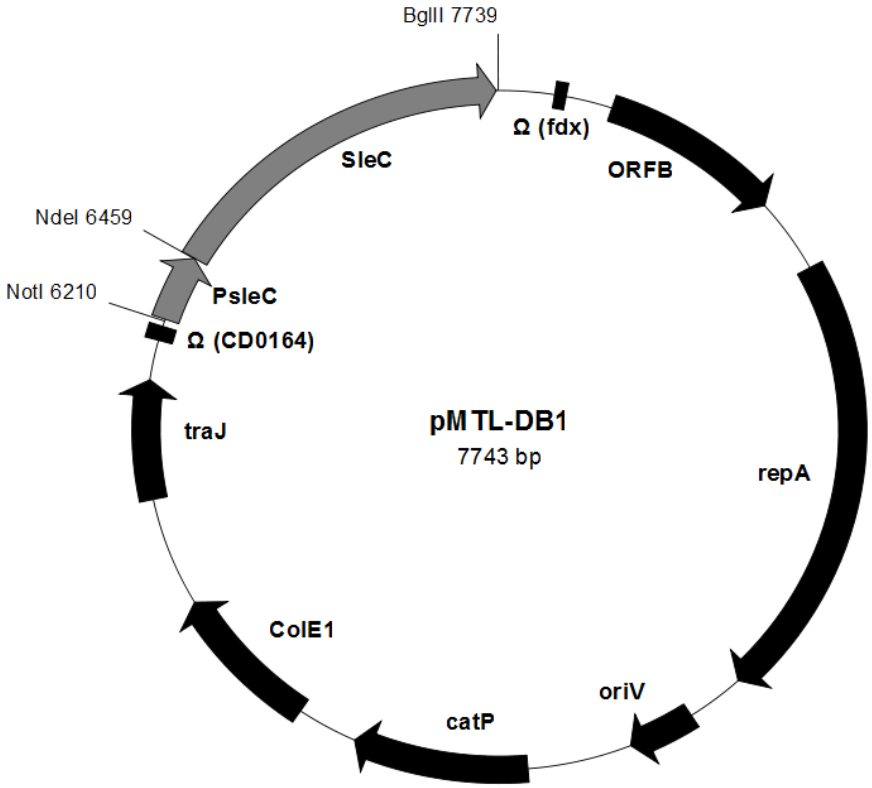

Figure 2.2. Vector map of plasmid pMTL-DB1. This vector conforms to the pMTL80000 modular system for *Clostridium* shuttle plasmids, based upon the pMTL84151 vector (Heap, *et al.*, 2009). The plasmid backbone consisted of the pCD6 Gram-positive replicon (repA and ORFB), the origin of replication gene *oriV*, *catP* conferring thiamphenicol resistance, the Gram-negative replicon ColE1, and the conjugal transfer function *traJ*. A 1,516 bp fragment encompassing the *sleC* structural gene and 5′ non-coding region presumed to contain the *sleC* promoter was cloned into the plasmid as described in the text. The transcriptional terminators (Ω) are identical in sequence to those found immediately downstream of the *fdx* gene of *Clostridium pasteurianum* and the CD0164 ORF of *C. difficile* 630.

2.7.5 Complementation of a C. difficile cspC mutant with parental CspC

For complementation studies of a *cspC* mutant strain of *C. difficile* 630Δ*erm*, a 1,976 bp fragment encompassing the *cspC* structural gene and a 5′ non-coding upstream region was cloned into plasmid pMTL84151 to generate plasmid pMTL-DB2 (Figure 2.3). The 302 bp non-coding region, located immediately upstream of *cspBA*, and the 1,674 bp region containing the *cspC* structural gene were spliced and amplified as a single fragment using SOEing PCR and primer pairs PcspC_F2/PcspC_R3 and cspC_F4/cspC_R2, respectively. The subsequent fragment was cleaved with NotI/XhoI and then ligated into plasmid pMTL84151 cut with NotI/XhoI, which yielded plasmid pMTL-DB2. Both the pMTL-DB2 plasmid and a pMTL84151 empty vector control were transferred into *C. difficile* by conjugation, using additional lincomycin (20 μg/ml) selection in ClosTron mutant strains.

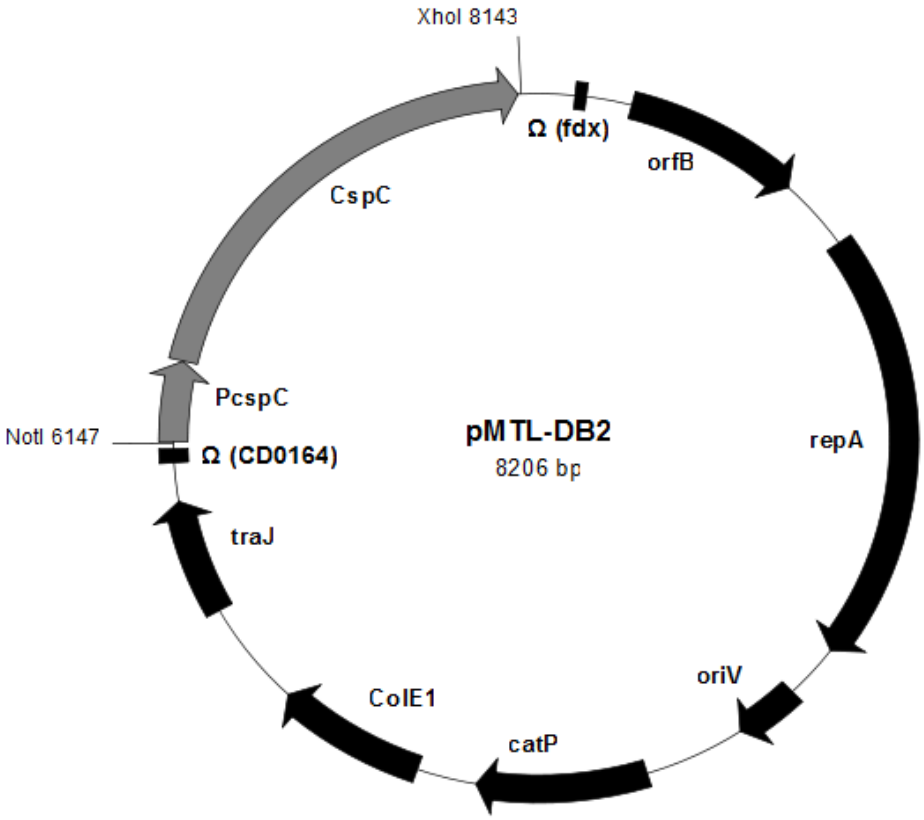

Figure 2.3. Vector map of plasmid pMTL-DB2. Similarly to pMTL-DB1, the plasmid backbone is based on the modular pMTL84151 plasmid (Heap, *et al.*, 2009). A 1,976 bp fragment encompassing the *cspC* structural gene and 5′ non-coding region presumed to contain the *cspBA*/*cspC* promoter was amplified by SOE PCR and cloned into the plasmid as described in the text.

Oligonucleotide	Sequence (5′-3′)
pSleC_F1	TAAAGAATGCGGCCGCAGATTATTTTCCTTTCAAAATTTTTGATTTATTTATGATTTATATCATCTAC
pSleC_R1	TAAAGAATCATATGATCACCCTTTCTTTAAATGAATTTAGTTCCC
SleC_F2	TAAAGAATCATATGCAAGATGGTTTCTTAACAGTAAGCATAATTGATGC
SleC_R2	TAAAGAATCTCGAGATCTCCATGGTTAAATTAAAGGATTTAAAGAAGCTATTCTAGTTGTAGC
pCspC_F2	TAAAGAATGCGGCCGCCCAGAACTTAGAGATGGATGGGATTCAATTA
pCspC_R2	TAAAGAATCATATGGACTTCTAAAATTATTATTAATGTATATGTTTTGTGTGA
pCspC_R3	ATACAATAAGATTTTTCCATCTCGACTTCTAAAATTATTATTAATGTATATGTTTTG
CspC_F2	TAAAGAATCATATGGAAAAATCTTATTGTATAATTTATCAAGGTGATAT
CspC_F4	TAATAATTTTAGAAGTCGAGATGGAAAAATCTTATTGTATAATTTATCAAGGTG
CspC_R2	TAAAGAATCTCGAGATCTCCATGGCTATAGAGTATTTGCTATCTGTTGAATCGTAT

Table 2.5. Oligonucleotides used for complementation studies. Oligonucleotides were used as described in the text.

Chapter Three

Development of assays to accurately measure the sporulation and germination characteristics of *C. difficile* mutants

3.1 Introduction

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 now important to have appropriate methods at hand for studying processes such as germination in the mutant strains we are now able to generate.

3.1.1 Methods for studying germination in other spore formers

Current knowledge of sporulation and germination has been principally derived from studying spores of the model organism, *B. subtilis*. Therefore, reproducible methods for preparing *B. subtilis* spores, measuring sporulation rates, and assessing germination properties have been in place for a number of years. When *B. subtilis* spore germination is initiated, subsequent events include the release of monovalent cations (H^+, Na^+ and K^+) and the large spore depot of dipicolinic acid (DPA) (Setlow, 2003). These events can be measured experimentally by observing the drop in optical density of spore samples, and by quantifying the release of DPA from spores during germination. Unfortunately, there have been very few published examples of using optical density measurements to quantitatively measure *C. difficile* germination (Sorg

and Sonenshein, 2008; Sorg and Sonenshein, 2009) and to-date, measurements of DPA release from *C. difficile* spores are not present in the literature.

3.1.2 Previous knowledge of sporulation and germination in C. difficile

Perhaps a consequence of the poor availability of tools to manipulate *C. difficile* genetically, studies of the sporulation and germination mechanisms of *C. difficile* have been infrequent. To effectively study sporulation and germination, one must understand (i) the conditions under which the organism forms spores efficiently; and (ii) the chemicals or compounds which can stimulate spore germination. In organisms such as *B. subtilis*, the mechanisms of sporulation are well understood, and growth medium which starves the organism of nutrients has been shown to efficiently induce sporulation (Schaeffer, *et al.*, 1965). However, the sporulation mechanisms of *C. difficile* are yet to be fully elucidated, and the precise conditions for optimal *C. difficile* sporulation are not known. Germination has also been well studied in *B. subtilis*, and the chemicals and compounds known to stimulate the germination of *B. subtilis* spores include nutrients such as amino acids and sugars, and non-nutrients such as exogenous CaDPA, lysozyme, salts and peptidoglycan (Paidhungat and Setlow, 2000; Setlow, 2003; Setlow, 2008; Shah, *et al.*, 2008). On the other hand, prior to the beginning of this study, only the bile salt taurocholate had been strongly linked to stimulating the germination of *C. difficile* spores, as it can improve spore recovery on solid media (Wilson, *et al.*, 1982; Wilson, 1983). These same studies also suggested media in which *C.*

difficile sporulates efficiently, and this has formed the vast majority of the fields' knowledge of *C. difficile* sporulation and germination *in vitro*.

3.1.3 Aim of this study

As a range of genetic tools are now available for studying *C. difficile*, it is imperative that appropriate procedures are put in place for analysing the phenotypes of mutants. This study aimed to develop and optimise effective assays for the analysis of sporulation and germination in the directed mutants of *C. difficile* to be studied in the following chapters. A principal aim was to ensure that the assay could accurately distinguish between sporulation and germination defects. As spores must complete germination in order to form colonies on solid medium, it was decided to develop a reproducible colony formation-based assay.

3.2 Results

3.2.1 Conditions suitable for efficient C. difficile sporulation

Detailed studies of *C. difficile* sporulation and germination characteristics had not been reported prior to the beginning of this study. As a result, it was necessary to gain a better understanding of the conditions in which *C. difficile* forms spores efficiently and reproducibly. It has been suggested that *C. difficile* can form spores efficiently in a Trypticase Peptone broth containing Proteose Peptone (12.5 mg/ml), $(NH_4)SO_4$ (2.5 mg/ml) and TrisHCl (3.75

mg/ml), at a pH of 7.4 (Wilson, *et al.*, 1982). It was, therefore, decided to prepare *C. difficile* 630Δ*erm* spores using this medium and enumerate spore titres based upon colony formation on BHI agar.

As the sporulation medium would likely contain a mixture of spores and vegetative cells, it is necessary to treat the culture in such a way to kill all vegetative cells but not the spores. Accordingly, after seven days incubation in the sporulation medium, a range of treatments were analysed to determine (i) their efficacy against *C. difficile* 630Δ*erm* vegetative cells; and (ii) the sporulation efficiency of *C. difficile* 630Δ*erm* in the Trypticase Peptone broth (Figure 3.1). In comparison to the colony formation of a negative control, in which cultures were incubated in PBS for 10 min before being plated onto BHI agar, colony formation appeared similar following treatment with chloroform (10%) or NaOH (0.25M), suggesting that both chloroform and NaOH may be ineffective at removing vegetative cells from the spore mixture. When cultures were heated at 80 °C for 10 min, or incubated in EtOH (100%) for 10 min, no colonies were observed after 24 h incubation on BHI agar. This suggested that while these treatments are suitable for removing vegetative cells from the spore mixture, these treatments may have killed/inactivated the spores or, more likely, there were no spores present following incubation in the sporulation medium. Indeed, when cultures from the sporulation medium were analysed by phase-contrast microscopy, no spores were observed. This suggests that the sporulation medium used in this assay is not suitable for reproducibly preparing *C. difficile* spores. Nonetheless, both heat treatment (80 °C for 10

min), and EtOH treatment (100% for 10 min) were effective at killing vegetative cells of *C. difficile* 630Δ*erm*.

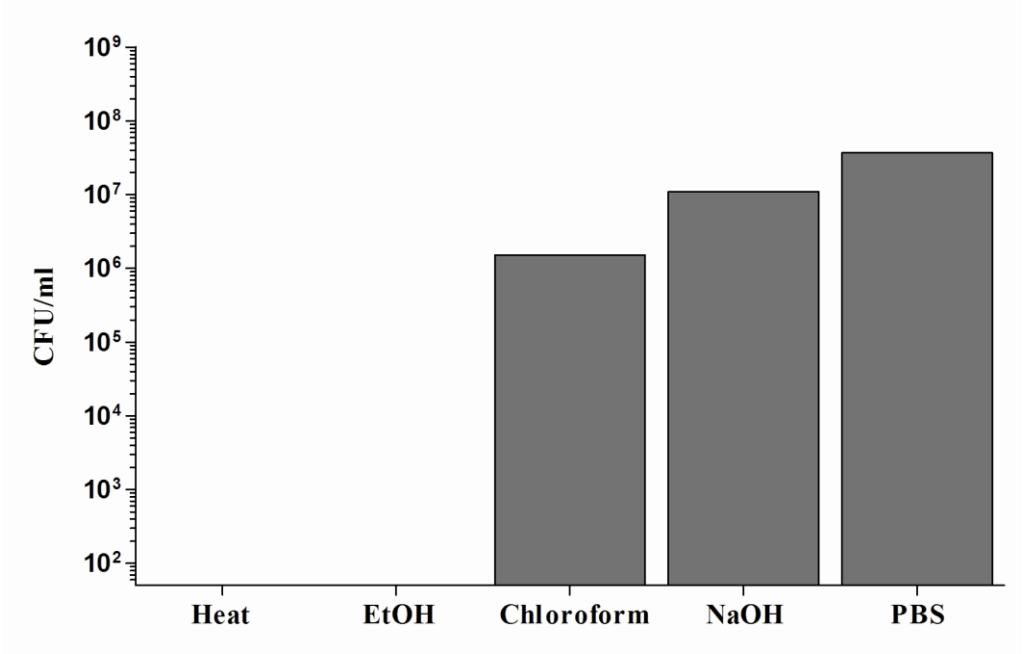

Figure 3.1. Colony formation of *C. difficile* 630Δ*erm* after seven days incubation in a Trypticase Peptone sporulation broth, and treatment with either heat (80 °C for 10 min), 100% EtOH, 10% Chloroform, 0.25M NaOH or PBS. The bars indicate a single experiment and the detection limit for the assay was 50 CFU/ml.

In order to improve the yield of *C. difficile* spores, and efficiently recover spores on BHI agar, the assay described above was altered in three main ways. First, it was attempted to induce sporulation on solid medium by incubating cultures of *C. difficile* on BHI plates for 10-14 days, or until a considerable number of spores were observed by phase-contrast microscopy. The spore mixture was then incubated in 100% EtOH for 10 min to kill vegetative cells

but not spores. Finally, the spore mixture was plated onto BHI agar supplemented with the bile salt taurocholate (0.1%), which has been shown to improve the recovery of *C. difficile* spores on solid medium (Wilson, *et al.*, 1982). A considerable number of spores were observed by phase-contrast microscopy after 10-14 days incubation, and the colony formation after EtOH treatment was then compared to the colony formation following incubation in PBS, with spore recovery expressed as the percentage of CFU survival following EtOH treatment (Figure 3.2). In comparison to a negative control, in which the master regulator of sporulation *spo0A* was inactivated (Heap, *et al.*, 2007), approximately 90% survival was observed in *C. difficile* 630Δ*erm*. This suggests that *C. difficile* is able to form spores efficiently on solid media. In addition, adding the bile salt taurocholate as a supplement allows for suitable recovery of *C. difficile* spores.

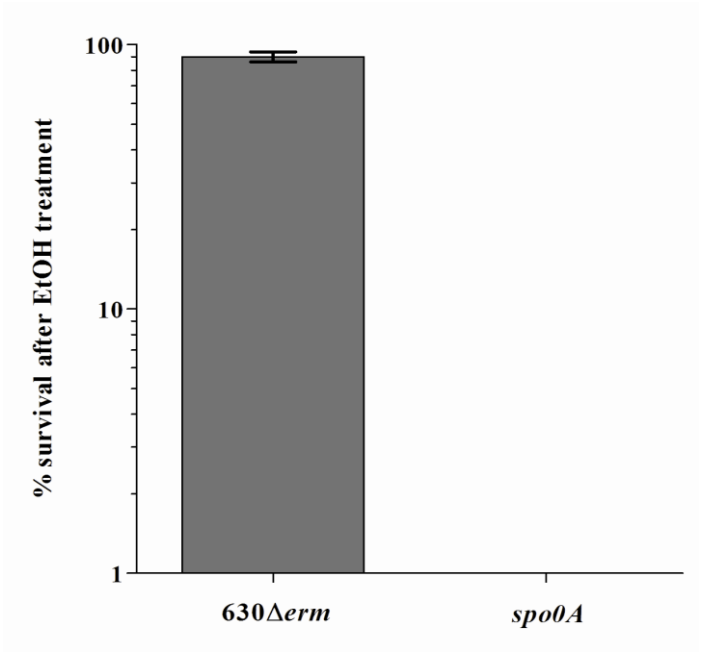

Figure 3.2. Recovery of *C. difficile* 630Δ*erm* after 10-14 days incubation on BHI plates. Spore recovery is the number of CFU following incubation in 100% EtOH for 10 min, as a percentage of the number of CFU observed following incubation in PBS. A *C. difficile* 630Δ*erm spo0A* mutant was used as a sporulation-negative control. The bars indicate the averages of three independent experiments and the error bars indicate the standard errors of the means.

3.2.2 Optimisation of C. difficile sporulation and spore recovery

The assay described above allows for measurements of *C. difficile* spore survival at a precise time-point. However, this study required an assay that could (i) determine the rate of *C. difficile* sporulation; (ii) accurately quantify sporulation, independent of colony formation; and (iii) assess the colony forming ability of *C. difficile* mutant spores when germination was induced with taurocholate.

In order to measure the rate of sporulation, samples must be analysed at multiple time-points, which requires the use of a liquid sporulation medium. Accordingly, sporulation of *C. difficile* was induced in a brain-heart infusion broth enriched by supplementation with L-cysteine and yeast extract (BHIS), as it had recently been described that *C. difficile* sporulates efficiently in nutrient-rich medium (Sorg and Sonenshein, 2008). Therefore, *C. difficile* was incubated anaerobically in BHIS broth and the CFU titre in a sample was enumerated every 24 h, following heat treatment at 60 °C for 25 min and plating onto BHIS agar supplemented with 0.1% taurocholate. Heat-resistant CFU were measured until the CFU titre no longer increased (Figure 3.3). In this case, heat treatment was preferred to EtOH incubation as the wash steps required to remove traces of EtOH following incubation were time consuming. The resulting colony formation clearly showed that *C. difficile* 630Δ*erm* sporulates efficiently in BHIS broth. Furthermore, incubation for five days appeared to be appropriate for a plateau of *C. difficile* sporulation.

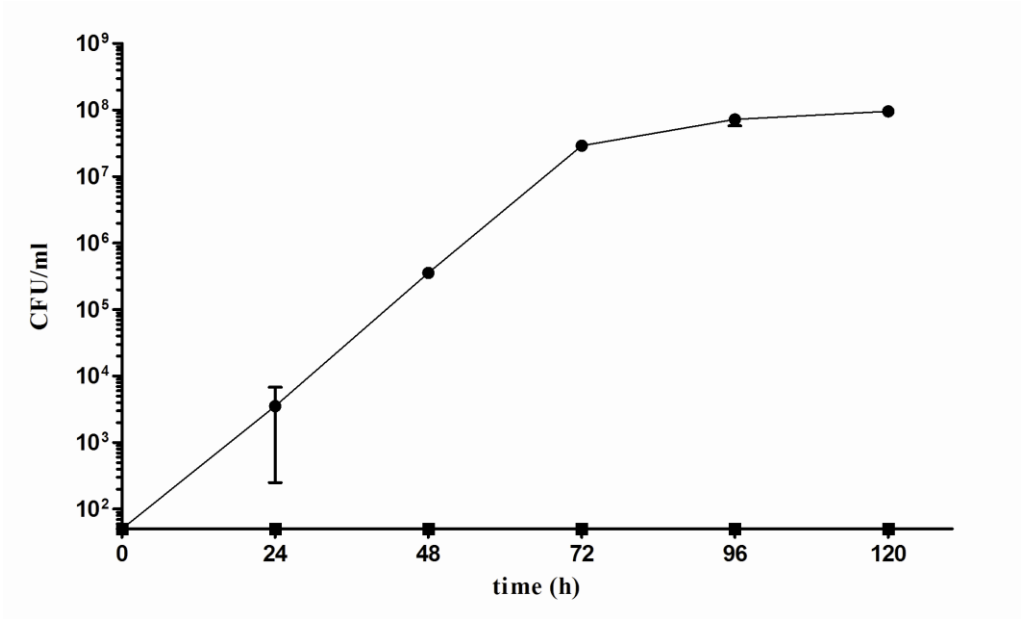

Figure 3.3. The development of heat-resistant CFU of ●, *C. difficile* 630Δ*erm*; and ■, CRG789 (*spo0A*) over a five-day period in BHIS broth. The symbols indicate the averages of three independent experiments, and the error bars indicate the standard errors of the means. The detection limit for the assay was 50 CFU/ml.

As heat-resistant CFU represent successfully sporulated vegetative cells, which were able to survive heat treatment, germinate and grow vegetatively, an assay to measure both sporulation and germination must be able to distinguish between these processes. Therefore, *C. difficile* 630Δ*erm* spore titres were enumerated after five days by using phase-contrast microscopy and then compared to the CFU observed, before and after heat treatment, at the same time-point (Figure 3.4). It was possible to recover more than 90% of the *C. difficile* 630Δ*erm* spores which were observed by microscopy. Taken together, these data demonstrate that this assay allows for efficient *C. difficile* sporulation in a liquid medium, allowing analysis at multiple time-points.

Furthermore, spore titres can be quantified independently of colony formation by phase-contrast microscopy, and the observed recovery of spores on BHIS medium supplemented with taurocholate indicates that *C. difficile* 630Δ*erm* germinates efficiently under these conditions.

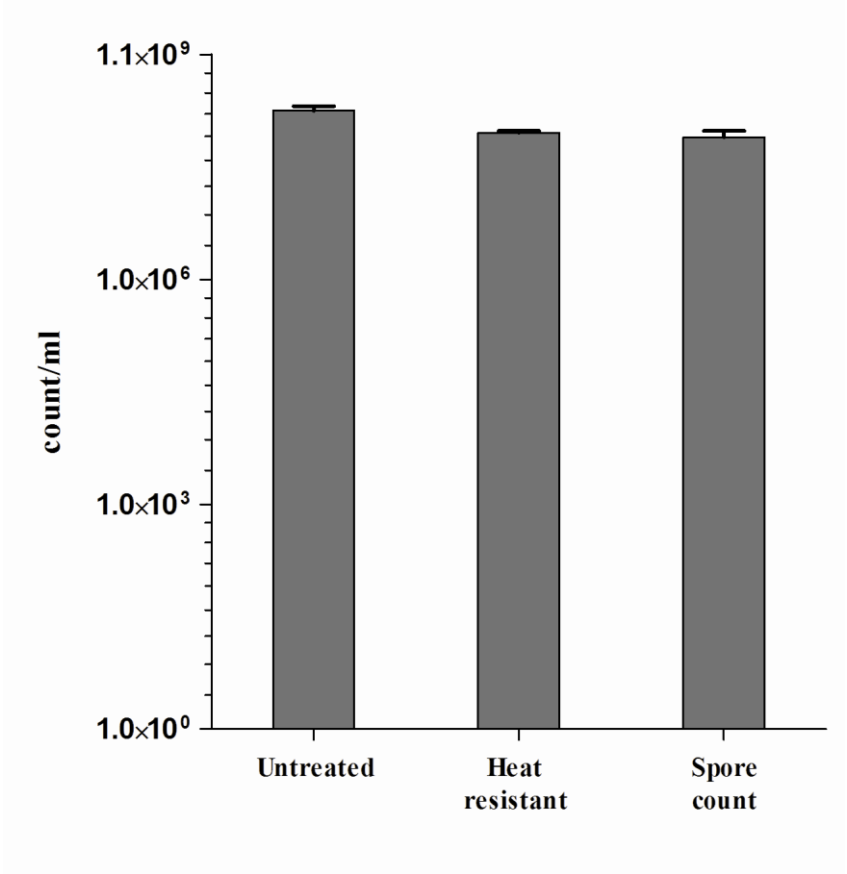

Figure 3.4. Numbers of untreated CFU, spores counted by phase-contrast microscopy, and heat-resistant CFU of *C. difficile* 630Δ*erm* after five days of incubation in BHIS broth. The bars indicate the averages of three independent experiments, and the error bars indicate the standard errors of the means. The detection limit for colony counts was 50 CFU/ml, and the detection limit for spore counts was 5×10^3 spores.

3.2.3 Comparison of sporulation and germination of C. difficile 630Δerm and R20291

It has been suggested that 'epidemic' BI/NAP1/027 strains of *C. difficile* may be more prolific in terms of sporulation than 'non-epidemic' *C. difficile* types (Wilcox and Fawley, 2000; Fawley, *et al.*, 2007; Akerlund, *et al.*, 2008; Merrigan, *et al.*, 2010), although the evidence for this is currently limited. Therefore, it was decided to showcase the applications of the assay developed in this chapter, by studying the sporulation and germination characteristics of a BI/NAP1/027 strain, R20291, and a non-epidemic strain, 630Δ*erm*.

First, the development of heat-resistant CFU was observed over a five-day period (Figure 3.5). The resultant colony formation clearly showed that *C. difficile* 630Δ*erm* developed heat-resistant spores more rapidly than R20291. 630Δ*erm* formed countable heat-resistant CFU after 24 h of incubation, while it was only after 48 h that this was the case for R20291. In addition, 630Δ*erm* cultures contained approximately 400-fold more heat-resistant CFU than R20291 cultures after five days.

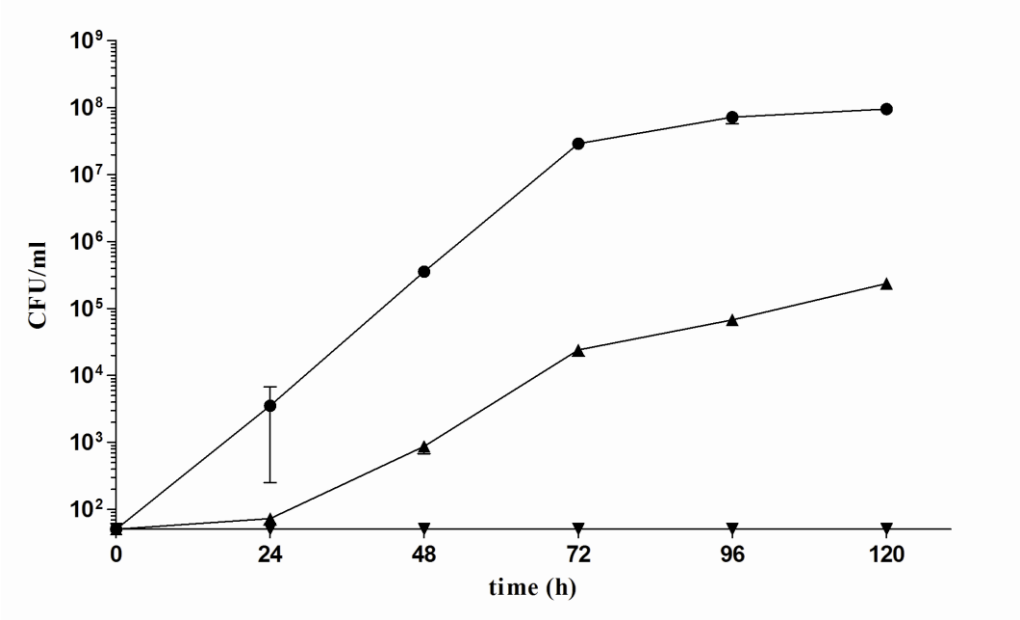

Figure 3.5. Development of heat-resistant CFU of ●, *C. difficile* 630Δ*erm*; ▲, *C. difficile* R20291; and ▼, CRG789 (*spo0A*) over a five-day period. The symbols indicate the averages of three independent experiments, and the error bars indicate the standard errors of the means. The detection limit for the assay was 50 CFU/ml.

It is possible that the observed difference in heat-resistant CFU was not due to sporulation or germination and instead was a result of growth differences between the two strains. The data for non-heat-treated CFU recovered on BHIS agar supplemented with taurocholate after five days were also compared, and as the colony formation was found to be 30-fold lower for R20291 than for 630Δ*erm* (Figure 3.7), the changes in OD_{600} for both 630Δ*erm* and R20291 were analysed over a five-day period. No difference was seen between the two strains (Figure 3.6), which ruled out an elementary growth difference between 630Δ*erm* and R20291. Therefore, it is possible that the observed difference in

the number of non-heat-treated CFU for R20291 could be due to the death of non-sporulating cells.

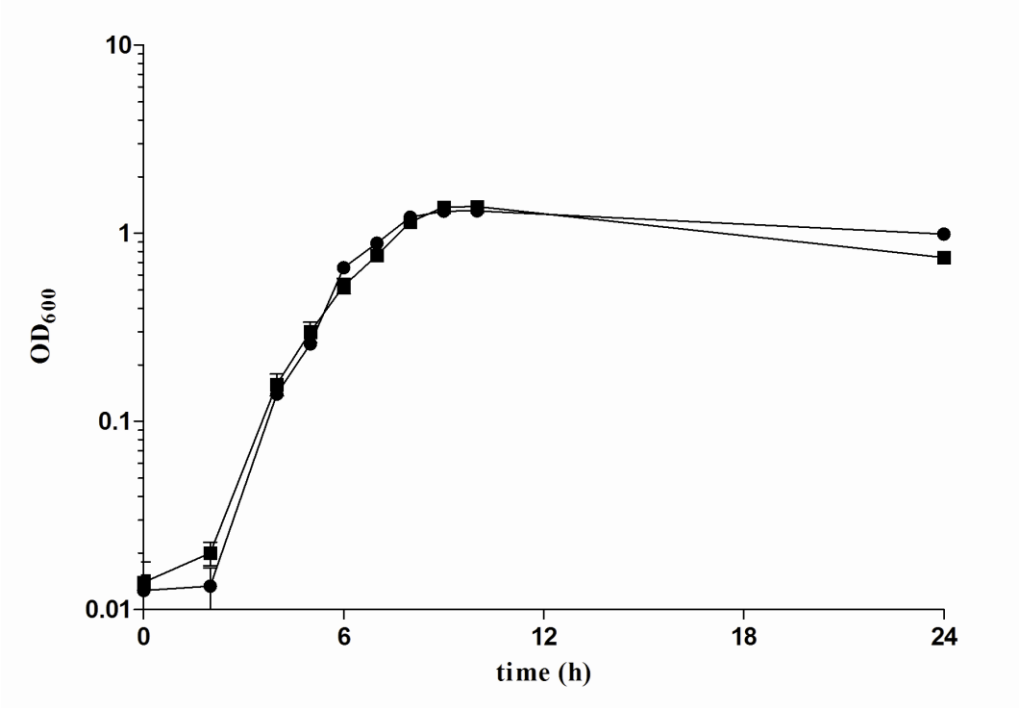

Figure 3.6. Growth of ●, *C. difficile* 630Δ*erm*, and ■, *C. difficile* R20291 in BHIS broth during a 24 h period. Strains were cultivated as described in Chapter Two, and the change in OD_{600} was measured. The symbols represent the average of three independent experiments, and error bars indicate the standard errors of the means.

To pinpoint any difference in sporulation between the two strains, the cultures used to measure heat-resistant CFU after five days were analysed by phase-contrast microscopy, and the numbers of spores/ml were determined. *C. difficile* 630Δ*erm* was found to produce approximately 100-fold more spores/ml than R20291 (Figure 3.7), suggesting that the previously observed

difference in heat-resistant CFU between the two strains was due in part to sporulation.

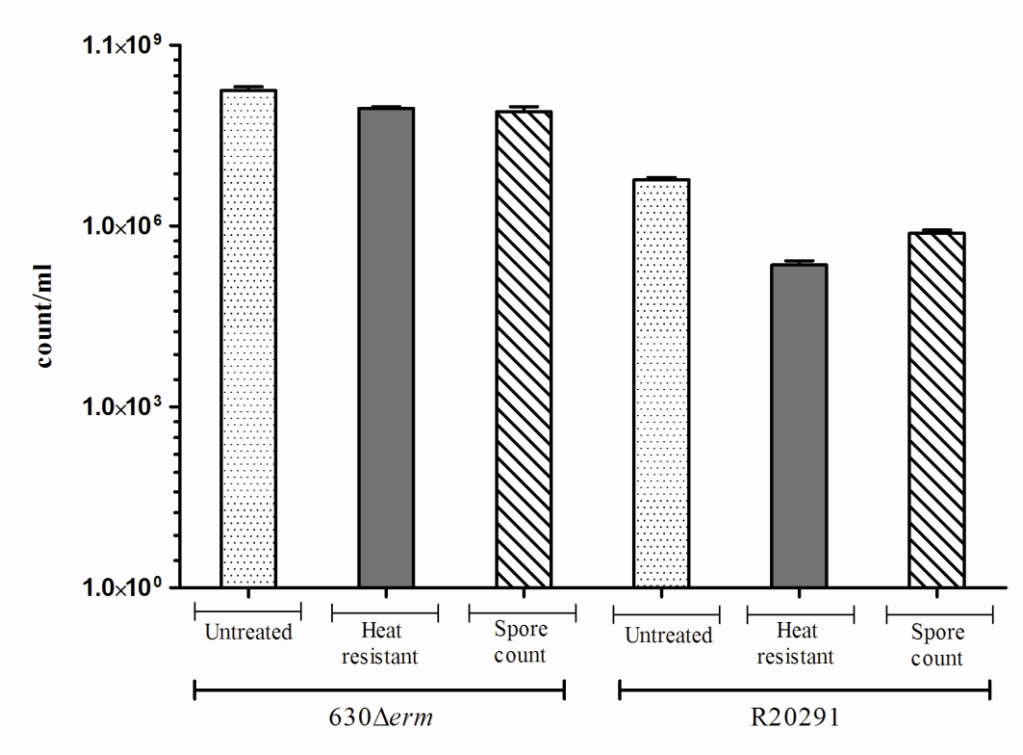

Figure 3.7. Numbers of untreated CFU, spores counted by phase-contrast microscopy, and heat-resistant CFU of *C. difficile* 630Δ*erm* and *C. difficile* R20291 after five days of incubation in BHIS broth. The bars indicate the averages of three independent experiments and error bars indicate the standard errors of the means. The detection limit for colony counts was 50 CFU/ml, and the detection limit for spore counts was 5×10^3 spores.

As 630Δ*erm* produced 400-fold more heat-resistant CFU than R20291, it was also hypothesised that fewer R20291 spores than 630Δ*erm* spores had completed germination and outgrowth under the conditions used in this assay. To test the hypothesised differences in germination and outgrowth between

630Δ*erm* and R20291, the observed spore titres were compared to the numbers of heat-resistant CFU on BHIS agar supplemented with taurocholate (Figure 3.8) after five days. It was found that a greater proportion of 630Δ*erm* spores (>95%) than of R20291 spores (<30%) formed colonies after heat treatment. Thus, the data suggest not only that the non-epidemic strain *C. difficile* 630Δ*erm* sporulates earlier and to a greater degree than the BI/NAP1/027 isolate R20291 under the growth conditions employed, but also that a higher proportion of 630Δ*erm* spores than of R20291 spore complete germination and form colonies in association with the bile salt taurocholate.

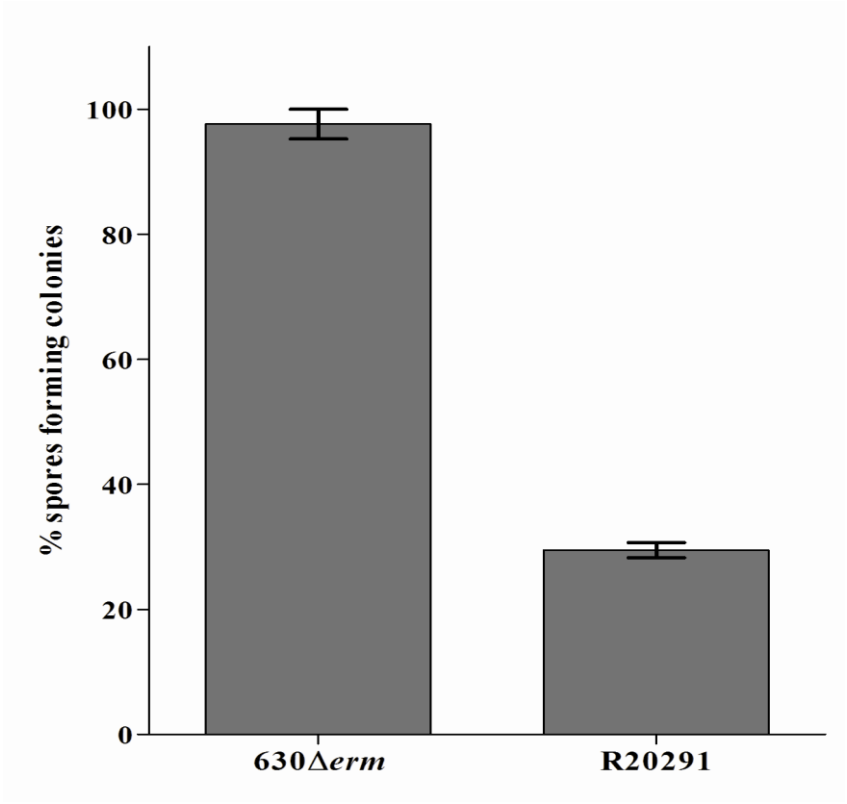

Figure 3.8. The proportion of *C. difficile* 630Δ*erm* and *C. difficile* R20291 spores, counted by phase-contrast microscopy, that formed colonies after heat treatment. The bars represent three independent experiments, and the error bars indicate the standard errors of the means.

3.3 Discussion

One of the most challenging aspects of studying *C. difficile* sporulation and germination has been developing suitable methods for analysing mutant phenotypes. A historic lack of genetic tools has, up until now, perhaps prevented research into this area, but a range of tools are now available (Heap, *et al.*, 2007; Heap, *et al.*, 2009; Cartman and Minton, 2010; Heap, *et al.*, 2010). This chapter described the development of a reproducible assay for studying both the sporulation and germination characteristics of *C. difficile*. Such an assay will provide the means by which genes required for *C. difficile* sporulation and germination can be identified in the future.

Unfortunately, the precise mechanisms by which *C. difficile* forms spores remain unclear. As a result, it is difficult to understand the specific conditions that are optimal for *C. difficile* sporulation. The data presented above suggest that *C. difficile* forms spores efficiently in a nutrient-rich liquid medium, which is interesting as nutrient starvation has long been described as a trigger of sporulation in bacilli (Schaeffer, *et al.*, 1965; Sonenshein, 2000). It was possible to reproducibly induce sporulation of *C. difficile* by incubating cultures in the nutrient-rich broth for five days. The rate of sporulation was then determined by observing the development of heat-resistant CFU at different time-points, with the bile salt taurocholate acting as a germinant to recover *C. difficile* spores. Importantly, spore titres were enumerated independently of colony formation after five days, using phase-contrast microscopy, and this spore titre was then compared to the observed CFU titre

at the same time-point. This made it possible to distinguish between *C. difficile* sporulation and germination.

The emergence of *C. difficile* BI/NAP1/027 strains (Dawson, *et al.*, 2009) has strengthened the need to understand *C. difficile* sporulation and germination characteristics. These strains are associated with more severe disease and greater virulence, and although it is still unclear how sporulation and germination in epidemic and non-epidemic strains compare, it has been suggested that the rate of sporulation is higher in particular BI/NAP1/027 isolates (Wilcox and Fawley, 2000; Fawley, *et al.*, 2007; Akerlund, *et al.*, 2008; Merrigan, *et al.*, 2010). The assay developed above was, therefore, used to determine the sporulation and germination characteristics of a BI/NAP1/027 isolate, R20291, and a non-epidemic strain, 630Δ*erm*. *C. difficile* R20291 formed spores slower (Figure 3.5) and formed fewer spores than *C. difficile* 630Δ*erm* over a five-day period (Figure 3.7). Furthermore, a lower number of the observed spores of *C. difficile* R20291 than of the observed spores of 630Δ*erm* completed germination and formed colonies after heat treatment. Given the current literature on the sporulation characteristics of different *C. difficile* types, these findings indicate that caution should be taken when general conclusions are drawn about the properties of types of *C. difficile* strains without an adequate sample size. Studies encompassing a larger number of *C. difficile* isolates are necessary to define what role sporulation and germination play in disease.

3.4 Key outcomes

- An assay was optimised to accurately assess the *in vitro* sporulation and germination/outgrowth characteristics of *C. difficile* strains based on colony formation after heat treatment, with the bile salt taurocholate acting as a germinant. Measurements were taken at multiple time-points to analyse the rate of sporulation, and total sporulation was enumerated, independent of colony formation, by phase-contrast microscopy.

- The *C. difficile* BI/NAP1/027 type strain R20291 was found to sporulate more slowly and produce fewer spores than the non-epidemic 630Δ*erm*. Furthermore, fewer R20291 spores appeared to germinate and form colonies under the conditions employed. These findings are in contrast to the previously published literature, which has suggested that BI/NAP1/027 strains are more prolific in terms of sporulation *in vitro*.

Chapter Four

The diverse sporulation and germination characteristics of *C. difficile* clinical isolates

4.1 Introduction

In the last decade, there has been an alarming rise in reported cases of CDAD across Europe and North America (Brazier, *et al.*, 2008; Rupnik, *et al.*, 2009). Outbreaks of severe disease, high rates of relapse, and increased mortality have all become associated with the so-called 'hypervirulence' of an emerging group of *C. difficile* strains. Cases of CDAD associated with the BI/NAP1/027 type have now been reported in 16 European countries (Kuijper, *et al.*, 2008). Furthermore, three countries have described imported disease associated with BI/NAP1/027 strains, where the patient acquired the *C. difficile* strain abroad (Kuijper, *et al.*, 2008).

Outbreaks of CDAD due to BI/NAP1/027 strains of *C. difficile* continue to be reported and these types are now the most clinically relevant type in the United Kingdom (Brazier, *et al.*, 2008). Consequently, as these strains are also associated with more severe disease, studies of the mechanisms behind the pathogenesis of BI/NAP1/027 strains are now at the forefront of *C. difficile* research worldwide. The first characteristic of BI/NAP1/027 strains to be associated with hypervirulence was the production of toxins, the major virulence factors of *C. difficile*. As described in Chapter One, 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.

4.1.1 Association of sporulation/germination with hypervirulence

The increased disease incidence and severity associated with *C. difficile* types such as BI/NAP1/027 have largely been attributed to the increase in toxin production described above. However, whilst this observed higher toxin production is doubtless important, perhaps the recent attention given to BI/NAP1/027 strains has focused too much on toxins. It seems likely that a number of other factors may play a role in the emergence of hypervirulent *C. difficile* strains, factors such as increased antibiotic resistance (Kuijper, *et al.*, 2006), increased adherence (Schwan, *et al.*, 2009), and possibly the rate of spore formation and the subsequent spore germination. It has recently been suggested that BI/NAP1/027 strains have a greater *in vitro* sporulation capacity than non-outbreak strains (Wilcox and Fawley, 2000; Fawley, *et al.*, 2007; Akerlund, *et al.*, 2008; Merrigan, *et al.*, 2010). As spores represent the infectious stage of *C. difficile*, and BI/NAP1/027 strains have become the most frequently clinically isolated type in the United Kingdom, a detailed understanding of the sporulation properties of such epidemic types is important and may have practical implications on future cleaning and treatment procedures. However, given the findings in Chapter Three that the BI/NAP1/027 isolate R20291 forms spores slower and to a lower degree *in vitro* than a non-epidemic PCR ribotype 012 isolate 630Δ*erm*, the differences in sporulation efficiency between *C. difficile* strains may not be as straightforward as has been previously suggested. To-date, there has been no convincing evidence published for or against the type-association of *C. difficile* sporulation rates. Consequently, until multiple isolates from many families of

C. difficile have been analysed, it may be premature to assume such differences exist between epidemic and non-outbreak strains.

As spores must germinate to cause disease following ingestion by susceptible individuals, it is also desirable to study any differences in germination between *C. difficile* types. Knowledge of diversity in the germination characteristics of *C. difficile* clinical isolates could help to develop a more detailed understanding of those types associated with more severe disease. The evidence presented in Chapter Three, that more 630Δ*erm* spores than R20291 spores appear to germinate and form colonies in association with the bile salt taurocholate, suggests that differences in germination may exist between epidemic and non-epidemic isolates. Consequently, a study of both the sporulation and germination characteristics of a large range of *C. difficile* isolates, from a variety of types, could help in understanding better the characteristics that affect the virulence of *C. difficile* strains.

4.1.2 Aim of this study

In order to gather more data and better understand any differences in sporulation and germination between different *C. difficile* types, the study presented in this chapter used a series of assays to measure these characteristics in a variety of *C. difficile* types, including several BI/NAP1/027 isolates from both North America and Europe (Table 4.1 lists the strains used in this study and their relevant properties).

4.2 Results

4.2.1 C. difficile strains used in this study

Previous studies of sporulation efficiencies between different *C. difficile* types have been limited to a few individual strains. It is, therefore, difficult to currently link processes such as sporulation (or germination) with increased disease severity. To investigate this subject in more detail, the sporulation and germination characteristics of *C. difficile* types were analysed using 14 strains. The group incorporated seven isolates belonging to the BI/NAP1/027 group, and seven isolates of a variety of other types, including isolates of PCR-ribotypes 017 and 078, which are also of interest due to their recent emergence and association with infection of animals used for food (Songer and Anderson, 2006). An isolate associated with particularly high levels of toxin production, VPI 10463, was also analysed as it has been suggested that those types producing high levels of toxin sporulate less prolifically than other types (Sullivan, *et al.*, 1982; Akerlund, *et al.*, 2008).

The BI/NAP1/027 isolates chosen for this study were all previously isolated from outbreaks across Europe and North America (Killgore, *et al.*, 2008). Interestingly, two of these isolates, CDC 32 and R12087, were examples of 'historical' BI/NAP1/027 strains, isolated as early as the 1980s (Killgore, *et al.*, 2008). The non-BI/NAP1/027 group included a wide range of PCR-ribotypes. Given the findings in Chapter Three that 630Δ*erm* sporulates and forms colonies after heat treatment to greater levels than the BI/NAP1/027

strain R20291, the *C. difficile* 630Δ*erm* parental strain was also selected for comparison with the other BI/NAP1/027 strains in this study. Table 4.1 lists the *C. difficile* strains selected for analysis, which also included a *C. difficile* 630Δ*erm spo0A* mutant to act as a sporulation-negative control.

Strain	Relevant properties	Source/reference
BI/NAP1/027		
R20291	Stoke Mandeville (2004/2005) isolate	Anaerobe Reference Laboratory, Cardiff
CDC 32	Historical USA isolate	(Killgore, *et al.*, 2008)
CDC 38	USA isolate	(Killgore, *et al.*, 2008)
M13042	Canada isolate	(Killgore, *et al.*, 2008)
DH326	Yorkshire and Humberside, Sheffield isolate (2005)	Anaerobe Reference Laboratory, Cardiff
DH1329	West Midlands, Coventry isolate (2007/8)	Anaerobe Reference Laboratory, Cardiff
R12087	Historical EU isolate (1980s)	Michel Popoff, Institut Pasteur, Paris
Non-BI/NAP1/027		
630Δ*erm*	PCR ribotype 012 Erythromycin sensitive strain of *C. difficile* 630	(Hussain, *et al.*, 2005)
630Δ*erm* spo0A::intron *ermB*	*C. difficile* sporulation-negative control	(Heap, *et al.*, 2007)
GAI 95601	PCR-ribotype 017, Japan	(van den Berg, *et al.*, 2007)
001-3	PCR-ribotype 001	ECDC – Cardiff collection
Serosubtype A2	PCR-ribotype 002	ECDC – Cardiff collection
Wilcox 078	PCR -ribotype 078	Mark Wilcox
R10459	PCR-ribotype 106	ECDC – Cardiff collection
VPI 10463	PCR-ribotype 003 Toxinotype 0 reference strain	(Sullivan, *et al.*, 1982)

Table 4.1. *C. difficile* strains selected for analysis of sporulation and germination characteristics.

4.2.2 Growth of C. difficile strains in BHIS medium

As sporulation and germination measurements may be affected by growth differences between strains, all *C. difficile* strains were first cultivated in BHIS broth for 24 h, and growth was monitored by measuring the change in OD_{600} (Figure 4.1 and Figure 4.2). All strains showed highly similar growth characteristics, which allows for the exclusion of growth differences when interpreting the subsequent experiments in this chapter. It was interesting to observe that there were no obvious growth differences between the selected *C. difficile* types, as it has been suggested in the past that a particular characteristic of the BI/NAP1/027 type is for the cell density at 24 h to be approximately 20% higher than that of other *C. difficile* types (Warny, *et al.*, 2005; Akerlund, *et al.*, 2008).

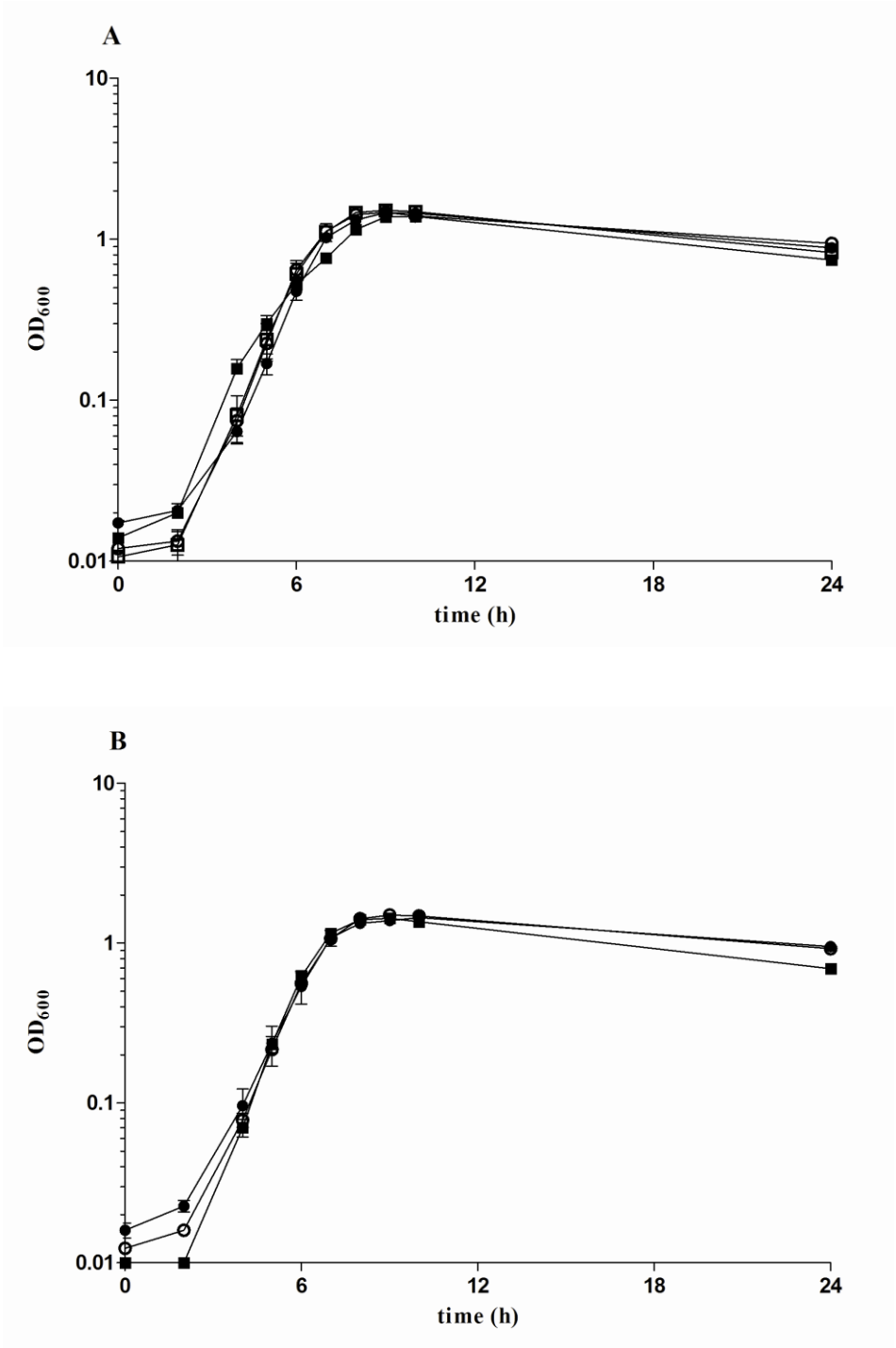

Figure 4.1. The growth of *C. difficile* BI/NAP1/027 strains in BHIS broth, as indicated by the change in OD_{600}, during a 24 h period. (A) ●, CDC 32; ■, R20291; ○, R12087; □, M13042; (B) ●, CDC 38; ■, DH1329; ○, DH326. The symbols represent the averages of three independent experiments, and error bars indicate standard errors of the means.

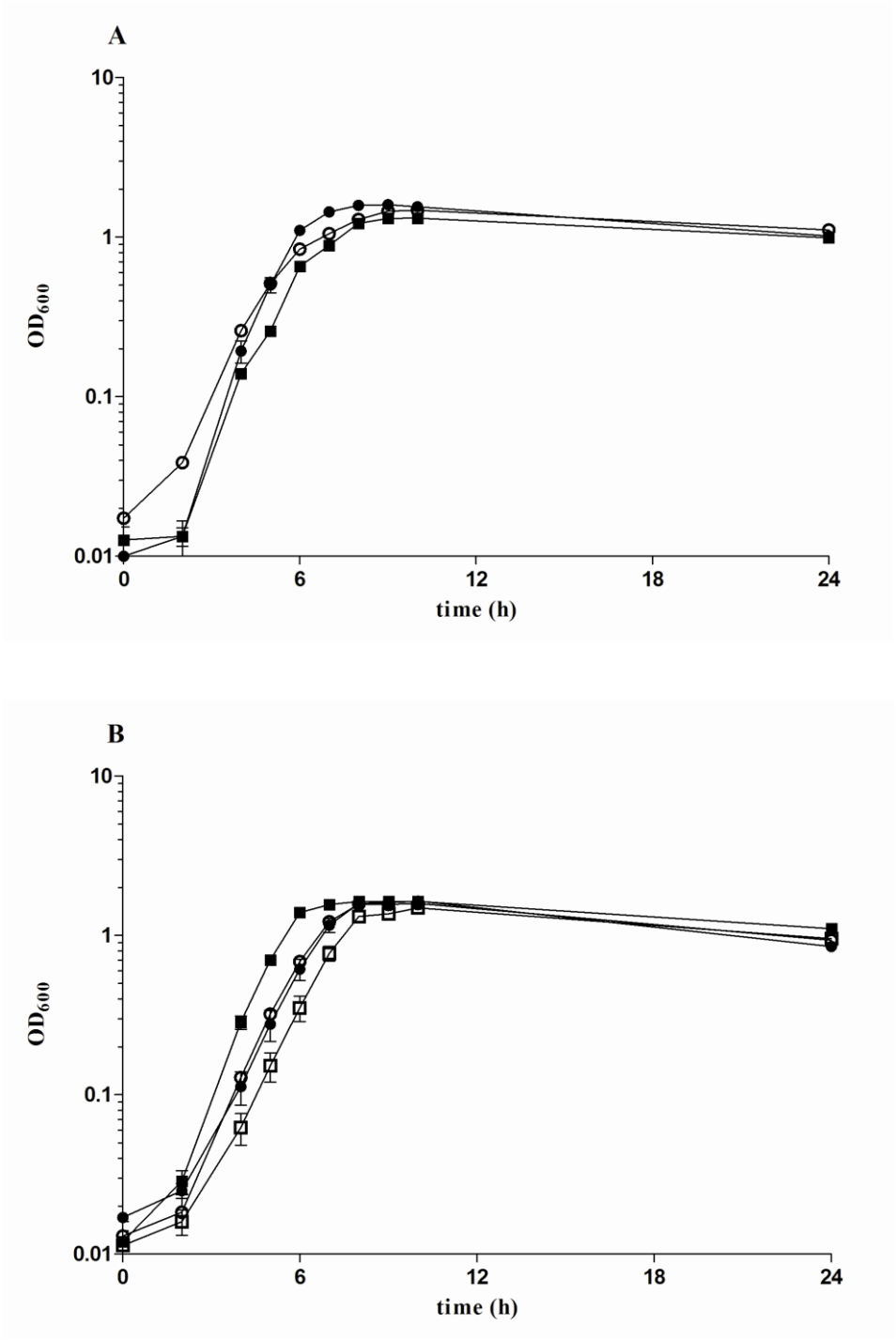

Figure 4.2. The growth of *C. difficile* non-BI/NAP1/027 strains in BHIS broth, as indicated by the change in OD_{600}, during a 24 h period. (A) ●, R10459; ■, 630Δ*erm*; ○, VPI 10463; (B) ●, GAI 95601; ■, Serosubtype A2; ○, 001-3; □, Wilcox 078. The symbols represent the averages of three independent experiments, and error bars indicate standard errors of the means.

4.2.3 Colony formation of C. difficile strains after heat treatment

In order to understand the rate at which the selected *C. difficile* strains formed spores *in vitro*, 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 large degree of variation within, but not necessarily between *C. difficile* types (Figure 4.3 and Figure 4.4), which suggests that the variation in sporulation rates between strains is not associated with type.

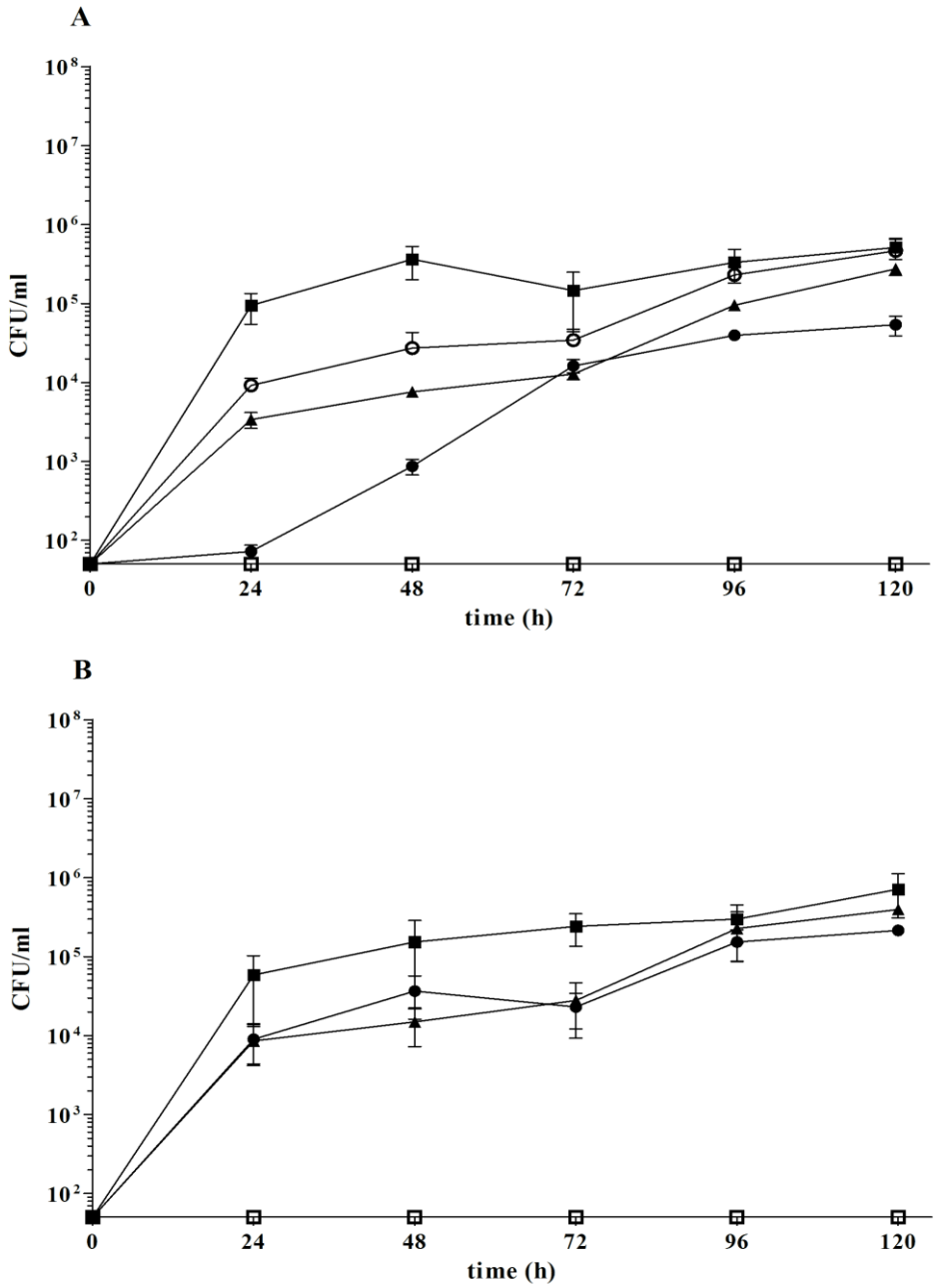

Figure 4.3. The development of heat-resistant CFU of BI/NAP1/027 *C. difficile* strains over five days. (A) ○, CDC 32; ■, M13042; ▲, R12087; ●, R20291; □ *C. difficile* 630Δ*erm spo0A*; (B) ●, CDC 38; ■, DH326; ▲, DH1329; ○, *C. difficile* 630Δ*erm spo0A*. The symbols represent the averages of three independent experiments, and error bars indicate standard errors of the means. The detection limit for colony counts was 50 CFU/ml.

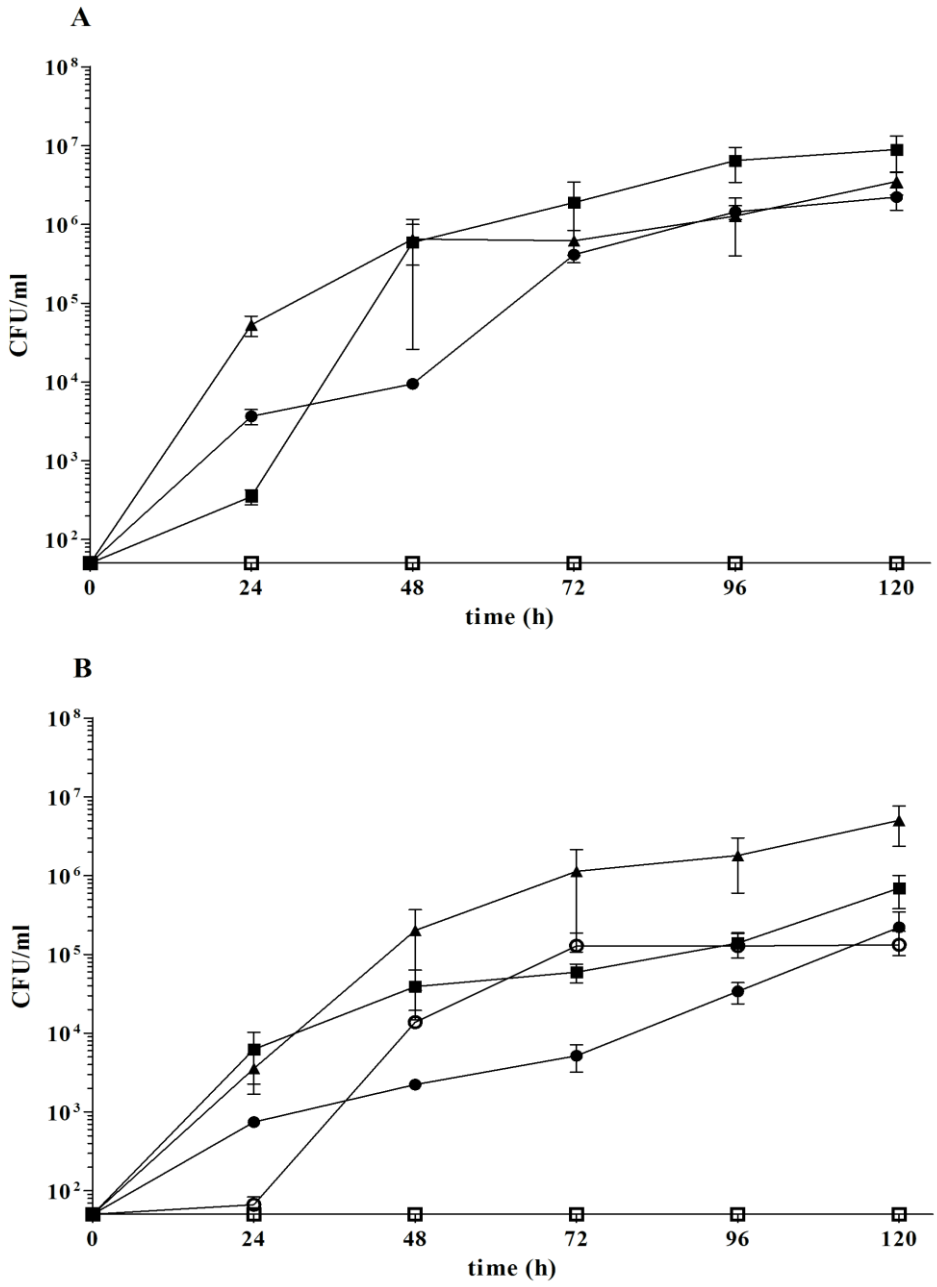

Figure 4.4. The development of heat-resistant CFU of non-BI/NAP1/027 *C. difficile* strains over five days. (A) ▲, R10459; ●, 630Δ*erm*; ■, VPI 10463; ○, *C. difficile* 630Δ*erm spo0A*; (B) ■, 001-3; ▲, Serosubtype A2; ●, GAI 95601; ○, Wilcox 078; □ *C. difficile* 630Δ*erm spo0A*. The symbols represent 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.

To allow for a better comparison of the sporulation rate of *C. difficile* strains, the levels of heat-resistant CFU for each strain at both 24 and 120 h were then plotted and compared separately (Figure 4.5). No obvious difference in the level of heat-resistant CFU was seen between the BI/NAP1/027 and non-BI/NAP1/027 groups after 24 h suggesting that, under the conditions of the assay, there is no association between *C. difficile* type and sporulation over a 24 h period. However, the BI/NAP1/027 group appeared to form lower levels of heat-resistant CFU after 120 h when compared to the non-BI/NAP1/027 group, although this difference was not quite statistically significant ($p = 0.054$).

Interestingly, a low level of heat-resistant CFU after 24 h in comparison to other isolates, such was the case with *C. difficile* VPI 10463, did not necessarily correlate to a low level of heat-resistant CFU after 120 h. To the contrary, it was noted that while VPI 10463 formed few heat-resistant CFU after 24 h, significantly more heat-resistant CFU were observed after 120 h than all tested BI/NAP1/027 isolates ($p < 0.05$). Likewise, when compared to the other individual strains, a high level of colony formation observed after 24 h, such was the case with the BI/NAP1/027 isolate *C. difficile* M13042, did not always result in high levels of colony formation after 120 h when compared to other strains. This suggests that in a case where a strain initiates sporulation particularly early, such a strain may not necessarily form a higher total of spores than a strain which initiates sporulation at a later stage.

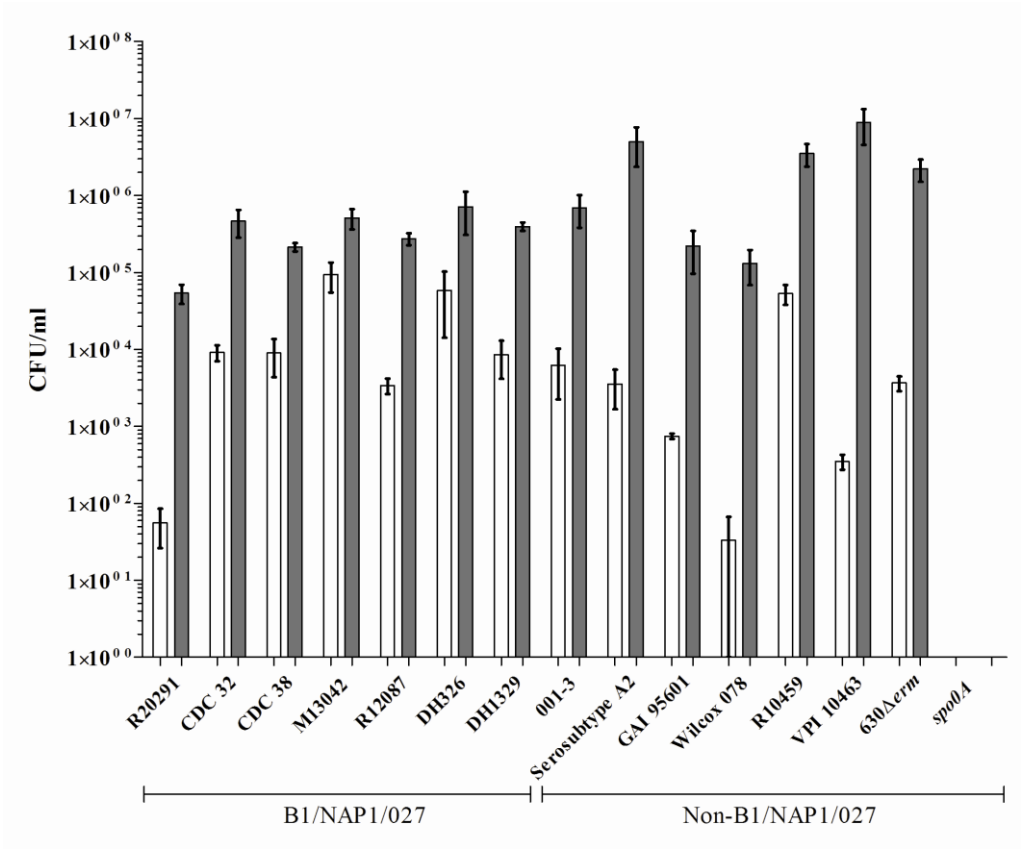

Figure 4.5. The colony formation following heat treatment of *C. difficile* strains after 24 h (open bars) and 120 h (filled bars). Heat-resistant CFU were enumerated from the same cultures as analysed in Figure 4.3 and Figure 4.4. The bars represent the averages of three independent experiments, and error bars indicate standard errors of the means. The detection limit for colony counts was 50 CFU/ml.

4.2.4 Sporulation of C. difficile strains after 5 days

As the BI/NAP1/027 group appeared to form fewer heat-resistant CFU after five days than the non-BI/NAP1/027 group, it is conceivable that differences exist in the sporulation characteristics of these groups. However, as heat-resistant CFU represent successfully sporulated vegetative cells that were also able to germinate and grow vegetatively, a discrepancy between CFU of

different strains may also due to the *in vitro* germination characteristics of the strains. In order to accurately compare the sporulation of *C. difficile* strains, spores titres were therefore enumerated independently of colony formation, by phase-contrast microscopy (Figure 4.6). Spore titres varied significantly among the 14 isolates studied ($p < 0.05$), and although the BI/NAP1/027 group appeared to form fewer spores than the non-BI/NAP1/027 group, this difference was not statistically significant. These data suggest that while spore titres varied greatly between individual strains, there was no obvious type-associated variation in the spore titres obtained from the *C. difficile* strains in this study. However, in a number of cases there was a discrepancy between the microscopically-observed spore titre and heat-resistant CFU.

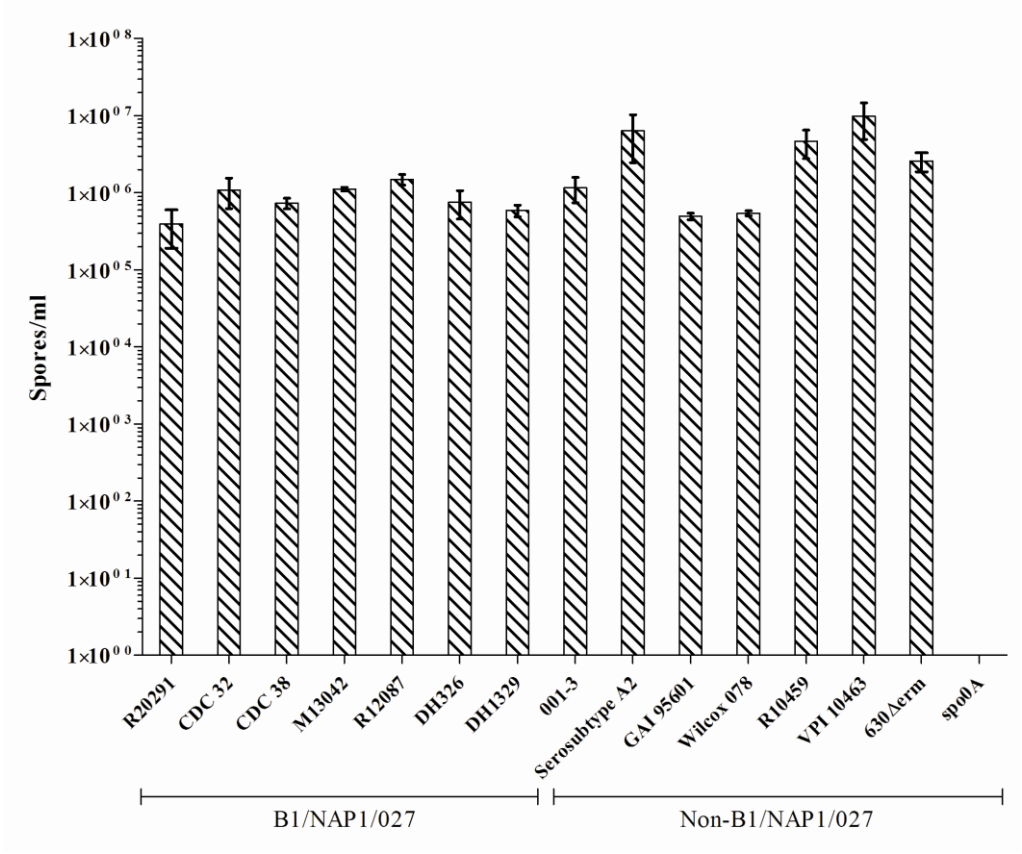

Figure 4.6. *C. difficile* spore titres enumerated by phase-contrast microscopy following five days of incubation in BHIS broth. The bars represent the averages of three independent experiments, and error bars indicate standard errors of the means. The detection limit for spore counts was 5×10^3 spores.

4.2.5 Colony forming characteristics of C. difficile spores

As spores must complete germination in order to form colonies, an inconsistency between spore titre and colony formation may be caused by a difference in the frequency of germination and spore outgrowth. Accordingly, the proportion of spores counted by phase-contrast microscopy that formed heat-resistant CFU at 120 h, when germination was induced by plating onto BHIS agar supplemented with taurocholate, were analysed (Figure 4.7). This

colony formation varied significantly among the 14 *C. difficile* isolates tested ($p < 0.001$), and it was also noted that spores of a number of non-BI/NAP1/027 strains formed more colonies than the spores of certain BI/NAP1/027 strains. In particular, a significantly higher proportion of VPI 10463 and 630Δ*erm* spores formed colonies than the BI/NAP1/027 strains R20291, R12087, CDC 32 and CDC 38 ($p < 0.05$). Furthermore, spores of the non-BI/NAP1/027 isolates Serosubtype A2 and R10459 formed significantly more colonies than R12087 and CDC 38 ($p < 0.05$). However, it was also noted that spores of a PCR-ribotype 078 isolate (Wilcox 078) formed significantly fewer colonies in comparison to VPI 10463 ($p < 0.05$). Not all BI/NAP1/027 strains formed proportionally fewer colonies than microscopically-observed spores, as a significantly higher proportion of DH326 spores formed colonies when compared to R12087 ($p < 0.05$), while not showing any significant differences to VPI 10463, R10459, Serosubtype A2 or 630Δ*erm* (non-BI/NAP1/027). Additionally, when the BI/NAP1/027 and non-BI/NAP1/027 groups were directly compared, there was no significant variation observed in the proportion of spores which formed colonies ($p = 0.14$). Taken together, these data indicate that following heat treatment, spores of a number of *C. difficile* BI/NAP1/027 isolates appear to form colonies at a lower frequency than certain non-BI/NAP1/027 isolates. However, a large degree of variation was observed between individual *C. difficile* strains, and the smaller proportion of spores forming colonies was not specific to the BI/NAP1/027 type.

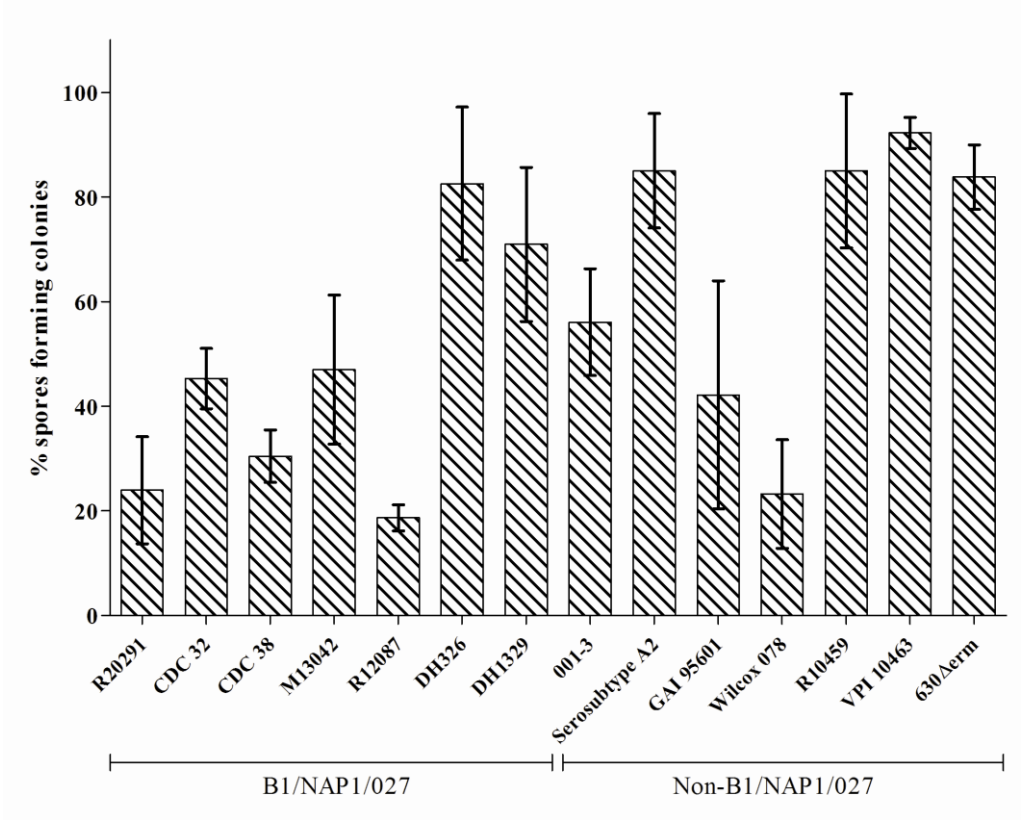

Figure 4.7. The proportion of *C. difficile* spores, counted by phase-contrast microscopy, that formed colonies after heat treatment. The bars represent the averages of three independent experiments, and error bars indicate standard errors of the means.

4.3 Discussion

Given the recent suggestions that *C. difficile* strains belonging to the BI/NAP1/027 type are more prolific in terms of sporulation than spores of other *C. difficile* types, the data presented in this chapter are perhaps surprising. This study used 14 isolates to compare sporulation and germination characteristics *in vitro*, the largest sample-size used to-date in such a study, and it was observed that the substantial variation in sporulation and germination properties between isolates does not seem to be type-associated.

A particularly interesting difference between this study and previous work was noted in *C. difficile* VPI 10463. It has been suggested by Akerlund et al. that VPI 10463 sporulates poorly over a 48 h period (Akerlund, *et al.*, 2008), but the data presented here show the opposite. While few heat-resistant CFU were seen after 24 h (Figure 4.5), VPI 10463 formed spores just as prolifically as any other strain tested from 48 h onwards (Figure 4.3, Figure 4.4 and Figure 4.6). Two notable differences between this study and the one by the Åkerlund laboratory are the growth conditions employed and the methods of assaying sporulation. Akerlund and co-workers cultivated *C. difficile* strains in a broth containing peptone-yeast and without cysteine and glucose (Akerlund, *et al.*, 2008), while the *C. difficile* strains in this study were cultivated in BHIS broth as described in Chapter Two. Furthermore, Akerlund *et al*. (2008) expressed sporulation rate as a ratio of spores to vegetative cells within the population after 48 h, a method that has previously been used to compare the sporulation of *C. difficile* isolates (Wilcox and Fawley, 2000; Fawley, *et al.*, 2007). However, this relative measure can be affected by growth differences between strains and also by the survival of non-sporulating cells. Additionally, without an actual spore titre it is difficult to accurately quantify sporulation. Based on the data presented in this chapter, it may be more appropriate to (i) measure the change in OD_{600} to evaluate growth differences; (ii) observe the development of heat-resistant CFU to assess the rate of sporulation; and (iii) enumerate spore titres independently of colony formation, by microscopy, to allow for a direct comparison of *C. difficile* sporulation with germination and outgrowth.

The most obvious follow-up to this study would be a comprehensive analysis of the sporulation and germination characteristics of 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 the sporulation and germination properties of *C. difficile* strains and types, and the subsequent role in disease incidence and severity.

Overall, the findings in this chapter show that the variation in *C. difficile* sporulation and germination frequency is far greater within PCR-ribotypes than between PCR-ribotypes. The obvious question remains of how the *in vitro* sporulation characteristics described in this study relate to sporulation proficiency *in vivo*. Consequently, until our understanding of *in vivo C. difficile* sporulation improves, it will be difficult to associate the sporulation characteristics of *C. difficile* BI/NAP1/027 with the severity of disease caused by this type. Furthermore, the observed inconsistency between spore counts and heat-resistant CFU of a number of *C. difficile* strains in this study also raises questions about the germination rates of different *C. difficile* strains. If differences do exist in the germination rates of different *C. difficile* types, future studies analysing these characteristics may provide a greater understanding of what factors contribute to the incidence and severity of *C. difficile* disease.

4.4 Key outcomes

- The sporulation characteristics of fourteen *C. difficile* strains were analysed, incorporating a variety of PCR-ribotypes, including several BI/NAP1/027 isolates from both North America and Europe.

- The rate of sporulation and total sporulation after five days varied substantially between individual isolates, but this variation did not appear to be PCR-ribotype-associated.

- Spores of a number of BI/NAP1/027 formed colonies at a lower frequency than spores of some non-BI/NAP1/027 strains, suggesting differences exist in the germination properties of *C. difficile* strains. However, the observed lower colony formation was not specific to the BI/NAP1/027 type, and substantial variation was observed between individual isolates.

- Taken together, these data cast doubt on the suggestions that BI/NAP1/027 strains of *C. difficile* sporulate more prolifically than other PCR-ribotypes. Careful experimental design may therefore be required when measuring the *in vitro* sporulation characteristics of *C. difficile*, and larger sample sizes should be studied before generalisations are made.

Chapter Five

SleC is essential for germination of *C. difficile* spores in nutrient-rich medium supplemented with the bile salt taurocholate

5.1 Introduction

The precise mechanisms of germination in *C. difficile* have not been studied in great depth, due in part to an absence of genetic tools. However, the ClosTron now provides an efficient means to study *C. difficile* spore germination mechanisms using a reverse genetics approach (Heap, *et al.*, 2007; Heap, *et al.*, 2010), by identifying homologues of genes important for germination in other spore formers, inactivating them, and inferring their function from mutant phenotypes.

5.1.1 Spore cortex-lytic enzymes

In spores of *B. subtilis*, the spore core, which contains the spore DNA, RNA and most enzymes, is surrounded by the germ cell wall, a thin layer of PG identical to that of vegetative cell PG. The germ cell wall is surrounded by the cortex, a thicker layer of spore-specific PG which, as described in Chapter One, is chemically distinct from vegetative cell PG. One such modification in spore PG is that approximately 50% of muramic acid residues are converted to muramic-δ-lactam (Warth and Strominger, 1969; Warth and Strominger, 1972). It is believed that this muramic-δ-lactam serves as a recognition component for germination-specific spore cortex-lytic enzymes (SCLEs) (Popham, *et al.*, 1996c), which are responsible for hydrolysis of the PG cortex. It is during this hydrolysis that the previously low water content of the spore core is restored to the water content of a normal vegetative cell and the core is

able to expand, which in turn allows enzyme activity, metabolism, and spore outgrowth (Setlow, 2003).

B. subtilis encodes two semi-redundant SCLEs, CwlJ and SleB, which are involved in cortex hydrolysis, breaking down PG containing muramic-δ-lactam (Popham, *et al.*, 1996a). SleB has been shown to localise in both the inner and outer layers of *B. subtilis* spores through interaction of the enzyme peptidoglycan-binding motif and the δ-lactam structure of the cortex (Masayama A, 2006) and in association with YpeB, which is required for *sleB* expression during sporulation (Boland, *et al.*, 2000; Chirakkal, *et al.*, 2002). It has been demonstrated that CwlJ is localised to the spore coat during sporulation in association with GerQ (Bagyan and Setlow, 2002; Ragkousi, *et al.*, 2003), and is required for CaDPA-induced germination (Paidhungat, *et al.*, 2001). Activation of CwlJ can be due to either CaDPA released from the spore core at the onset of germination or exogenous CaDPA (Paidhungat, *et al.*, 2001). This mechanism of germination is interesting, as it completely by-passes the *B. subtilis* germinant receptors (Paidhungat and Setlow, 2000), and this provides strong evidence for alternative germination pathways which may allow for germination of the spore under a range of conditions. While CwlJ is essential for CaDPA-mediated germination in *B. subtilis*, neither CwlJ nor SleB is essential for complete cortex hydrolysis during nutrient germination, although inactivation of both *cwlJ* and *sleB* results in a spore unable to complete this process (Ishikawa, *et al.*, 1998). The role of a third SCLE, SleL,

has recently been studied in *Bacillus anthracis*. A *sleL* mutant in *B. anthracis* is still able to complete germination, although this process is retarded.

The SCLEs of *Clostridium* are less well studied than those of *Bacillus*. The SCLEs SleC (Miyata, *et al.*, 1995) and SleM (Chen, *et al.*, 1997) have been identified in *C. perfringens*, and a study demonstrated that SleC is absolutely required during germination for complete cortex hydrolysis (Paredes-Sabja, *et al.*, 2009b). Although SleM has been shown to degrade spore cortex peptidoglycan, and inactivation of both *sleC* and *sleM* decreased the ability of spores to germinate more than inactivation of *sleC* alone, SleM was not essential (Paredes-Sabja, *et al.*, 2009b). In addition, it has recently been shown that the germination-specific serine protease CspB is essential for cortex hydrolysis and functions to convert the inactive pro-SleC found in dormant spores to an active enzyme (Shimamoto, *et al.*, 2001; Paredes-Sabja, *et al.*, 2009c). The action of these proteases represents a significant difference between the germination of *C. perfringens* spores and the *Bacillus* paradigm as *Bacillus* encodes no known germination-specific proteases.

5.1.2 Germination targets in C. difficile

Thus far, there have been no detailed studies of any genes responsible for spore germination in *C. difficile*, although genes showing homology to *cwlJ* and *sleB* of *B. subtilis* (CD3563) and *sleC* of *C. perfringens* (CD0551, annotated as *sleC*) have now been identified in the *C. difficile* 630 genome (Sebaihia, *et al.*,

2006). The product of *sleC* shows 53% amino acid identity to the previously characterised SleC of *C. perfringens*, and the product of CD3563 shows 30% and 45% amino acid identity to *B. subtilis* CwlJ and SleB respectively. In addition, CD0552 has been annotated as *sleB* in the *C. difficile* 630 genome, and examination of an incomplete genome sequence available at http://www.sanger.ac.uk indicated that these equivalent homologues are also present in a BI/NAP1/027 strain, R20291.

5.1.3 Aim of this study

With a range of genetic tools now at our disposal, the way has been opened to understand more thoroughly the mechanisms of *C. difficile* spore germination. The study presented in this chapter, therefore, aimed to utilise the ClosTron system to inactivate *sleC*, CD3563 and CD0552 in both *C. difficile* 630Δ*erm* and R20291, and analyse the role played by these genes in the germination of *C. difficile* spores.

5.2 Results

5.2.1 Construction of mutant strains

To assess the importance of *sleC*, CD3563 and CD0552 in *C. difficile* germination, the ClosTron system (Heap, *et al.*, 2007; Heap, *et al.*, 2010) was used, as described in Chapter Two, to create independent insertional mutants

of *C. difficile* 630Δ*erm* in which either the CD3563, CD0552 or *sleC* gene was inactivated, yielding strains CRG878, CRG879 and CRG1115, respectively.

To confirm correct ClosTron insertion, putative mutant strains were analysed by PCR (Figure 5.1), and one erythromycin-resistant clone each of CRG878, CRG879 and CRG1115 were picked for sequencing at the intron insertion site (Table 5.1 shows intron insertion sites and the frequencies of the desired mutants obtained). In the case of *sleC*, it was not possible to isolate the desired mutant using the base 493 target site. Screening of a pool of genomic DNA from >100 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 128 with the antisense orientation) was chosen for *sleC*.

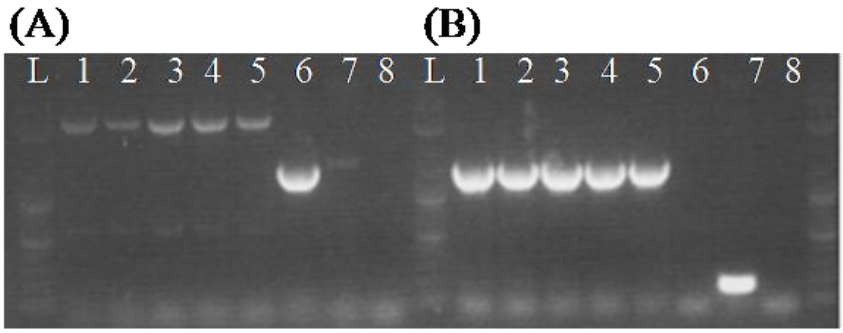

Figure 5.1. An example of PCR screening for putative *C. difficile* 630Δ*erm* *sleC* ClosTron insertion mutants. PCR was used initially to screen for intron insertion using (A) the gene-specific primer pairs SleC_F1 and SleC_R1 (630Δ*erm*), and (B) intron-specific EBS Universal primer and the gene-specific primer SleC_R1 (630Δ*erm*). Lane L, 2-log DNA ladder; Lanes 1-5, putative *sleC* mutant clones; Lane 6, *C. difficile* 630Δ*erm*; Lane 7, pMTL007C-E2::cdi-SleC-128a. The presence of a large band in (A) indicated putative intron insertion, and the 1,458 bp band in (B) was consistent with the size of band expected following 128a insertion of the ClosTron.

Strain and target site	Frequency of desired mutant among clones screened	
	%	No. positive/no. screened
C. difficile 630Δ*erm* CD3563 226s	100	8/8
C. difficile 630Δ*erm* CD0552 75a	90	9/10
C. difficile 630Δ*erm sleC* 493s	0	0/20
C. difficile 630Δ*erm sleC* 128a	100	5/5
C. difficile R20291 *sleC* 128a	100	4/4

Table 5.1. ClosTron insertion frequencies with erythromycin or lincomycin 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*) and lincomycin-resistant (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.

5.2.2 Colony formation by C. difficile mutant strains after heat treatment

Following inactivation of CD3563, CD0552 and *sleC* in *C. difficile* 630Δ*erm*, these 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 5.2). Although inactivation of CD3563 or CD0552 appeared to have no effect on *C. difficile* 630Δ*erm* colony formation, a *sleC* mutant was unable to form heat-resistant CFU at any point over the 5-day period. This suggests that *sleC* is absolutely required for sporulation and/or

taurocholate-induced germination in *C. difficile* 630Δ*erm*, while neither CD3563, nor CD0552, play an obvious role in either process.

In light of the findings in Chapter Three of sporulation and germination differences between *C. difficile* 630Δ*erm* and R20291, the effect of *sleC* inactivation was analysed in both strains. In this analysis, an equivalent ClosTron-derived *sleC* mutant of *C. difficile* R20291 (CRG1166) was constructed (Table 5.1 shows the intron insertion site and frequency of mutants obtained), and the effect on colony formation was determined using the conditions employed previously for 630Δ*erm*. This mutant exhibited the same phenotype as its 630Δ*erm* counterpart (Figure 5.3), suggesting that the role of *sleC* is the same in both strains.

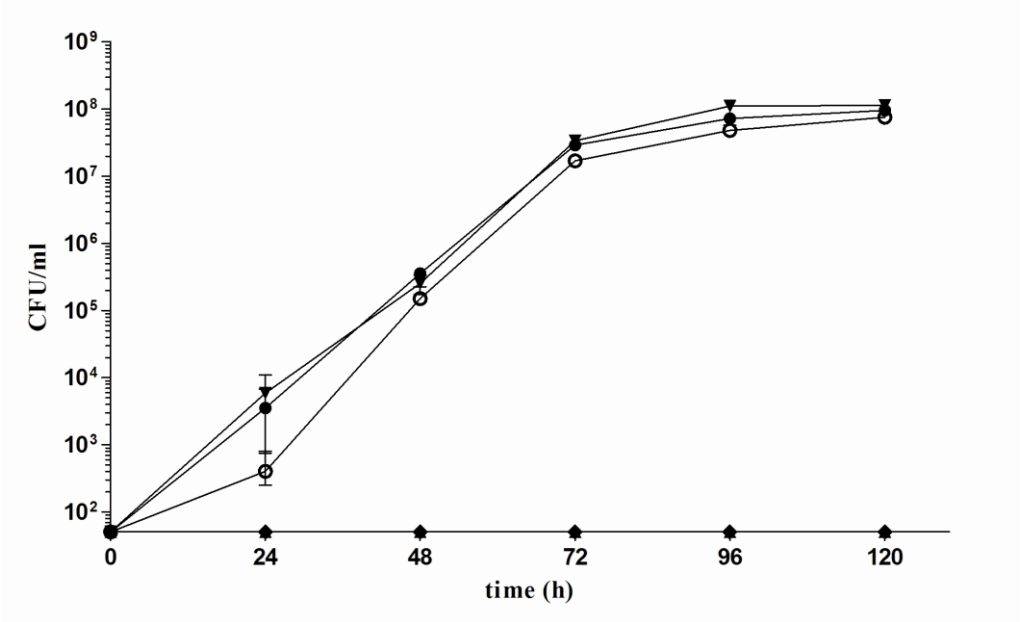

Figure 5.2. Development of heat-resistant CFU of *C. difficile* 630Δ*erm* mutants over 5 days on BHIS agar supplemented with 0.1% taurocholate. *C. difficile* strains were cultivated as described in Chapter Two. ●, *C. difficile* 630Δ*erm*; ▼, CRG878 (CD3563); ○, CRG879 (CD0552); ▲, CRG1115 (*sleC*); ♦, CRG789 (*spo0A*). 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. Data points for CRG1115 and CRG789 are overlaid because neither formed any colonies.

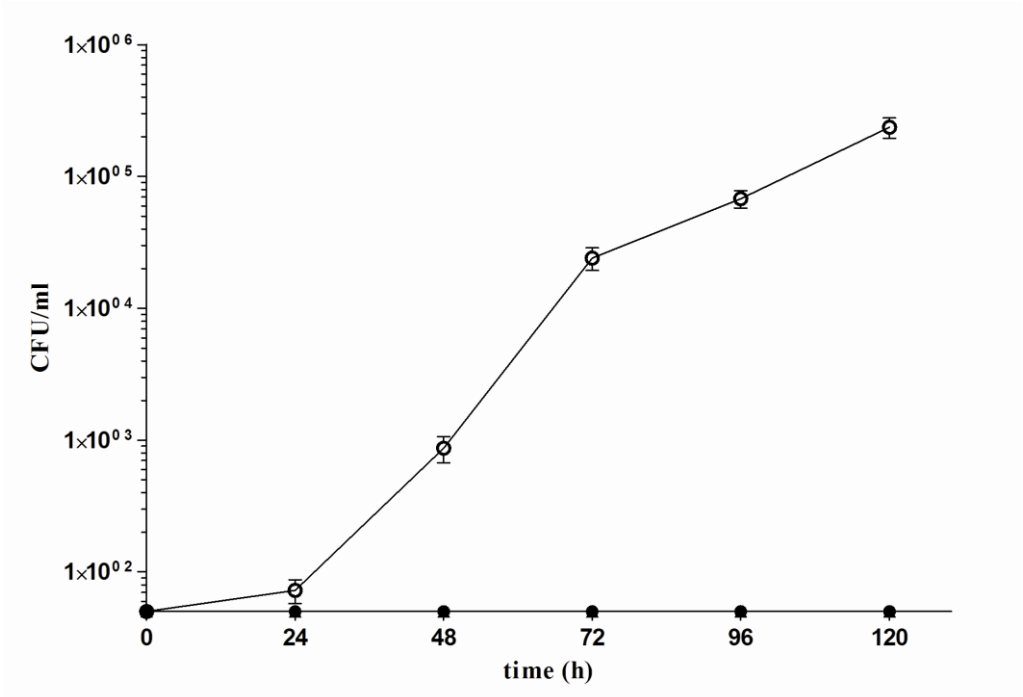

Figure 5.3. Development of heat-resistant CFU of *C. difficile* R20291 mutants over 5 days on BHIS agar supplemented with 0.1% taurocholate. *C. difficile* strains were cultivated as described in Chapter Two. ○, *C. difficile* R20291; ●, CRG1166 (*sleC)*; ▲, CRG1375 (*spo0A*). The symbols indicate the 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. Data points for CRG1166 and CRG1375 are overlaid because neither formed any colonies.

To ensure that the observed phenotypes of the *sleC* mutants of 630Δ*erm* and R20291 did not result from growth deficiencies, the change in OD_{600} was used to monitor growth over 5 days in BHIS broth (Figure 5.4 and Figure 5.5). The growth of both mutants was found to be indistinguishable from the growth of their respective parental strains, which suggests that there were no obvious growth defects following inactivation of *sleC* in either 630Δ*erm* or R20291.

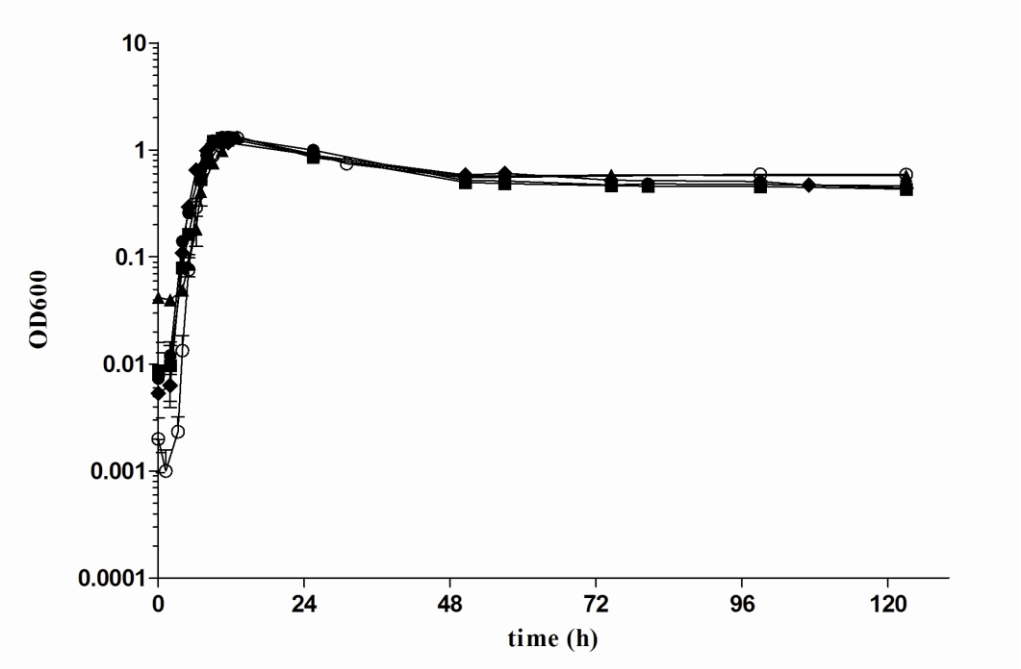

Figure 5.4. Growth of *C. difficile* 630Δ*erm* strains in BHIS broth over five days. Strains were cultivated as described in Chapter Two, and the OD_{600} measured. ●, *C. difficile* 630Δ*erm*; ▲, CRG1115 (*sleC*); ■, CRG878 (CD3563); ○, CRG879 (CD0552); ♦, CRG789 (*spo0A*). The symbols represent the averages of three independent experiments, and the error bars represent standard errors of the means.

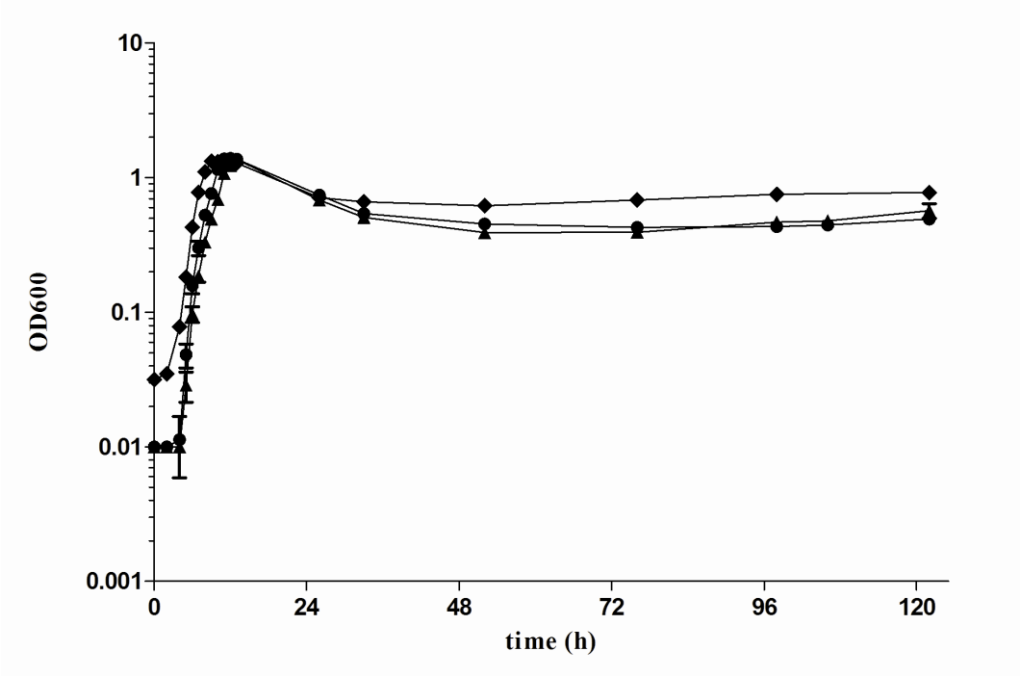

Figure 5.5. Growth of *C. difficile* R20291 strains in BHIS broth over five days. Strains were cultivated as described in Chapter Two, and the OD_{600} measured. ●, *C. difficile* R20291; ▲, CRG1166 (*sleC*); ♦, CRG1375 (*spo0A*). The symbols represent the averages of three independent experiments, and the error bars represent standard errors of the means.

5.2.3 Complementation of sleC mutant with parental SleC

In order to show that the observed phenotypes of CRG1115 and CRG1166 were a specific consequence of *sleC* inactivation, complementation studies were performed. Plasmid pMTL-DB1 was constructed, which contains the parental *sleC* structural gene and the 244 bp region immediately upstream of the open reading frame presumed to contain its promoter. Strains CRG1555 and CRG1634 were created by introducing this plasmid into *sleC* mutants of *C. difficile* 630Δ*erm* and *C. difficile* R20291, respectively. Empty vector control strains CRG1556 (630Δ*erm*) and CRG1628 (R20291) were also

created through introduction of pMTL84151 into the respective *sleC* mutant strains. 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 CRG1651 and CRG1652 were created by introducing pMTL84151 into parental strains 630Δ*erm* and R20291, respectively. 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. The levels of heat-resistant CFU associated with the taurocholate supplement were fully restored in the *sleC* mutant strains of both *C. difficile* 630Δ*erm* (Figure 5.6) and R20291 (Figure 5.7) carrying pMTL-DB1 to the levels of the parental strains carrying the control vector pMTL84151. In the case of 630Δ*erm*, there was a 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 630Δ*erm*. The *sleC* mutant controls containing the empty vector performed like the original mutant, with no observed colony formation over the five day period. Thus it was possible to complement the heat-resistant CFU defect, indicating that the observed phenotype was due solely to inactivation of *sleC*.

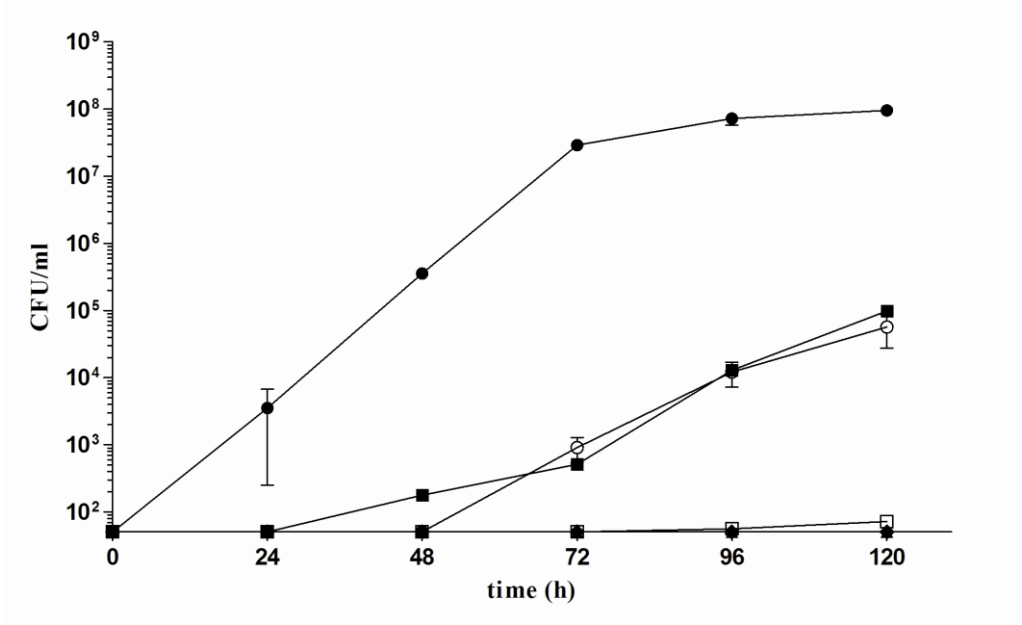

Figure 5.6. Development of heat-resistant CFU of *C. difficile* 630Δ*erm* mutants over 5 days on BHIS agar supplemented with 0.1% taurocholate. *C. difficile* strains were cultivated as described in Chapter Two. ●, *C. difficile* 630Δ*erm*; ▲, CRG1115 (*sleC*); ○, CRG1555 (CRG1115 complemented with parental *sleC*); □, CRG1556 (CRG1115 harbouring pMTL84151 empty vector control); ■, CRG1651 (*C. difficile* 630Δ*erm* harbouring pMTL84151). 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.

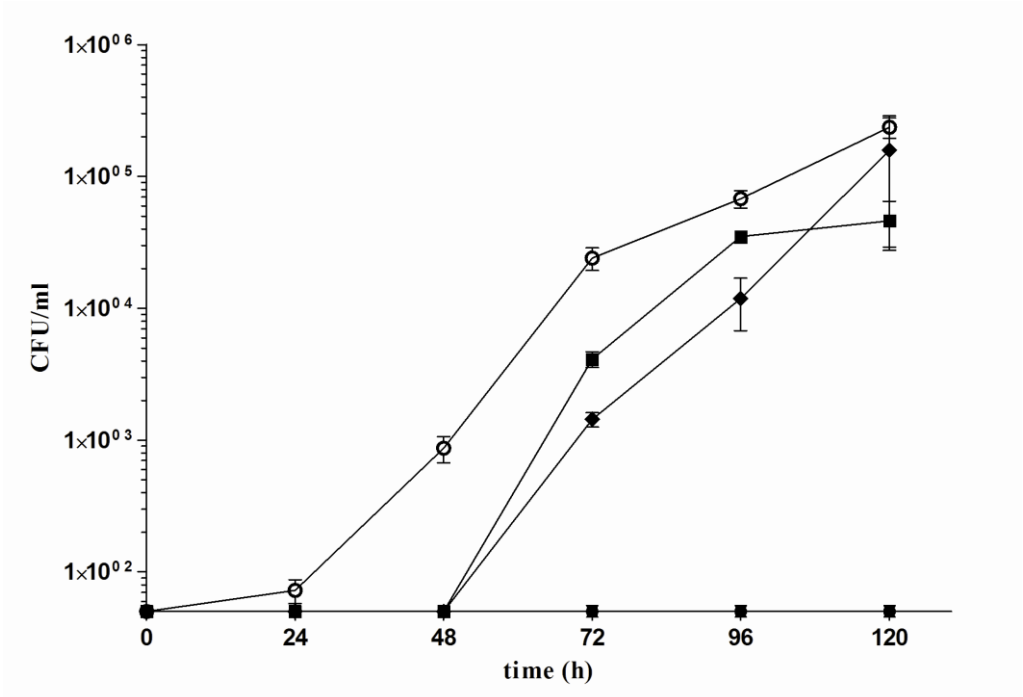

Figure 5.7. Development of heat-resistant CFU of *C. difficile* R20291 mutants over 5 days on BHIS agar supplemented with 0.1% taurocholate. *C. difficile* strains were cultivated as described in Chapter Two. ○, *C. difficile* R20291; ●, CRG1166 (*sleC)*; ■, CRG1634 (CRG1166 complemented with parental *sleC*); ▼, CRG1628 (CRG1166 harbouring pMTL84151); ♦, CRG1652 (*C. difficile* R20291 harbouring pMTL84151). 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. Data points for CRG1166 and CRG1628 are overlaid because neither formed any colonies.

5.2.4 Effect of sleC mutation on C. difficile sporulation

The inability of a *sleC* mutant of both 630Δ*erm* and R20291 to develop heat-resistant CFU could be a consequence of a number of possible defects. By definition, a heat-resistant CFU represents a successfully sporulated vegetative cell that was able to survive heat treatment, complete germination, and finally grow vegetatively. A reduction in the observed colony formation following

heat shock would suggest that the mutant strains are unable to complete one or more of these processes. To examine the sporulation efficiencies of the mutant strains, spores of all *C. difficile* 630Δ*erm* and R20291 strains were counted after five days by phase-contrast microscopy and the results were compared to the observed development of heat-resistant CFU (Figure 5.8 and Figure 5.9). Except for a sporulation negative control, in which the master regulator of sporulation *spo0A* was insertionally inactivated (Heap, *et al.*, 2007), the sporulation frequencies of all mutant strains were found to be equivalent to those of the corresponding parental strains. These data confirm that the *sleC* mutants of both *C. difficile* 630Δ*erm* and R20291 sporulate at parental levels but are unable to form colonies after heat treatment, even in the presence of the bile salt taurocholate.

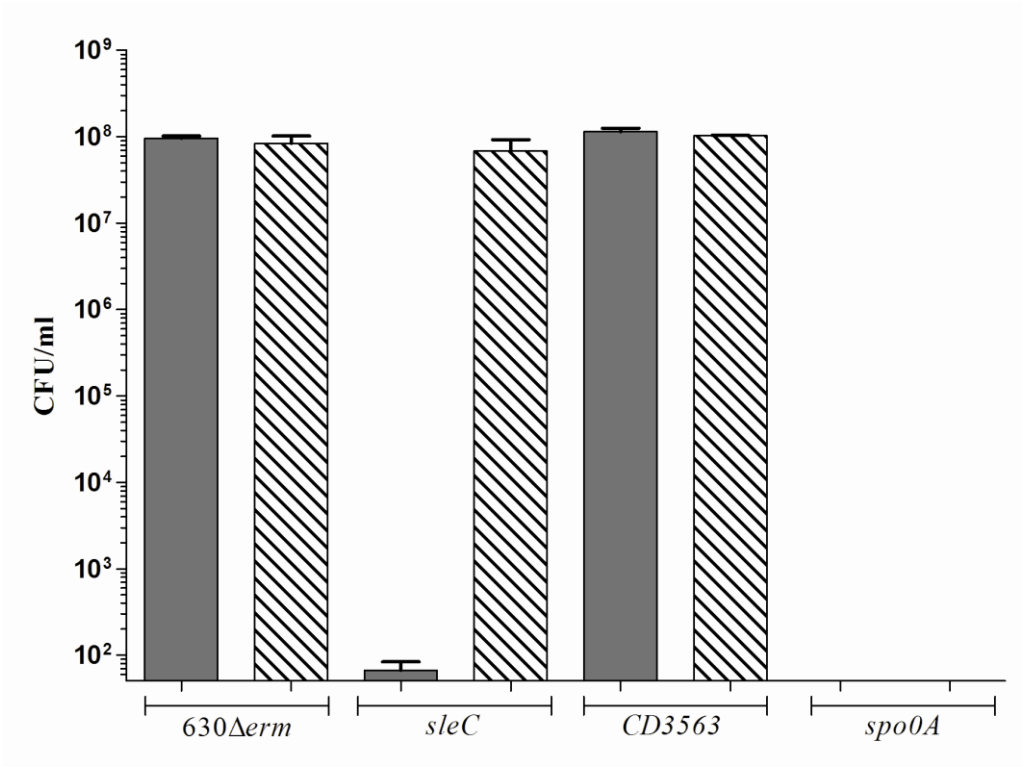

Figure 5.8. Numbers of heat-resistant CFU and spore titres after five days incubation in BHIS broth. Spore counts (bars with diagonal lines) and heat-resistant CFU (filled bars) were determined for *C. difficile* 630Δ*erm*, CRG1115 (*sleC*), CRG878 (CD3563), and CRG789 (*spo0A*). Spore titres and heat-resistant CFU were enumerated as described in Chapter Two. The bars represent the averages of three independent experiments, and the error bars indicate the standard errors of the means. The detection limit for colony counts was 50 CFU/ml, and the detection limit for spore counts was 5×10^3 spores.

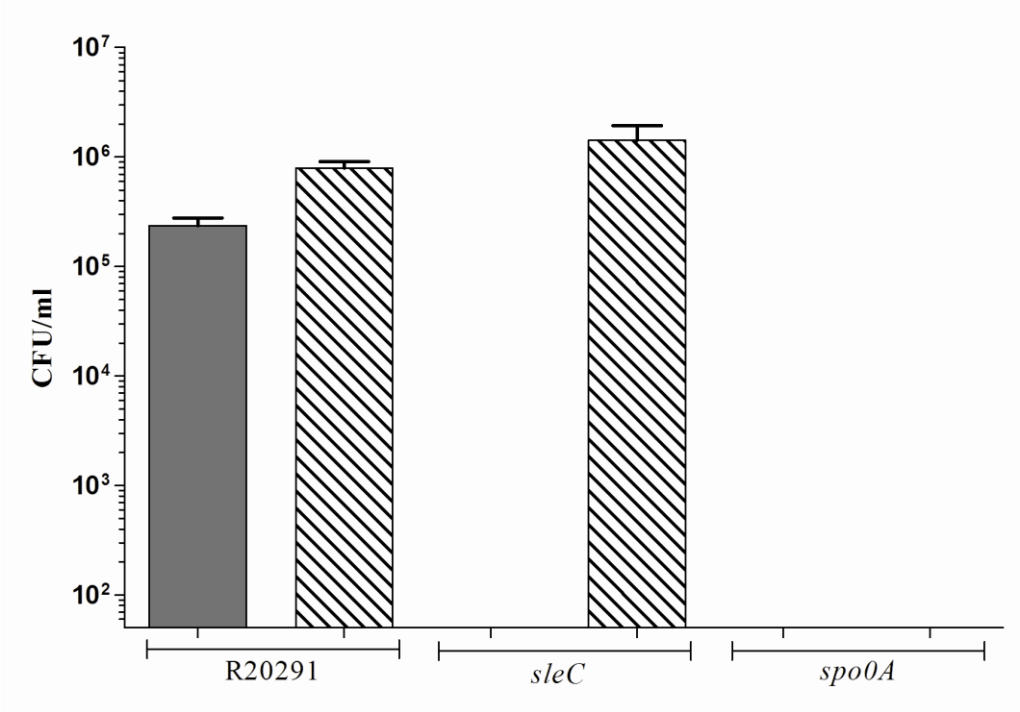

Figure 5.9. Numbers of heat-resistant CFU and spore titres after five days incubation in BHIS broth. Spore counts (bars with diagonal lines) and heat-resistant CFU (filled bars) were determined for *C. difficile* R20291, CRG1166 (*sleC*) and CRG1375 (*spo0A*). Spore titres and heat-resistant CFU were enumerated as described in Chapter Two. The bars represent the averages of three independent experiments, and the error bars indicate the standard errors of the means. The detection limit for colony counts was 50 CFU/ml, and the detection limit for spore counts was 5×10^3 spores.

5.2.5 Effect of sleC mutation on C. difficile spore viability

Having shown that the rate of sporulation of *sleC* mutant strains of *C. difficile* 630Δ*erm* and R20291 was not affected compared to the rate of sporulation of the respective parental strain, it was important to demonstrate that the previously noted reduction in heat-resistant CFU was not a result of altered spore heat resistance properties. Decoating of spores has previously been used

to distinguish between spore viability and germination phenotypes (Popham, *et al.*, 1995; Paredes-Sabja, *et al.*, 2009b) as decoating and plating with supplemental lysozyme allow a germination-deficient spore to form heat-resistant CFU. Purified spores of both *C. difficile* 630Δ*erm* and R20291 plated without taurocholate formed few heat-resistant CFU compared to spores plated with taurocholate (Figure 5.10 and Figure 5.11). Decoating spores of the parental strains resulted in much higher numbers of heat-resistant CFU even in the absence of taurocholate, when compared to plating dormant spores without taurocholate. Purified spores of *sleC* mutants of both *C. difficile* 630Δ*erm* and R20291 were unable to form heat-resistant CFU on BHIS agar, with or without taurocholate supplement, but decoated *sleC* spores formed colonies to parental levels when plated with lysozyme supplement (Figure 5.10 and Figure 5.11), confirming that they were viable.

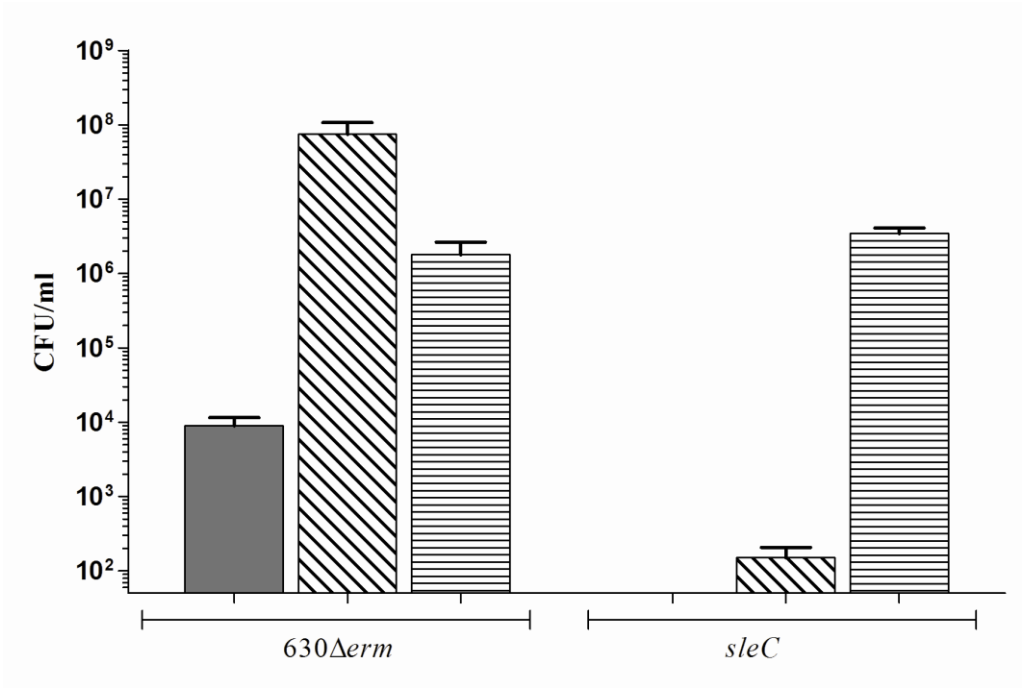

Figure 5.10. Viability of *C. difficile* 630Δ*erm* and CRG1115 (*sleC*) spores. Heat-resistant CFU were determined as described in Chapter Two. Dormant spore cultures were heated before being plated onto BHIS agar (filled bars) or BHIS agar supplemented with 0.1% taurocholate (bars with diagonal lines). Spore cultures were also decoated before they were plated onto BHIS agar supplemented with 1 μg/ml lysozyme (bars with horizontal lines). The bars 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.

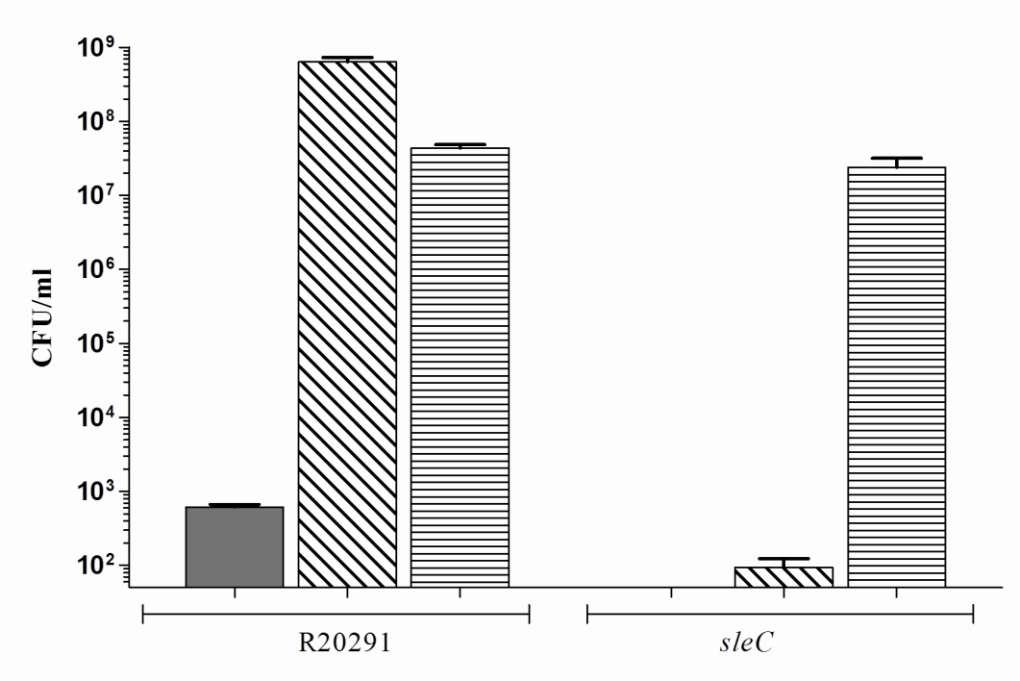

Figure 5.11. Viability of *C. difficile* R20291 and CRG1166 (*sleC*) spores. Heat-resistant CFU were determined as described in Chapter Two. Dormant spore cultures were heated before being plated onto BHIS agar (filled bars) or BHIS agar supplemented with 0.1% taurocholate (bars with diagonal lines). Spore cultures were also decoated before they were plated onto BHIS agar supplemented with 1 μg/ml lysozyme (bars with horizontal lines). The bars 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.

Finally, spores of *C. difficile* 630Δ*erm*, R20291 and their respective *sleC* mutant strains were observed by phase-contrast microscopy after heat treatment and incubation in BHIS broth supplemented with taurocholate. Spores of *sleC* mutants of both 630Δ*erm* and R20291 appeared to lose their 'phase-bright' characteristics to the same degree as parental spores after 2 h of incubation. This is consistent with the characterisation of *sleC* in *C.*

perfringens (Paredes-Sabja, *et al.*, 2009b) and suggests that *C. difficile sleC* mutant spores undergo the initial stages of germination in association with taurocholate, but do not complete the process and go on to grow as vegetative cells.

5.3 Discussion

This study has identified an apparent homologue of *sleC* from *C. perfringens* that is essential for complete germination and spore outgrowth (in the presence of the bile salt taurocholate) of *C. difficile* spores in both the non-epidemic strain 630Δ*erm* and the BI/NAP1/027 isolate R20291. Loss of SleC eliminated the development of heat-resistant CFU over five days despite normal spore formation by the parental strain, and the viability of *sleC* mutant spores was proven as their colony-forming ability after heat shock was restored by decoating the dormant *sleC* spores and supplementing the growth media with lysozyme.

It is interesting that SleC is essential for *C. difficile* spore germination and vegetative cell outgrowth. While *Bacillus* has traditionally been thought of as the model genus for spore germination studies and previous work has identified three SCLEs, the *cwlJ*, *sleB*, and *sleL* products (Ishikawa, *et al.*, 1998; Boland, *et al.*, 2000; Lambert and Popham, 2008), these enzymes are not individually essential for complete cortex lysis (Ishikawa, *et al.*, 1998; Paidhungat, *et al.*, 2001). On the other hand, recent work with *C. perfringens*

suggests that the mechanisms of germination are somewhat different in *Clostridium* (Paredes-Sabja, *et al.*, 2008; Paredes-Sabja, *et al.*, 2009b). The identification of SleC as an essential *C. perfringens* SCLE underlined the difference between this clostridial species and the *Bacillus* paradigm (Paredes-Sabja, *et al.*, 2009b), and the findings discussed in this chapter support the hypothesis that there is a generic difference between the germination of *Bacillus* spores and the germination of *Clostridium* spores.

It has been shown in *B. subtilis* that CwlJ is important for CaDPA-induced germination (Paidhungat, *et al.*, 2001), providing evidence that a CaDPA-mediated germination signalling pathway exists in *B. subtilis*. In contrast, this study has found that an apparent homologue of *B. subtilis cwlJ*, CD3563, plays no obvious role in taurocholate-induced *C. difficile* spore germination. Indeed, a recent study has suggested that SCLEs in *C. perfringens* are not triggered by CaDPA (Paredes-Sabja, *et al.*, 2009b), although further analysis of CD3563 is required to understand its importance, if any, to *C. difficile* spore germination. As CD3563 shares homology with both *B. subtilis cwlJ* and *sleB*, it is worthwhile to consider the possibility that a variety of functions are possible, although under our assay conditions CD3563 does not seem to play any role. This becomes more important with the knowledge that although CD0552 has been annotated as *sleB* in the *C. difficile* 630 genome (Sebaihia, *et al.*, 2006), further analysis has shown that the 238 residue protein shares no similarity with any known SCLE. The annotation of CD0552 as *sleB* may, therefore, be a mistake.

The absolute requirement for SleC for *C. difficile* to complete bile-induced spore germination could represent a therapeutic target in the healthcare environment. Spore germination in the patient could be prevented through inhibition of SleC, and this could reduce the ability of *C. difficile* to cause disease. Conversely, activating SleC could force spores to germinate on surfaces. As the resulting vegetative cells are exquisitely sensitive to traditional decontamination procedures, this may allow for a second or combined disinfection step.

5.4 Key outcomes

- SleC was found to be essential for the complete germination and outgrowth of *C. difficile* spores in both the non-epidemic 630Δ*erm* and the BI/NAP1/027 isolate R20291.

- CD3563 was found to play no obvious role in the germination and outgrowth of *C. difficile* spores, suggesting that there may be a generic difference between the germination of *Bacillus* and *Clostridium* spores.

Chapter Six

Analysis of further *C. difficile* targets which show homology to genes important for germination in other spore formers

6.1 Introduction

The study in the previous chapter described the absolute requirement of SleC for complete germination and outgrowth of *C. difficile* spores, while an apparent homologue of *B. subtilis* CwlJ and SleB plays no obvious role in this process. As SleC is also essential for germination in *C. perfringens*, this suggests that the germination mechanisms of *Clostridium* spores may be somewhat different to the germination mechanisms of *Bacillus* spores. It is, therefore, important to further investigate the germination mechanisms of *C. difficile*, in an effort to better understand how they relate to the germination mechanisms of organisms such as *C. perfringens* and *B. subtilis*.

6.1.1 Germination-specific proteases

In dormant spores of *C. perfringens*, SleC exists in an inactive form, which is cleaved into an active enzyme during germination (Urakami, *et al.*, 1999; Okamura, *et al.*, 2000; Shimamoto, *et al.*, 2001). During sporulation, a SleC precursor is synthesised consisting of four domains: an N-terminal pre-sequence (113 amino acid residues); an N-terminal pro-sequence (35 amino acid residues); a mature enzyme (264 amino acid residues); and a C-terminal pro-sequence of 25 residues (Miyata, *et al.*, 1995; Urakami, *et al.*, 1999; Okamura, *et al.*, 2000; Shimamoto, *et al.*, 2001). During the later stages of sporulation, both the N-terminal pre-sequence and the C-terminal pro-sequence are cleaved generating an inactive pro-enzyme with a mass of 35 kDa (termed "pro-SleC)", consisting of the N-terminal pro-sequence and mature enzyme

domains (Okamura, *et al.*, 2000). The inactive pro-SleC is located on the outside of the cortex layer in the dormant spore, presumably in the spore outer membrane or inner coat layer (Miyata, *et al.*, 1997). In the pro-SleC form the enzyme cannot degrade spore PG, but requires cleavage of the N-terminal pro-sequence to yield an active SleC (Miyata, *et al.*, 1995; Okamura, *et al.*, 2000).

Work in *C. perfringens* has identified three germination-specific proteases, CspA, CspB and CspC. These proteins are thought to be important for cleaving the N-terminal pro-sequence and generating active SleC, as a germination-specific protease consisting of CspA, CspB and CspC can accomplish this process (Shimamoto, *et al.*, 2001). Recently, mutagenesis studies have shown that CspB is essential for converting pro-SleC to an active enzyme (Paredes-Sabja, *et al.*, 2009c). Consequently, *C. perfringens cspB* mutant spores do not generate active SleC, are unable to initiate cortex hydrolysis, and cannot complete germination and outgrowth. Whilst this information would suggest that the sole function of CspB is to activate pro-SleC upon initiation of *C. perfringens* spore germination, in which case the phenotype of a *cspB* mutant would be identical to the phenotype of a *sleC* mutant, there is evidence to suggest that CspB has some other function. It was noted that *cspB* mutant spores were not able to release CaDPA during germination, while a *sleC* mutant does release CaDPA, albeit slowly (Paredes-Sabja, *et al.*, 2009c). This observation of a more severe germination defect in *cspB* mutant strains compared to *sleC* mutant strains is consistent with CspB having another germination-related function other than to activate pro-SleC.

Given that the role of SleC appears to be similar in *C. perfringens* and *C. difficile*, it is desirable to evaluate if germination-specific proteases are also involved in *C. difficile* spore germination. Genome analysis has shown that apparent homologues of these Csp proteases are present in both *C. difficile* 630Δ*erm* and *C. difficile* R20291. The product of CD2246 (annotated as *cspC*) shows 32% amino acid identity to both CspA and CspC of *C. perfringens*, and the product of CD2247 (annotated as *cspBA*) shows 36% and 35% identity to *C. perfringens* CspA and CspB, respectively.

6.1.2 Spore-specific peptidoglycan

As described in Chapter One, cortex PG has a unique, spore-specific structure. There are two main differences between the structure of spore PG and vegetative cell PG. First, spore cortex PG is less highly cross-linked compared to vegetative cell PG, which may play a role in properties such as heat resistance (Atrih and Foster, 1999). The second main difference is that approximately half of NAM residues in spore cortex PG have been converted to muramic-δ-lactam, a modification not found in vegetative cell PG (Atrih and Foster, 1999). It has been shown that the delta lactam of cortex PG likely allows for substrate recognition by SCLEs (Popham, *et al.*, 1996a), and in turn prevents SCLE-associated damage to vegetative cell PG following the completion of germination. In *B. subtilis*, the CwlD protein is required for muramic-δ-lactam formation during sporulation (Sekiguchi, *et al.*, 1995;

Popham, *et al.*, 1996a). Spores of a *cwlD* mutant are able to complete the earliest stages of germination, including the release of CaDPA and partial spore core rehydration, but cortex lysis and, therefore, spore outgrowth does not occur (Moir, 2003). An apparent homologue of *B. subtilis cwlD* has been identified in *C. difficile*, CD0106 (annotated as *cwlD*), the product of which shows 37% amino acid identity to *B. subtilis* CwlD.

6.1.3. Bile salts and C. difficile germination

It is not yet known how *C. difficile* spores sense a suitable environment for germination, although there is evidence to suggest that this involves the interaction of *C. difficile* spores with bile salts. As described in Chapter One, the primary bile acids cholate and chenodeoxycholate are synthesised in the liver and further metabolised via conjugation to glycine or taurine. Taurocholate is well known to stimulate the germination of *C. difficile* spores (Wilson, *et al.*, 1982; Wilson, 1983; Sorg and Sonenshein, 2008; Giel, *et al.*, 2010), while chenodeoxycholate acts as an inhibitor of *C. difficile* spore germination (Sorg and Sonenshein, 2009). Various members of intestinal microflora can modify these primary bile acids, converting them to secondary bile acids (Coleman and Hudson, 1995; Ridlon, *et al.*, 2006). The conversion of cholate derivatives (which can stimulate *C. difficile* germination) to the secondary bile acid deoxycholate (which can inhibit *C. difficile* vegetative cell growth) may be important for prevention of CDAD in healthy individuals (Sorg and Sonenshein, 2008). Analysis of the *C. difficile* 630 genome has indicated the presence of CD0065, a putative bile acid inducible NADP-

dependent 7-alpha-hydroxysteroid dehydrogenase, hypothesised to convert chenodeoxycholate into the secondary bile acid 7-keto-lithocholate (Sebaihia, *et al.*, 2006). As chenodeoxycholate has been shown to inhibit *C. difficile* germination, but lithocholate is not known to affect the germination of *C. difficile* spores or vegetative cell growth (Sorg and Sonenshein, 2008; Sorg and Sonenshein, 2009), such a system may allow vegetative cells of *C. difficile* to 'create' a suitable environment for the germination of surrounding spores.

6.1.4 Aim of this study

The previous chapter described the first use of reverse genetics to study the germination of *C. difficile* spores. This study aimed to inactivate and characterise CD0065, *cspBA*, *cspC* and *cwlD* in both *C. difficile* 630Δ*erm* and *C. difficile* R20291, in an effort to further understand how the germination mechanisms of *C. difficile* compare with the germination mechanisms of *Bacillus* and *C. perfringens* spores.

6.2 Results

6.2.1 Construction of mutant strains

To assess the role of CD0065, *cspBA*, *cspC* and *cwlD* in *C. difficile* spore germination, the ClosTron system (Heap, *et al.*, 2007; Heap, *et al.*, 2010) was used, as described in Chapter Two, to create insertional mutants of *C. difficile* 630Δ*erm*, in which either CD0065, *cspBA*, *cspC* or *cwlD* were inactivated,

yielding strains CRG1720, CRG1894, CRG1718 and CRG1719, respectively (Table 6.1 shows intron insertion sites and the frequencies of the desired mutants obtained). Correct ClosTron insertion of putative mutant strains was confirmed as described in Chapter Five. In the case of *cspBA*, it was not possible to isolate the desired mutant in 630Δ*erm* using the base 825s target site. Further screening of >100 erythromycin-resistant clones indicated that no desired mutants were present. The intron site was therefore judged to be inefficient (as occasionally occurs when using group II intron technology), so another target site (base 1844 with the antisense orientation) was chosen to inactivate *cspBA*.

Strain and target site	Frequency of desired mutant among clones screened	
	%	No. positive/no. screened
C. difficile 630Δ*erm* CD0065 440a	100	2/2
*C. difficil*e 630Δ*erm cspBA* 825s	0	0/100+
C. difficile 630Δ*erm cspBA* 1844a	12.5	1/8
C. difficile 630Δ*erm cspC* 737a	100	2/2
C. difficile 630Δ*erm cwlD* 198s	100	2/2
C. difficile R20291 *cwlD* 198s	100	5/5

Table 6.1. ClosTron insertion frequencies with erythromycin or lincomycin 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*) and lincomycin-resistant (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 Colony formation of C. difficile mutant strains after heat treatment

To assess the importance of CD0065, *cspBA*, *cspC* and *cwlD* in *C. difficile* spore germination, the *C. difficile* 630Δ*erm* mutant strains described above were tested to determine their ability to form heat-resistant CFU on BHIS agar supplemented with taurocholate (Figure 6.1). Inactivation of CD0065 had no effect on colony formation, but those strains with mutations in either *cspBA*,

cspC or *cwlD* were unable to form colonies at any point over the five-day period. These data suggest that *cspBA*, *cspC* and *cwlD* are essential for sporulation and/or taurocholate-induced germination in *C. difficile* 630Δ*erm*, while CD0065 plays no obvious role in either process.

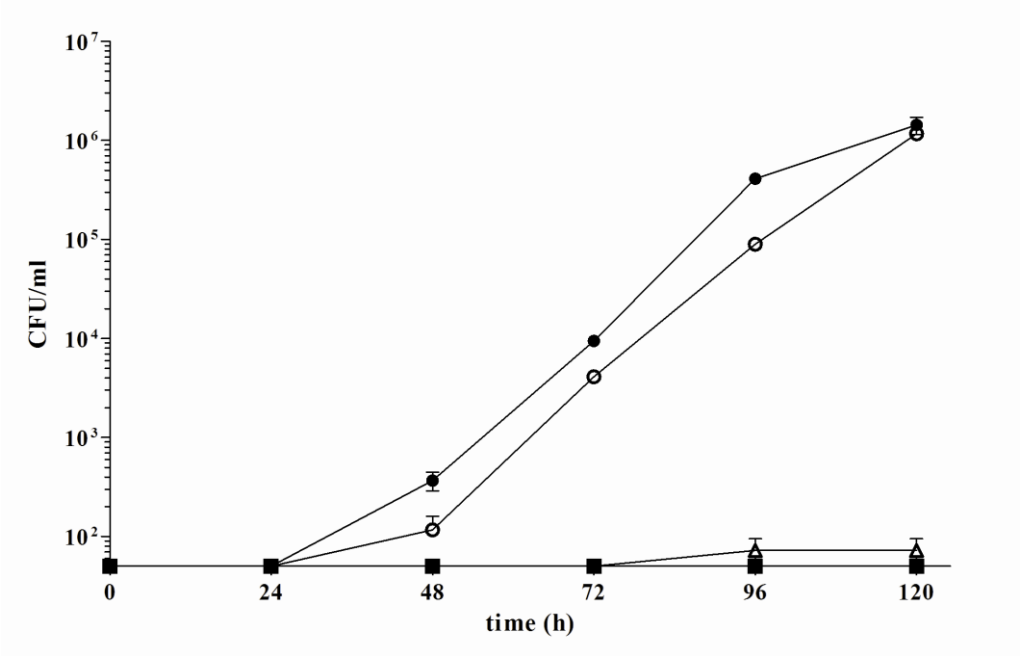

Figure 6.1. Development of heat-resistant CFU of *C. difficile* 630Δ*erm* mutant strains over a five-day period on BHIS agar supplemented with 0.1% taurocholate. ●, *C. difficile* 630Δ*erm*; ○, CRG1720 (CD0065); Δ, CRG1894 (*cspBA*); ■, CRG1718 (*cspC*); ▲, CRG1719 (*cwlD*); and □, CRG789 (*spo0A*) strains 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. Data points for CRG1718, CRG1719 and CRG789 are overlaid due to no colony formation.

As described in Chapter Three and Chapter Four, differences appear to exist in the sporulation and germination characteristics of different *C. difficile* types. Consequently, it was decided to study the effect of *cspBA*, *cspC* and *cwlD* inactivation in both *C. difficile* 630Δ*erm* and *C. difficile* R20291. An equivalent ClosTron-derived *cwlD* mutant of *C. difficile* R20291 (CRG1948) has been isolated and confirmed (Table 6.1 shows the intron insertion site and the frequency of mutants obtained), and the effect on colony formation was analysed as described for *C. difficile* 630Δ*erm* (Figure 6.2). This mutant exhibited the same phenotype as its 630Δ*erm* counterpart, which suggests that the role of *cwlD* is the same in both 630Δ*erm* and R20291.

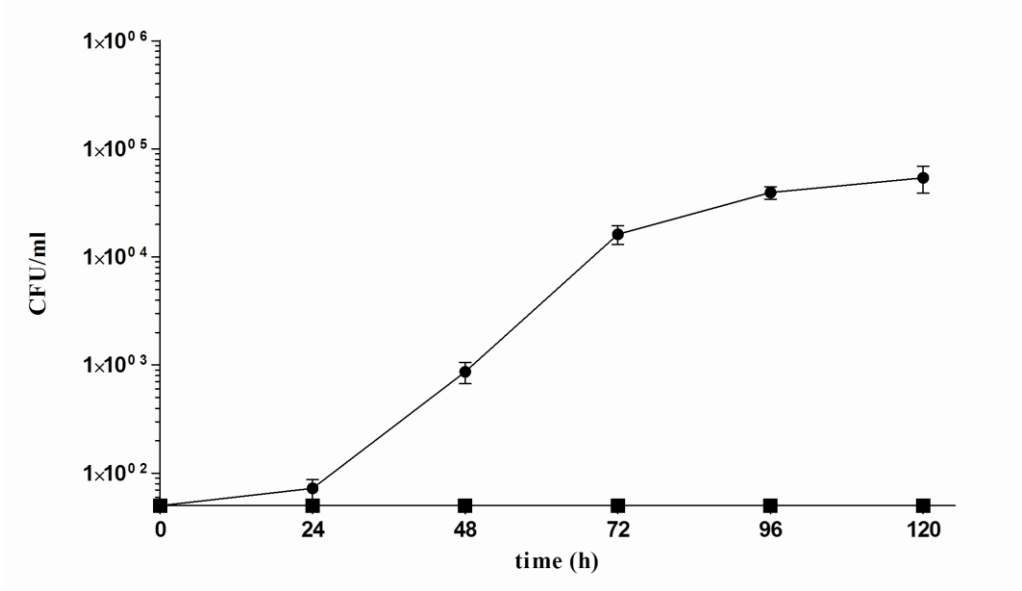

Figure 6.2. Development of heat-resistant CFU of *C. difficile* R20291 mutant strains over a five-day period on BHIS agar supplemented with 0.1% taurocholate. ●, *C. difficile* R20291; ■, CRG1948 (*cwlD*); and □, CRG1375 (*spo0A*) strains were cultivated as described in Chapter Two. The symbols indicate 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. Data points for CRG1948 and CRG1375 are overlaid because neither formed any colonies.

In order to determine if the observed phenotypes of *cspBA*, *cspC* and *cwlD* mutants were the result of growth deficiencies, the change in OD_{600} was used to monitor growth over 24 h. The growth of all mutants was indistinguishable from the growth of their parental strains (Figure 6.3 and Figure 6.4), which suggests that there were no obvious growth defects following inactivation of *cspBA*, *cspC* or *cwlD* in either *C. difficile* 630Δ*erm* or *C. difficile* R20291.

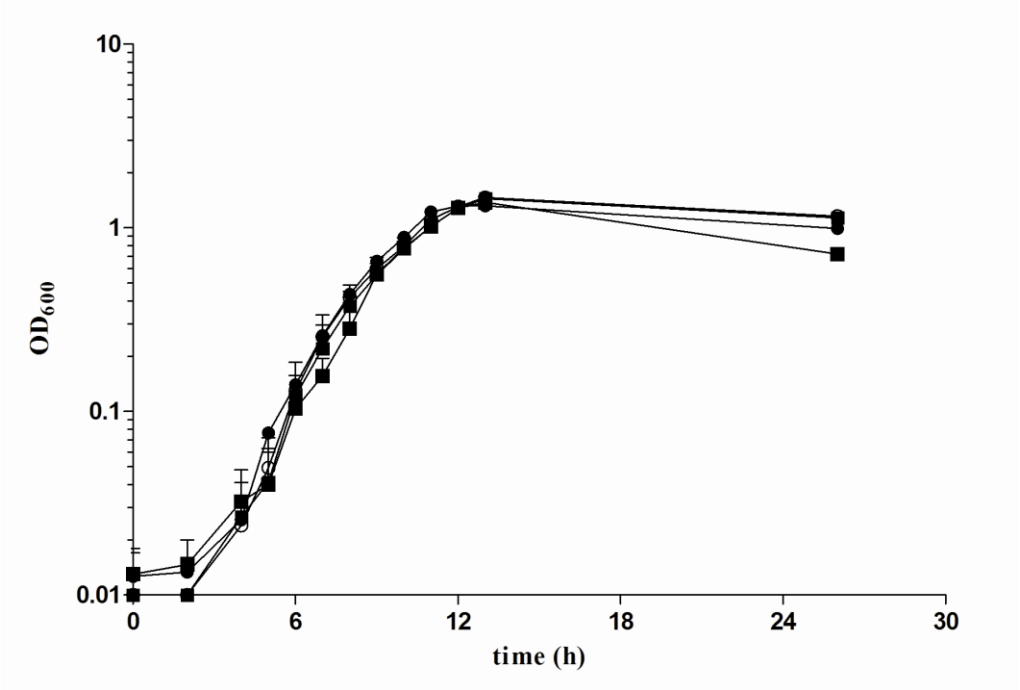

Figure 6.3. Growth of *C. difficile* 630Δ*erm* strains in BHIS broth over 24 h. ●, *C. difficile* 630Δ*erm*; ■, CRG1894 (*cspBA*); ○, CRG1718 (*cspC*); and ■, CRG1719 (*cwlD*) strains were cultivated as described in Chapter Two, and the OD_{600} measured. The symbols represent the averages of three independent experiments and error bars indicate the standard errors of the means.

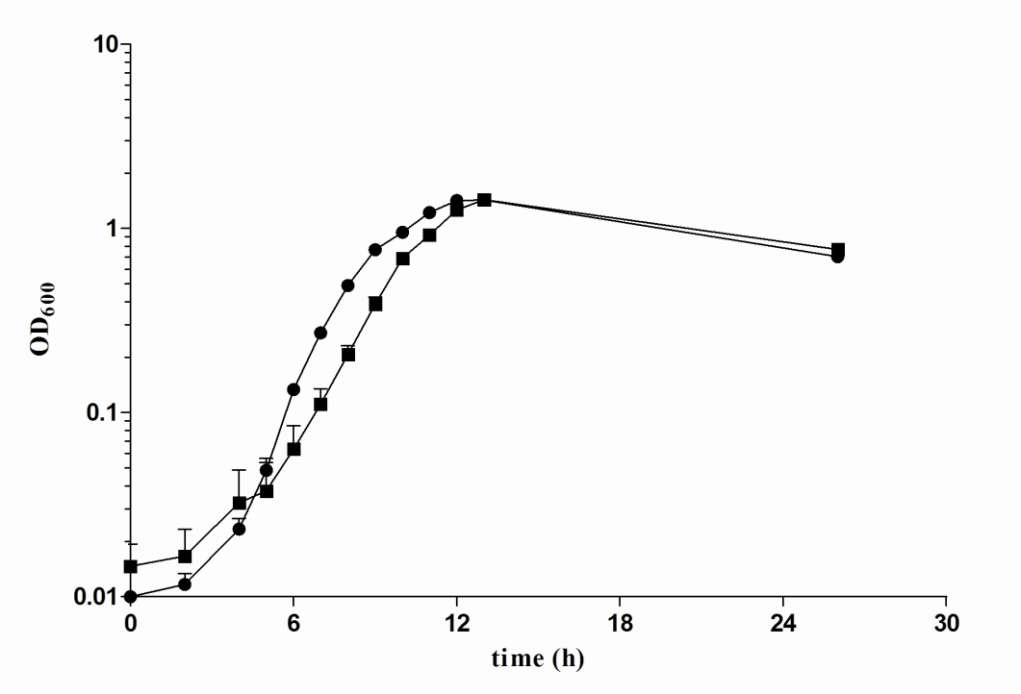

Figure 6.4. Growth of *C. difficile* R20291 strains in BHIS broth over 24 h. ●, *C. difficile* R20291; and ■, CRG1948 (*cwlD*) strains were cultivated as described in Chapter Two, and the OD_{600} measured. The symbols represent the averages of three independent experiments and error bars indicate the standard errors of the means.

6.2.3 Effect of cspBA, cspC and cwlD inactivation on C. difficile sporulation

As described in the previous chapter, an inability to develop heat-resistant CFU could be the result of a sporulation defect, altered spore heat-resistance properties and/or the inability to complete germination and outgrowth. To study efficiencies of the mutant strains, spores of all *C. difficile* strains were enumerated using phase-contrast microscopy after five days (Figure 6.5 and Figure 6.6). Aside from a sporulation-negative control, a *spo0A* mutant, the spore titres of all mutant strains were the same as their respective parental strain. This suggests that inactivating *cspBA*, *cspC* or *cwlD* does not affect *C.*

difficile spore formation, but that these mutant spores are unable to form colonies after heat treatment in association with the bile salt taurocholate.

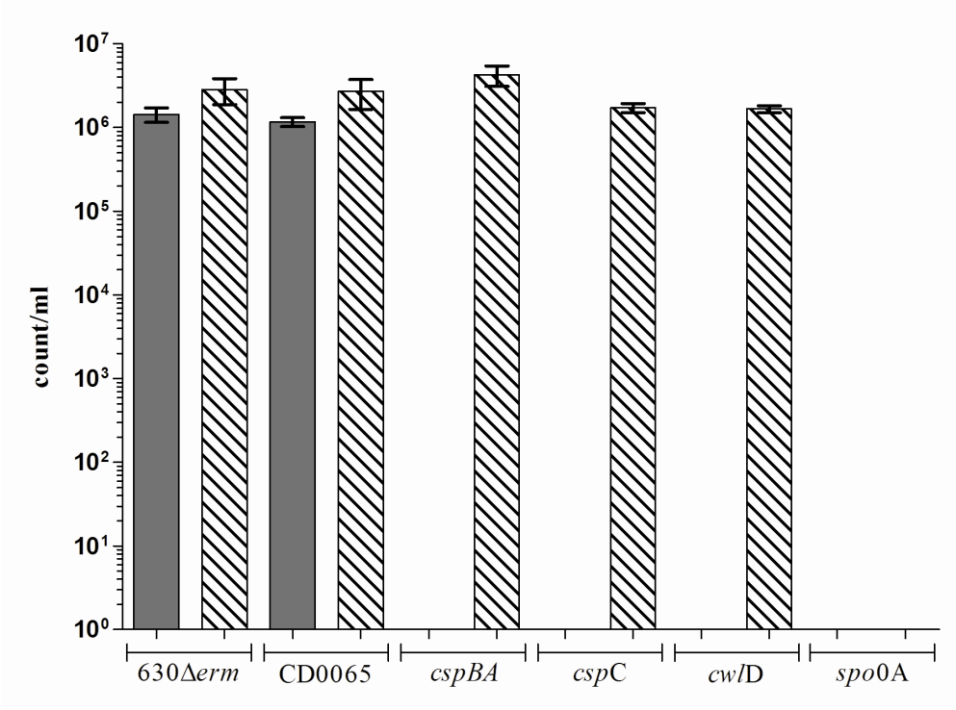

Figure 6.5. Numbers of heat-resistant CFU and spore titres after five days incubation in BHIS broth. Spore counts (bars with diagonal lines) and heat-resistant CFU (filled bars) were determined for *C. difficile* 630Δ*erm*, CRG1720 (CD0065), CRG1894 (*cspBA*), CRG1718 (*cspC*), CRG1719 (*cwlD*) and CRG789 (*spo0A*). Spore titres and heat-resistant CFU were enumerated as described in Chapter Two. The bars represent the averages of three independent experiments and error bars indicate the standard errors of the means. The detection limit for colony counts was 50 CFU/ml, and the detection limit for spore counts was 5×10^3 spores.

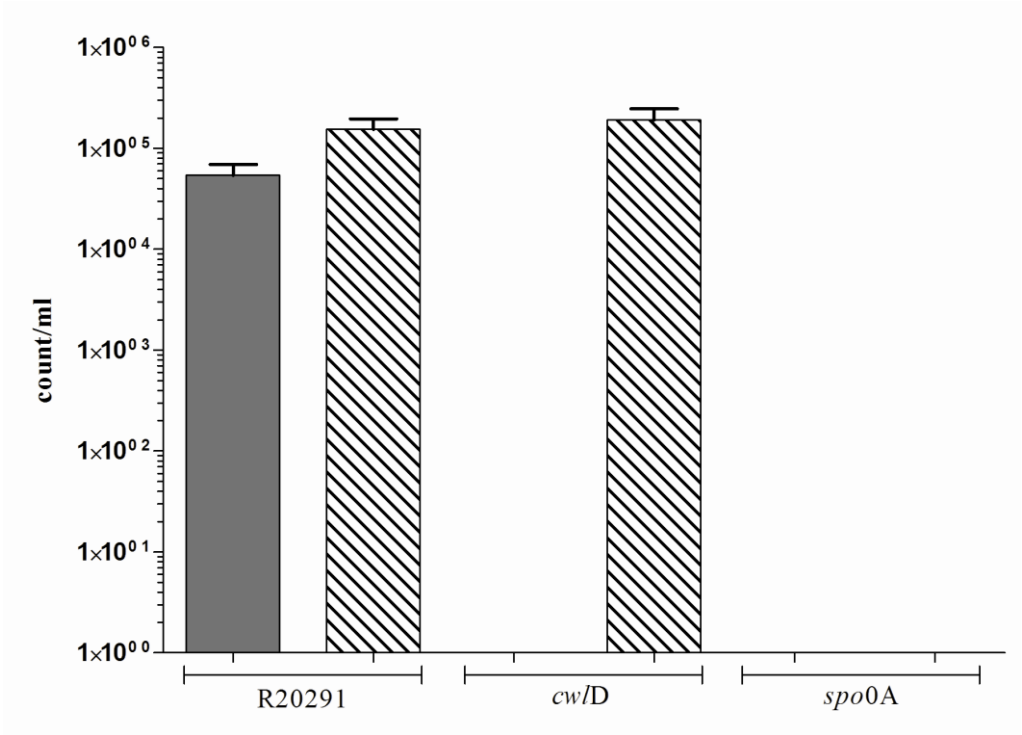

Figure 6.6. Numbers of heat-resistant CFU and spore titres after five days incubation in BHIS broth. Spore counts (bars with diagonal lines) and heat-resistant CFU (filled bars) were determined for *C. difficile* R20291, CRG1948 (*cwlD*) and CRG1375 (*spo0A*). Spore titres and heat-resistant CFU were enumerated as described in Chapter Two. The bars represent the averages of three independent experiments and error bars indicate the standard errors of the means. The detection limit for colony counts was 50 CFU/ml, and the detection limit for spore counts was 5×10^3 spores.

6.2.4 Effect of cspBA, cspC and cwlD inactivation on C. difficile spore viability

In order to show that the observed phenotype of a *cspBA*, *cspC* or *cwlD* mutant was not a consequence of altered spore heat resistance, spores of all *C. difficile* strains were decoated and plated with lysozyme supplement, as described in Chapter Two and Chapter Five. This treatment is important, as it allows a viable, germination-deficient spore to form colonies after heat treatment

(Popham, *et al.*, 1995; Paredes-Sabja, *et al.*, 2009b). Purified spores of *cspBA*, *cspC* and *cwlD* mutants of *C. difficile* 630Δ*erm* formed very few heat-resistant CFU on BHIS agar supplemented with taurocholate, but decoated *cspBA*, *cspC* and *cwlD* spores formed parental levels of colonies after heat treatment, confirming that these mutant spores were viable (Figure 6.7).

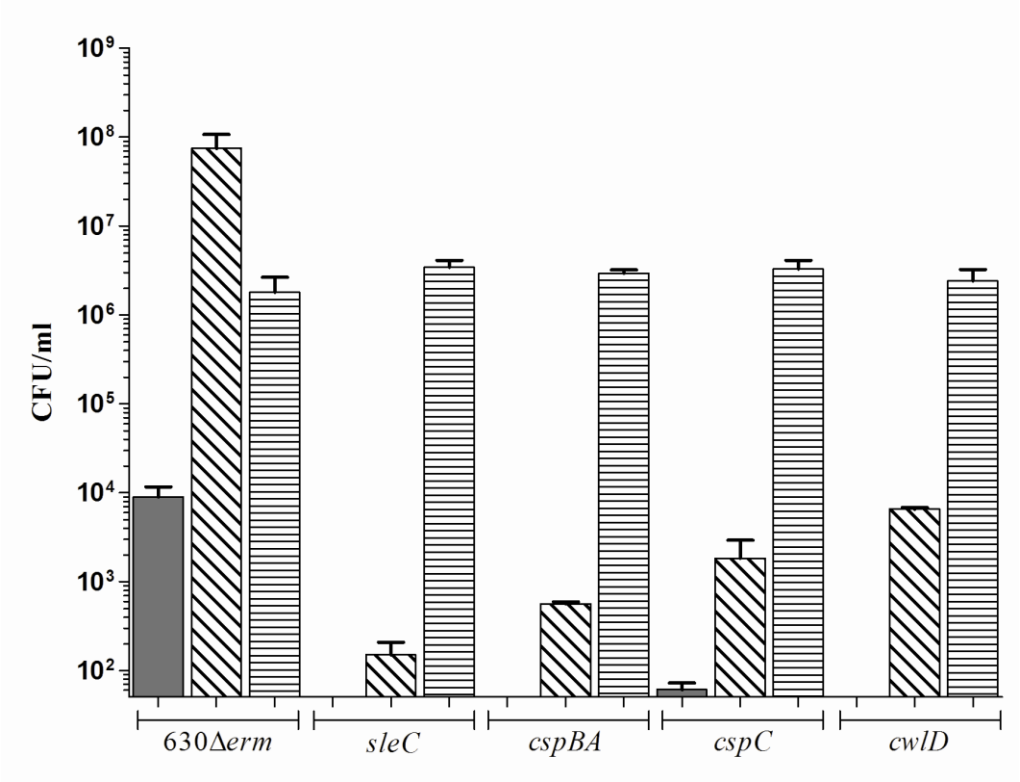

Figure 6.7. Viability of *C. difficile* 630Δ*erm*, CRG1115 (*sleC*), CRG1894 (*cspBA*), CRG1718 (*cspC*) and CRG1719 (*cwlD*) spores. Heat-resistant CFU were determined as described in Chapter Two. Dormant spore cultures were heated before being plated onto BHIS agar (filled bars) or BHIS agar supplemented with 0.1% taurocholate (bars with diagonal lines). Spore cultures were also decoated and plated with 1 μg/ml lysozyme supplement (bars with horizontal lines). The bars indicate the averages of three independent experiments and error bars indicate the standard errors of the means. The detection limit for colony counts was 50 CFU/ml.

Together, the data presented in this chapter show that *cspBA*, *cspC* and *cwlD* mutant strains are able to form parental levels of spores at a normal rate. The spores produced by these mutant strains are viable and heat resistant, but are unable to complete germination and return to vegetative cell growth. This suggests that CspBA, CspC and CwlD may all be essential for the germination and outgrowth of *C. difficile* spores.

6.3 Discussion

In this study, a number of apparent homologues of genes important for germination in other spore formers have been analysed for their role in *C. difficile* sporulation and germination. Loss of *cspBA*, *cspC* and *cwlD* did not affect the sporulation ability of *C. difficile* 630Δ*erm*, but the spores were unable to germinate and return to vegetative cell growth following heat treatment, in association with the bile salt taurocholate. Decoating mutant spores and plating with lysozyme supplement restored colony formation, confirming their viability. This suggests that *cspBA*, *cspC* and *cwlD* may all be essential for the germination and outgrowth of *C. difficile* 630Δ*erm* spores, providing an interesting follow-up to the previous chapter, which suggested that there may be a generic difference between the germination of *Bacillus* and *Clostridium* spores. While the germination-specific protease CspB has been shown to be essential for SleC processing in *C. perfringens* (Paredes-Sabja, *et al.*, 2009c), these proteases, or in fact proteases of any kind, are not known to play any role in the regulation of SCLE activity during the germination of *Bacillus* spores.

The hypothesised necessity of a *B. subtilis cwlD* homologue for complete germination and outgrowth of *C. difficile* 630Δ*erm* spores appears to provide evidence that the role of *cwlD* may be the same in both *B. subtilis* and *C. difficile*. CwlD in *B. subtilis* is required during sporulation for the formation of delta lactam of muramic acid, a component of spore-specific peptidoglycan. In *cwlD* mutant strains, germinant receptors are active and the earliest stages of germination are completed, however, cortex lysis does not occur. Such a role of CwlD in *C. difficile* could account for the heat-resistant CFU defect observed in this study.

It was observed that inactivation of CD0065 appeared to have no affect on the sporulation or germination characteristics of *C. difficile* 630Δ*erm*. This finding is not entirely unexpected, as a protein important for the conversion of chenodeoxycholate into lithocholate may not play any direct role as such in the sporulation or germination mechanisms of *C. difficile*. However, it is expected that future studies will analyse in more detail the interaction of *C. difficile* with bile salts such as chenodeoxycholate, as this is clearly an important aspect of *C. difficile* colonisation.

The characterisation of *cspBA*, *cspC* and *cwlD* described in this chapter has suggested a possible role of each gene in *C. difficile* germination and outgrowth, although it is obvious that these targets must be further analysed to pinpoint the role that they may be playing in *C. difficile* spore germination. In

particular, it is necessary to complement the heat-resistant CFU defect in these three mutant strains, in order to show that the observed phenotypes were a specific consequence of the inactivation of the respective target gene. However, initial attempts to complement the defect in a *cspC* mutant were not successful. As described in Chapter Two, plasmid pMTL-DB2 was constructed, carrying the parental *cspC* structural gene and a 302 bp non-coding region immediately upstream of *cspBA*, presumed to contain the promoter for *cspC* (Figure 2.3). This plasmid was introduced into the *cspC* mutant strain of *C. difficile* 630Δ*erm*, but no colony formation was observed over five days, suggesting that pMTL-DB2 was not suitable for complementing the heat-resistant CFU defect. There are a number of reasons why the heat-resistant CFU defect was not restored following expression of pMTL-DB2. Identification of a putative *cspC* promoter region was principally based on speculation that the promoter would be located immediately upstream of *cspBA* (Figure 6.8). However, approximately 150 bp upstream of *cspBA* are CD2248 and CD2249 and it is therefore possible that a suitable promoter lies further upstream of CD2249 (Figure 6.8). 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 *cspC* when attempting complementation studies in this mutant strain.

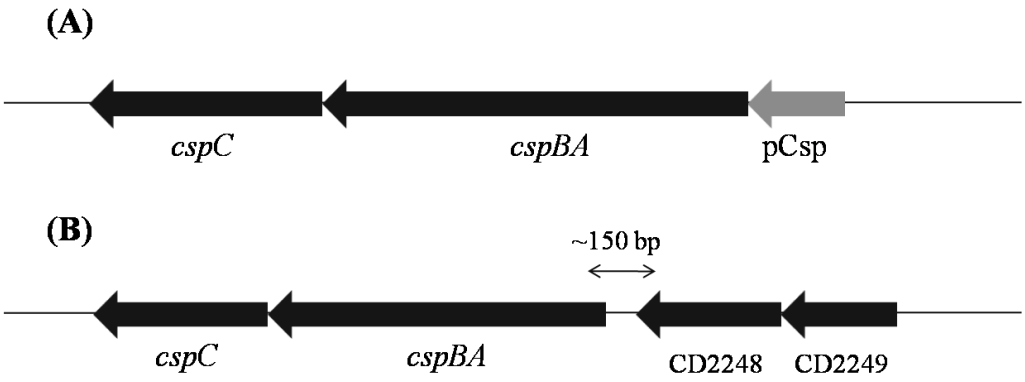

Figure 6.8. (A) Arrangement of *cspBA* and *cspC*, with an annotated 302 bp region upstream of *cspBA*, presumed to contain the promoter for both *cspBA* and *cspC*. (B) The arrangement of *cspBA*, *cspC*, CD2248 and CD2249 in the *C. difficile* 630Δ*erm* genome.

Other explanations for the failure of pMTL-DB2 expression to restore colony formation in a *cspC* mutant include the loss of pMTL-DB2 during cultivation in the sporulation medium, altered sporulation frequency of mutant strains carrying pMTL-DB2 (an effect that could be analysed by observing spores under phase-contrast microscopy), or indeed the possibility that the observed mutant phenotype is not a consequence of *cspC* inactivation, instead a result of a potential second ClosTron insertion. The latter hypothesis could be clarified by using Southern blot analysis to identify intron insertions in the *cspC* mutant strain. The observed mutant phenotype could also be the result of a spontaneous mutation, which cannot be confirmed by any method. In such a case, the obvious strategy would be to create further identical mutants and then attempt to complement their respective mutant phenotypes.

A *cwlD* mutant in *C. difficile* R20291 was also unable to form heat-resistant CFU over five days, despite forming a parental spore titre. However, in order to confirm spore viability, it will be necessary in the future to analyse heat-resistant CFU after decoating the mutant spores, as has been described for 630Δ*erm* mutants. Future studies will also aim to study *cspBA* and *cspC* mutants in *C. difficile* R20291, in order to determine if the mutant phenotype is similar in 630Δ*erm*. Interestingly, during the recent development of a transposon system for *in vivo* random mutagenesis of *C. difficile*, a *C. difficile* R20291 *cspBA* mutant was isolated (Cartman and Minton, 2010). This mutant strain was unable to form heat-resistant CFU after five days incubation in BHIS broth, which suggests that the phenotype of a *C. difficile* R20291 *cspBA* mutant is the same as the phenotype of a *cspBA* mutant in 630Δ*erm*. However, it will be necessary to analyse spore titres and, if necessary, observe colony formation after decoating mutant spores, to clarify the mutant phenotype. In addition, it is important that it is possible to complement this colony formation defect.

6.4 Key outcomes

- *C. difficile* strains with mutations in *cspBA*, *cspC* and *cwlD* formed parental spore titres, but were unable to form heat-resistant CFU on BHIS agar supplemented with the bile salt taurocholate. Furthermore, a *C. difficile* R20291 *cwlD* mutant was also unable to form colonies after heat-treatment at any point over five days, despite a parental spore titre.

- Spore viability of *C. difficile* 630Δ*erm cspBA*, *cspC* and *cwlD* mutants was confirmed by decoating, which suggests that CspBA, CspC and CwlD may all be required for complete germination and outgrowth of *C. difficile* spores.

Chapter Seven

General discussion

7.1 Key findings of this work

7.1.1 Genes required for germination of C. difficile spores

To-date, the precise mechanisms by which *C. difficile* spores germinate have not been studied in great depth. However, a number of genetic tools have now been developed for use with *C. difficile* and have provided a means for the reverse genetics studies presented in this thesis (Heap, *et al.*, 2007; Heap, *et al.*, 2009; Heap, *et al.*, 2010). Apparent homologues of genes important for germination in other spore formers have been identified in the *C. difficile* genome and this work has described the initial characterisation of six of these homologues, in both *C. difficile* 630Δ*erm* and the BI/NAP1/027 isolate R20291 (a type associated with outbreaks of increased disease severity).

A homologue of *C. perfringens sleC*, known to be essential for spore-cortex lysis during *C. perfringens* germination (Paredes-Sabja, *et al.*, 2009b), was found to be essential for complete germination of both *C. difficile* 630Δ*erm* and R20291 spores in nutrient-rich medium, when germination was induced with a supplement of the bile salt taurocholate. By contrast, CD3563, an apparent homologue of both *B. subtilis cwlJ* and *sleB*, which encode the two major SCLEs of *B. subtilis* spores, CwlJ and SleB, was found to play no obvious role in the germination of *C. difficile* spores. This work, described in Chapter Five, is the first report of using reverse genetics to study the germination of *C. difficile* spores and the first gene characterisation by mutagenesis in a BI/NAP1/027 isolate of *C. difficile*.

This study has also identified potential roles in the germination and outgrowth of *C. difficile* spores for CspBA and CspC, which show homology to the *C. perfringens* germination-specific proteases CspA, CspB and CspC, which are associated with the cleavage and activation of pro-SleC upon initiation of germination. While inactivation of *cspBA* and *cspC* in *C. difficile* 630Δ*erm* did not affect sporulation or spore viability, the spores produced by either mutant strain were unable to germinate and return to vegetative cell growth with taurocholate supplement. In addition, spores derived from mutants of *C. difficile* 630Δ*erm* and R20291 in which the gene equivalent to the *B. subtilis cwlD* gene was inactivated were unable to complete germination and outgrowth. CwlD is required during the sporulation of *B. subtilis* for formation of the characteristic muramic-δ-lactam of spore-cortex PG and is, therefore, an important pre-requisite for substrate recognition of SCLEs (Sekiguchi, *et al.*, 1995; Popham, *et al.*, 1996a). *B. subtilis* spores lacking CwlD are viable, but are unable to degrade the PG cortex and complete germination, presumably as SCLEs only degrade PG containing muramic-δ-lactam. The preliminary evidence presented in Chapter Six suggests that CspBA, CspC and CwlD may all be essential for germination and outgrowth of *C. difficile* spores. To date, however, it has not proven possible to complement the germination defect in these mutant strains. It is, therefore, not currently possible to definitively ascribe the observed phenotype to the ClosTron-mediated inactivation of the target gene.

7.1.2 Differences between the germination of Bacillus and Clostridium spores

Given some of the recent studies that have enhanced our understanding of *C. perfringens* germination mechanisms (Paredes-Sabja, *et al.*, 2009b; Paredes-Sabja, *et al.*, 2009c), perhaps the main outcome of the reverse genetics work presented here is the support for the hypothesis that there is a generic difference between the germination mechanisms of *Bacillus* spores and the germination mechanisms of *Clostridium* spores. The absolute requirement for SleC for complete germination of *C. difficile* spores is consistent with the role of SleC during *C. perfringens* germination (Paredes-Sabja, *et al.*, 2009b). In contrast, none of the three identified SCLEs of *B. subtilis*, CwlJ, SleB and SleL, are individually essential for complete cortex lysis (Ishikawa, *et al.*, 1998; Paidhungat, *et al.*, 2001). Furthermore, while CwlJ is required for CaDPA-induced germination in *B. subtilis* (Paidhungat, *et al.*, 2001), a *C. difficile* homologue, CD3563, appeared to play no obvious role in *C. difficile* germination, and it has been suggested that SCLEs of *C. perfringens* are not activated by CaDPA (Paredes-Sabja, *et al.*, 2009b).

The preliminary characterisation of *cspBA* and *cspC* in this work has also provided evidence that differences exist in the germination of *Bacillus* and *Clostridium* spores. CspBA and CspC show amino acid similarity to the germination specific proteases of *C. perfringens*. It was recently shown that CspB is essential for cortex hydrolysis during germination of *C. perfringens* spores, acting to cleave and activate pro-SleC upon initiation of germination (Paredes-Sabja, *et al.*, 2009c). The findings in Chapter Six, that CspBA and

CspC may be required for *C. difficile* germination, appear to support this hypothesis. On the other hand, there is no known involvement of Csp proteases, or indeed any proteases, in the regulation of SCLE activity in *Bacillus* spores.

The work described above has suggested that the activity and regulation of *C. perfringens* SCLEs is considerably different to the activity and regulation of *Bacillus* SCLEs. Taken together, the reverse genetics studies presented in Chapter Five and Chapter Six suggest that the germination mechanisms of *C. difficile* spores appear to be similar to *C. perfringens*.

7.2 Implications of findings for future experimental designs

7.2.1 Mutant studies

One of the most important aims of this work was to optimise an assay appropriate for analysing the sporulation and germination efficiencies of *C. difficile* mutant strains. The historical lack of genetic tools has contributed to the sparse literature on media in which *C. difficile* sporulates efficiently, and the methods by which sporulation and germination can be independently quantified. Chapter Three described the development of such an assay, which was subsequently used to analyse ClosTron mutants in following chapters. Use of this assay allows one to distinguish between (i) rate of sporulation; (ii) total sporulation; (iii) spore viability; and (iv) spore germination and outgrowth of *C. difficile* strains, all within a timeframe of approximately one week, with

the potential for high-throughput screening of a mutant library if required. Furthermore, as *C. difficile* appears to sporulate efficiently in BHIS broth over a five day period, the large *C. difficile* spore titre present in the culture after five days means that spores may then be purified from the same culture used to assay these sporulation and germination characteristics, without the need to prepare new stocks, for further analysis using other methods when they become available. Figure 7.1 shows a flowchart of the steps required to pinpoint a particular defect when analysing *C. difficile* mutant strains. Such an algorithm should be appropriate for initial screening of mutants in the future, to identify sporulation and/or germination defects.

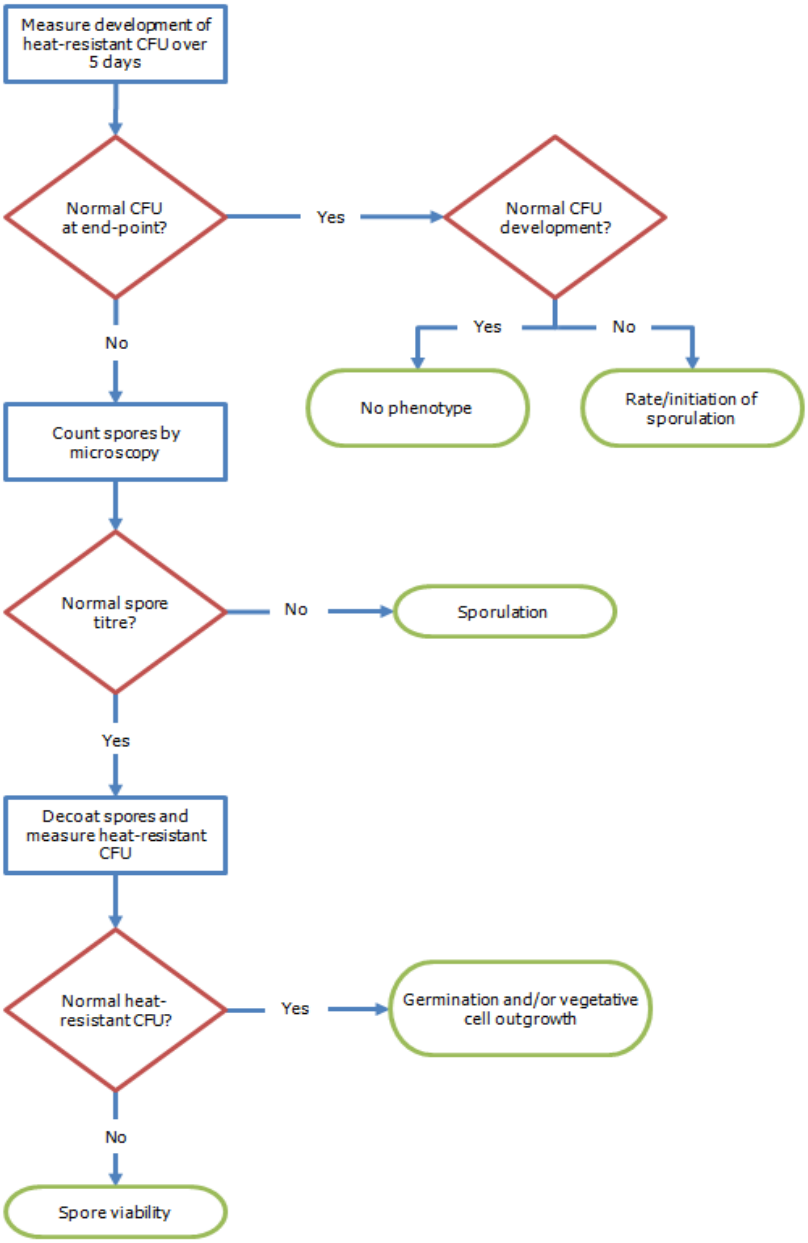

Figure 7.1. A diagrammatic representation of the algorithm used in this work to characterise the sporulation and germination characteristics of *C. difficile* mutants. This flowchart details the experimental procedures that, when applied together, can identify defects in (i) rate of sporulation; (ii) total sporulation at a defined end-point; (iii) viability of spores following heat treatment; and (iv) germination and outgrowth of spores.

7.2.2 Studying C. difficile sporulation and germination characteristics in vitro

The 'hypervirulence' associated with BI/NAP1/027 strains of *C. difficile* has been largely attributed to an increase in toxin production (Warny, *et al.*, 2005), although recent studies have argued that an increased rate of sporulation may play a role in the incidence and severity of disease (Wilcox and Fawley, 2000; Fawley, *et al.*, 2007; Akerlund, *et al.*, 2008). However, the hypothesis that BI/NAP1/027 strains of *C. difficile* sporulate more prolifically *in vitro* than other types has essentially been based on studies encompassing very few individual isolates. Furthermore, the experimental procedures employed to quantify sporulation rates in these studies can be called into question, as it is not clear how sporulation was measured independently of aspects such as growth rates and the death of non-sporulating cells.

It was shown in Chapter Four that sporulation rates and total sporulation after five days varied significantly among individual *C. difficile* strains, although this variation did not appear to be type-associated. Based on this evidence, and contrary to previously published work, the *in vitro* sporulation rate of *C. difficile* BI/NAP1/027 strains does not appear to be significantly higher than non-BI/NAP1/027 strains. It was also observed that the proportion of microscopically-enumerated spores which formed colonies with taurocholate supplement varied significantly among *C. difficile* strains. However, these differences were again not type-associated. This suggests that the frequency by which dormant spores are converted to vegetative cells *in vitro* varies among individual *C. difficile* isolates, when germination is stimulated on

nutrient-rich medium supplemented with the bile salt taurocholate. This finding is not entirely surprising, as it has been suggested that the germination capacity of different *C. difficile* isolates may vary (Baines, *et al.*, 2009), although this study only incorporated two individual strains.

There are two main outcomes of the study presented in Chapter Four; that (i) current evidence does not support the hypothesis that BI/NAP1/027 strains of *C. difficile* sporulate more prolifically than other types; and (ii) careful experimental design is required when analysing *C. difficile* sporulation rates, to avoid over-interpreting data. This work opens the question of sporulation and germination rates, and it is hoped that future studies will further investigate these characteristics in many more *C. difficile* strains. However, it may be premature to link any *in vitro* differences in sporulation or germination with differences in the disease incidence and severity caused by different *C. difficile* types, as it is likely that *in vitro* models are not truly representative of *in vivo* events.

7.3 The future of *C. difficile* germination research

7.3.1 C. difficile germinant receptors

Arguably, the most important aspect of *C. difficile* spore germination to understand is the mechanism(s) by which dormant spores sense a suitable environment for germination. As outlined previously, this is typically due to germinants binding to receptors located in the spore inner membrane, and such

receptors have been identified in a number of other spore formers. The most well known germinant receptor system is that of *B. subtilis*, which possesses three tricistronic operons, *gerA*, *gerB* and *gerK* (termed *gerA* operon homologues), and similar systems have been identified in spores of other *Bacillus* species (Clements and Moir, 1998; Paidhungat and Setlow, 2000; Hudson, *et al.*, 2001; Setlow, 2003; Pelczar, *et al.*, 2007; Mongkolthanaruk, *et al.*, 2009). Recent characterisation of *C. perfringens* has indicated the presence of monocistronic *gerA* and *gerKB* operons and a bicistronic *gerKA-gerKC* operon, which appear to play an important role in the initiation of germination, while a tricistronic *gerA* operon has also been identified in *C. botulinum* and *C. sporogenes* (Setlow, 2007). In addition, many other spore formers have been shown to possess various receptors from the Ger family. However, while recent studies of the kinetics of *C. difficile* germination have suggested that receptors of taurocholate and glycine are present within *C. difficile* spores (Ramirez, *et al.*, 2010), *C. difficile* encodes no known homologues of any of the systems present in other spore formers and as a result, the genetic basis through which *C. difficile* spores initiate germination remains unknown. As bile salts have not been shown to stimulate germination in any other spore formers, it is tempting to speculate that the interaction of *C. difficile* spores with bile salts may result in distinct mechanisms by which *C. difficile* spores initiate germination.

One avenue to explore regarding *C. difficile* germinant receptors may lie with a novel mechanism in *B. subtilis*, where germination can proceed independent of Ger receptors, in response to peptidoglycan in the surrounding environment

(Setlow, 2008; Shah, *et al.*, 2008). This involves binding of small muropeptides to a spore inner membrane-bound kinase, a process for which PrkC is required. *C. difficile* 630Δ*erm* encodes a homologue of *B. subtilis prkC*, CD2578 (Sebaihia, *et al.*, 2006), and it would be interesting to evaluate what role it plays in the germination of *C. difficile* spores. Given our knowledge of the role of taurocholate in *C. difficile* germination, it would be particularly interesting to study whether inactivation of CD2578 impairs the ability of *C. difficile* spores to germinate in response to taurocholate.

7.3.2 Forward genetics studies

Reverse genetic approaches allow for systematic identification and inactivation of target genes presumed to play a role in *C. difficile* spore germination, based on homology to genes important for germination in other spore formers. However, where no apparent homologues are present, a problem posed in the search for *C. difficile* germinant receptors, it is necessary to use another approach. Forward genetics aims to identify the genetic basis of a particular phenotype without assumptions about the genes involved. Based upon this principal, a novel *mariner*-based transposon system for *C. difficile* has recently been developed, which allows for the generation of libraries of random mutants (Cartman and Minton, 2010). Phenotypic screening of a random mutant library in *C. difficile* R20291 yielded a putative sporulation/germination defective clone with an insertion in the *cspBA* gene, showcasing the potential of the system in the detection of genes important for *C. difficile* germination. It is hoped that this system will now form a basis for identification of genes encoding putative *C. difficile* germinant receptors.

7.3.3 Genetic requirements for C. difficile spore germination in vivo

This work has identified SleC as essential for *in vitro* germination of *C. difficile* spores in association with the bile salt taurocholate. It is currently not known how the *in vitro* role of taurocholate in *C. difficile* germination relates to germination events *in vivo*, and this makes a *C. difficile sleC* mutant an appealing strain to study in an animal infection model. If *C. difficile sleC* mutant spores were unable to germinate (and therefore unable to cause disease) in a Golden Syrian hamster, SleC could become a therapeutic target, as inhibition of SleC could potentially prevent *C. difficile* spore germination in susceptible individuals.

7.4 Proposed future research

7.4.1 Genetics of C. difficile spore germination

Chapter Five described the identification of SleC as essential for germination of *C. difficile* spores with taurocholate. Obvious follow-up studies to this one would describe the precise role of SleC during germination. Is SleC required for cortex hydrolysis in the same way as SleC of *C. perfringens*? Is *C. difficile* SleC synthesised as an inactive pro-enzyme, which must be cleaved and activated upon initiation of germination events? Furthermore, is SleC regulated by other proteins within the spore, or can bile salts such as taurocholate directly activate SleC and drive germination?

The evidence presented in Chapter Six suggests that *cspBA*, *cspC* and *cwlD* may both be essential for germination and outgrowth of *C. difficile* spores. Further work, however, will be required to clarify the effect of inactivating these genes on *C. difficile* germination. In particular, it is necessary to complement the germination defect in these mutant strains. This is especially important with *cspBA*, as the positioning of *cspBA* and *cspC* in the genome could potentially lead to the *cspBA* mutant phenotype being the result of a polar effect on *cspC* when using the ClosTron (Figure 6.9). Finally, given the close-relatedness of CspBA and CspC to the germination-specific proteases CspA, CspB and CspC of *C. perfringens*, it would then be interesting to study whether these proteins interact with SleC in a similar way to what has been described in *C. perfringens* (Paredes-Sabja, *et al.*, 2009c).

Pinpointing the role of CwlD in germination and outgrowth of *C. difficile* spores will require a detailed knowledge of the PG structure of *C. difficile* spores. Analysis of spore PG structure by mass spectrometry and high-performance liquid chromatography could show whether or not CwlD functions to modify *C. difficile* spore PG and, therefore, allow for substrate recognition of SCLEs.

7.4.2 Establishment of useful assays to study C. difficile germination

The studies presented in this thesis have relied on measurements of colony formation. While such a procedure is appropriate for identifying a defect in

spore germination and outgrowth, it is worth considering that a number of questions will need to be answered in the future. Where a particular mutation prevents colony formation, one such question is whether or not the mutant spore can undergo the initial stages of germination. The most obvious procedure in this case is to analyse the loss of spore optical density following addition of germinants to a spore suspension. Such an assay has been briefly described in *C. difficile* (Sorg and Sonenshein, 2008; Sorg and Sonenshein, 2009), but may require further optimisation in order to improve its sensitivity and therefore identify small differences between strains. A further measure of the initiation of *C. difficile* germination is quantifying the release of DPA from spores during germination. This procedure has been widely used in studying *Bacillus* spores and in *C. perfringens*, but to-date there have been no published examples of such an assay in *C. difficile*.

7.5 Concluding Remarks

It is clear that our knowledge of *C. difficile* spore germination is rudimentary. However, it has now been shown that bile salts play a role in both *in vitro* and *in vivo* germination of *C. difficile* spores (Wilson, *et al.*, 1982; Wilson, 1983; Sorg and Sonenshein, 2008; Sorg and Sonenshein, 2009; Giel, *et al.*, 2010). In addition, our understanding of the genetic basis of *C. difficile* spore germination has been enhanced through the reverse genetics studies described in this thesis, and this work has also uncovered evidence that variation in germination characteristics exist among *C. difficile* clinical isolates. Reports to-date have only scratched the surface of *C. difficile* spore germination, but

with a range of genetic tools now at our disposal (Heap, *et al.*, 2007; Heap, *et al.*, 2009; Cartman and Minton, 2010; 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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SleC Is Essential for Germination of *Clostridium difficile* Spores in Nutrient-Rich Medium Supplemented with the Bile Salt Taurocholate[▽]

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Clostridium difficile **is the major cause of infectious diarrhea and a major burden to health care services. The ability of this organism to form endospores plays a pivotal role in infection and disease transmission. Spores are highly resistant to many forms of disinfection and thus are able to persist on hospital surfaces and disseminate infection. In order to cause disease, the spores must germinate and the organism must grow vegetatively. Spore germination in *Bacillus* is well understood, and genes important for this process have recently been identified in *Clostridium perfringens*; however, little is known about *C. difficile*. Apparent homologues of the spore cortex lytic enzyme genes *cwlJ* and *sleB* (*Bacillus subtilis*) and *sleC* (*C. perfringens*) are present in the *C. difficile* genome, and we describe inactivation of these homologues in *C. difficile* 630Δ*erm* and a B1/NAP1/027 clinical isolate. Spores of a *sleC* mutant were unable to form colonies when germination was induced with taurocholate, although decoated *sleC* spores formed the same number of heat-resistant colonies as the parental control, even in the absence of germinants. This suggests that *sleC* is absolutely required for conversion of spores to vegetative cells, in contrast to CD3563 (a *cwlJ*/*sleB* homologue), inactivation of which had no effect on germination and outgrowth of *C. difficile* spores under the same conditions. The B1/NAP1/027 strain R20291 was found to sporulate more slowly and produce fewer spores than 630Δ*erm*. Furthermore, fewer R20291 spores germinated, indicating that there are differences in both sporulation and germination between these epidemic and nonepidemic *C. difficile* isolates.**

The Gram-positive anaerobe *Clostridium difficile* causes diarrheal diseases ranging from asymptomatic carriage to a fulminant, relapsing, and potentially fatal colitis (8, 30). This organism is resistant to various broad-spectrum antibiotics and capitalizes on disruption of the normal intestinal flora to colonize and cause disease symptoms through the action of toxins A and B (16, 40). While these toxins are the principal virulence factors, the ability of the organism to produce endospores is necessary for disease transmission.

Clostridial 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 (35). Thus, the spores shed in fecal matter are very difficult to eradicate and can persist on contaminated surfaces in health care facilities for extended periods of time (35). This leads to infection or reinfection of cohabitating individuals through inadvertent ingestion of infected material (10, 32). Once in the anaerobic environment of the gut, spores presumably germinate to form toxin-producing vegetative cells and, in susceptible individuals, diarrheal disease.

Spore germination is defined as the events that result in the irreversible loss of spore characteristics. However, current mechanistic knowledge of the germination process is based principally on data derived from studying *Bacillus subtilis.* Little is known about spore germination in clostridia and, in particular, in *C. difficile.* Germination is initiated when the bacterial spore senses specific effectors, termed germinants. These effectors can include nutrients, cationic surfactants, peptidoglycan, and a 1:1 chelate of pyridine-2,6-dicarboxylic acid (dipicolinic acid) and Ca^{2+} (CaDPA) (23, 34, 36). Spores of *B. subtilis* can germinate in response to nutrients through the participation of three sensory receptors located in the spore inner membrane, GerA, GerB, and GerK (23). After activation, the events include the release of monovalent cations (H^+, K^+, and Na^+) and CaDPA (accounting for approximately 10% of the spore dry weight) (36). The third major step in germination involves hydrolysis of the spore peptidoglycan (PG) cortex. It is during this hydrolysis that the previously low water content of the spore is restored to the water content of a normal vegetative cell and the core is able to expand, which in turn allows enzyme activity, metabolism, and spore outgrowth (36).

CwlJ and SleB are two specific spore cortex-lytic enzymes (SCLEs) involved in *Bacillus* cortex hydrolysis, which break down PG containing muramic-δ-lactam (28). SleB has been shown to localize in both the inner and outer layers of *B. subtilis* spores through interaction of the enzyme peptidoglycan-binding motif and the δ-lactam structure of the cortex (7, 19) and in association with YpeB, which is required for *sleB* expression during sporulation (4, 7). SleB is a lytic transglycosylase muramidase, but so far its mode of activation is unknown (21). CwlJ is localized to the spore coat during sporulation (3) and is required for CaDPA-induced germination in *B. subtilis*. Activation can be due to either CaDPA released from the spore core at the onset of germination or exogenous CaDPA (22). Neither enzyme is individually essential for com-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source or reference
Strains		
E. coli TOP10		Invitrogen
E. coli CA434	Conjugation donor	31
CRG789	*C. difficile* 630Δ*erm spo0A*::intron *ermB*	12
C. difficile 630Δ*erm*		14
C. difficile R20291	Stoke Mandeville (2004-2005) isolate	Anaerobe Reference Laboratory, Cardiff, Wales
CRG878	*C. difficile* 630Δ*erm* CD3563::intron *ermB*	This study
CRG1115	*C. difficile* 630Δ*erm sleC*::intron *ermB*	This study
CRG1166	*C. difficile* R20291 *sleC*::intron *ermB*	This study
CRG1375	*C. difficile* R20291 *spo0A*::intron *ermB*	S. A. Kuehne and N. P. Minton, unpublished data
CRG1555	CRG1115 containing pMTL-DB1 (*sleC* complementation plasmid)	This study
CRG1556	CRG1115 containing pMTL84151	This study
CRG1628	CRG1166 containing pMTL84151	This study
CRG1634	CRG1166 containing pMTL-DB1 (*sleC* complementation plasmid)	This study
CRG1651	*C. difficile* 630Δ*erm* containing pMTL84151	This study
CRG1652	*C. difficile* R20291 containing pMTL84151	This study
Plasmids		
pMTL007C-E2	ClosTron plasmid containing *catP* and intron containing *ermB* RAM	11
pMTL84151	*Clostridium* modular plasmid containing *catP*	13
pMTL-DB1	pMTL84151 containing 1,272-bp SleC coding region and 244-bp upstream promoter region	This study

plete cortex hydrolysis during nutrient germination, although inactivation of both *cwlJ* and *sleB* in *B. subtilis* results in a spore unable to complete this process (15). The role of SleL has recently been studied in *Bacillus anthracis.* Mutants unable to produce this enzyme are still able to germinate, but the process is retarded (18).

The SCLEs of *Clostridium* are less well studied than those of *Bacillus*. The SCLEs SleC (20) and SleM (6) have been identified in *Clostridium perfringens*, and a recent study demonstrated that SleC is required during germination for complete cortex hydrolysis (26). Although SleM can degrade spore cortex peptidoglycan and inactivation of both *sleC* and *sleM* decreased the ability of spores to germinate more than inactivation of *sleC* alone did, SleM was not essential (26). It has also been shown that the germination-specific serine protease CspB is essential for cortex hydrolysis and converts the inactive pro-SleC found in dormant spores to an active enzyme (24). So far, there has been no detailed study of any gene responsible for spore germination in *C. difficile*, although genes showing homology to *cwlJ* and *sleB* of *B. subtilis* (CD3563) and *sleC* of *C. perfringens* (CD0551) have now been identified in the *C. difficile* 630 genome (33).

With germinant receptors in *C. difficile* yet to be identified, the mechanism by which the spores sense a suitable environment for germination is unclear. Recent studies have suggested that this process may involve the interaction of *C. difficile* with bile. Taurocholate has been shown to enhance recovery of *C. difficile* spores in nutrient-rich medium (42), and it has been proposed that glycine and taurocholate act as cogerminants (38), while chenodeoxycholate inhibits *C. difficile* spore germination (39).

The emergence of *C. difficile* B1/NAP1/027 strains has increased the burden on health care services worldwide. Such strains have been shown to produce higher levels of toxin in the laboratory than many other types of strains (41), although the mechanism behind this production is not fully understood. However, while the observed higher levels of toxin production is doubtless important, perhaps the recent attention given to B1/NAP1/027 strains has focused too much on toxins. As spores represent the infectious stage of *C. difficile*, processes such as spore germination may also contribute to the greater virulence of these strains. In this study we evaluated the sporulation and germination efficiencies of an "epidemic" B1/NAP1/027 *C. difficile* strain (R20291, isolated from the Stoke Mandeville outbreak in 2004 and 2005) and the "nonepidemic" strain 630Δ*erm* (14). We then constructed strains with mutations in CD3563 (a *cwlJ*/*sleB* homologue) and a *sleC* homologue to analyze the role of these genes in the germination of *C. difficile* spores.

MATERIALS AND METHODS

Strains and growth conditions. Unless indicated otherwise, all *C. difficile* strains were grown at 37°C in an anaerobic workstation (Don Whitley, United Kingdom) in BHIS (brain heart infusion supplemented with L-cysteine [0.1%; Sigma, United Kingdom] and yeast extract [5 mg/ml; Oxoid]) broth or agar, which has been shown to aid *C. difficile* sporulation (38). All *Escherichia coli* strains were grown using Luria-Bertani broth or agar at 37°C. Plasmid DNA was transferred into *C. difficile* 630Δ*erm* and R20291 by conjugation from an *E. coli* donor, as previously described (13, 31). Table 1 shows the strains used in this study.

Sporulation of *C. difficile* was achieved by incubating cultures anaerobically in BHIS broth for 5 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% inoculum of a *C. difficile* culture and incubated until the optical density at 600 nm (OD_{600}) was between 0.2 and 0.5. The sporulation medium was then inoculated with 0.01 volume of this exponential starter culture. To measure the growth rates of *C. difficile* strains, 1-ml samples were removed from the sporulation medium at different time points and the OD_{600} was measured (Biomate 3; Thermo Scientific).

Measurement of *C. difficile* heat-resistant colony formation. Sporulating cultures of *C. difficile* were prepared as described above. At different times, samples (500 μl) were removed from the anaerobic chamber and heated at 60°C for 25 min to kill the vegetative cells but not the spores. To control for any effects of

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′–3′)
Intron retargeting	
Cdi-CD3563-226s-IBS	AAAAAAGCTTATAATTATCCTTAATGAGCGACAGGGTGCGCCCAGATAGGGTG
Cdi-CD3563-226s-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCGACAGGTTTAACTTACCTTTCTTTGT
Cdi-CD3563-226s-EBS2	TGAACGCAAGTTTCTAATTTCGGTTCTCATCCGATAGAGGAAAGTGTCT
Cdi-*sleC*-493s-IBS	AAAAAAGCTTATAATTATCCTTAGTAGTCCCTGAAGTGCGCCCAGATAGGGTG
Cdi-*sleC*-493s-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCCTGAATTTAACTTACCTTTCTTTGT
Cdi-*sleC*-493s-EBS2	TGAACGCAAGTTTCTAATTTCGATTACTACTCGATAGAGGAAAGTGTCT
Cdi-*sleC*-128a-IBS	AAAAAAGCTTATAATTATCCTTACATTACTTCTTAGTGCGCCCAGATAGGGTG
Cdi-*sleC*-128a-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCTTCTTAGGTAACTTACCTTTCTTTGT
Cdi-*sleC*-128a-EBS2	TGAACGCAAGTTTCTAATTTCGGTTTAATGTCGATAGAGGAAAGTGTCT
EBS Universal	CGAAATTAGAAACTTGCGTTCAGTAAAC
ClosTron sequencing	
Spofdx_F1	GATGTAGATAGGATAATAGAATCCATAGAAAATATAGG
pMTL007_R1	AGGGTATCCCCAGTTAGTGTTAAGTCTTGG
Mutant screening	
CD3563_F1	CTTTTAGAACTGTTAATCCACCTAATCCCG
CD3563_R1	CTTTACATTTTTGTGTTTAACAACAACTTATTTATCGC
*sleC*_F1	GGGAACTAAATTCATTTAAAGAAAGGGTG
*sleC*_R1 (630Δ*erm*)	GGCTGTTATGAACTAATATATACCATAAGTATTAC
*sleC*_R1 (R20291)	GTATTTTTACTTATAAGTATTGTAGTCTTAACAGCC
Complementation	
pSleC_F1	TAAAGAATGCGGCCGCAGATTATTTTCCTTTCAAAATTTTTGATTTATTTATGATTTATATCATCTAC
pSleC_R1	TAAAGAATCATATGATCACCCTTTCTTTAAATGAATTTAGTTCCC
SleC_F2	TAAAGAATCATATGCAAGATGGTTTCTTAACAGTAAGCATAATTGATGC
SleC_R2	TAAAGAATCTCGAGATCTCCATGGTTAAATTAAAGGATTTAAAGAAGCTATTCTAGTTGTAGC

oxygen exposure during heat treatment, a nonheated sample was also removed from the anaerobic chamber for 25 min. Samples were then returned to the anaerobic chamber, serially diluted in phosphate-buffered saline (PBS), and plated onto BHIS agar supplemented with 0.1% taurocholate (Sigma, United Kingdom). *C. difficile spo0A* mutants of both 630Δ*erm* and R20291 were used as sporulation-negative controls (12). Plates were incubated for 24 h before CFU were enumerated. Samples were analyzed in the same way every 24 h for 5 days. The total sporulation after 5 days was measured by counting spores by phase-contrast microscopy using a Bright-Line hemocytometer (Sigma, United Kingdom).

Assaying the viability of *C. difficile* spores by decoating. Sporulation cultures were set up as described above. To prepare pure *C. difficile* spores following 5 days of incubation, cultures were repeatedly washed with ice-cold distilled H_2O (dH_2O) until they contained no cell debris or vegetative cells as observed by phase-contrast microscopy. Spores were stored at −20°C in dH_2O.

Purified spores were decoated by resuspending a spore pellet in 1 ml of 50 mM Tris-HCl (pH 8.0)-8 M urea-1% (wt/vol) sodium dodecyl-sulfate-50 mM dithiothreitol and incubating the preparation at 37°C for 90 min as described by Popham and coworkers (29). Following incubation, decoated spores were washed three times in PBS. Samples were then heat treated as described above and plated onto BHIS medium supplemented with lysozyme (1 μg/ml).

Strain construction. Target genes were insertionally inactivated with the ClosTron system as previously described (12), using modular ClosTron plasmid pMTL007C-E2 (11). Table 2 lists the oligonucleotides used for construction of insertional mutants. Erythromycin (2.5 μg/ml) was used to select *C. difficile* 630Δ*erm* integrants, while lincomycin (20 μg/ml) was used for selection of *C. difficile* R20291 integrants (Table 3). Mutants were confirmed by PCR and sequencing.

For complementation studies, a 1,516-bp fragment encompassing the *sleC* structural gene and 5′ noncoding region was cloned into plasmid pMTL84151 (13) to generate plasmid pMTL-DB1. The 244-bp 5′ noncoding region likely encompassing the *sleC* promoter and the 1,272-bp region containing the *sleC* structural gene were independently amplified by PCR using oligonucleotide primer pairs pSleC_F1/pSleC_R1 and SleC_F2/SleC_R2, respectively. The primers were designed to allow subsequent cleavage of the two fragments generated with NotI/NdeI and NdeI/XhoI, respectively, where the ATG of the NdeI site was synonymous with the translational start codon of *sleC*. The two cleaved fragments were subsequently ligated with plasmid pMTL84151 cut with NotI and XhoI, which yielded plasmid pMTL-DB1, in which the two fragments were inserted contiguously. The strategy of separating the two regions into two independent fragments was adopted to allow subsequent use of a heterologous promoter if the presence of the 5′ noncoding region did not result in expression of *sleC*. As the protein encoded by *sleC* is the same in *C. difficile* 630Δ*erm* and R20291, only one plasmid (pMTL-DB1) was constructed for use with both mutants.

Both the pMTL-DB1 complementation plasmid and a pMTL84151 empty vector control were transferred into *C. difficile* by conjugation, using additional lincomycin (20 μg/ml) selection in *sleC* mutant strains.

TABLE 3. ClosTron insertion frequencies with erythromycin or lincomycin selection

Strain	Target site[a]	Frequency of desired mutant among clones screened[b]	
		%	No. positive/no. screened
C. difficile 630Δ*erm* CD3563	226s	100	8/8
C. difficile 630Δ*erm sleC*	493s	0	0/20[c]
C. difficile 630Δ*erm sleC*	128a	100	5/5
C. difficile R20291 *sleC*	128a	100	4/4

[a] 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.

[b] 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 insertion site was verified by sequencing.

[c] Further screening of a pool of genomic DNA from >100 erythromycin-resistant clones indicated that no desired mutants were present using the base 493 target site. The intron or target site was therefore judged to be inefficient (as occasionally occurs using group II intron technology), so another target (base 128 with the antisense orientation) was chosen for *sleC*.

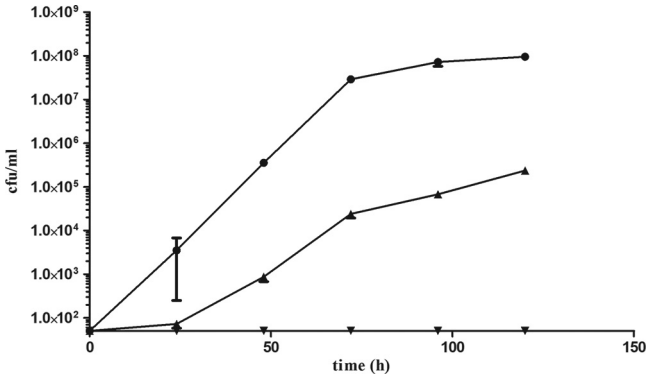

FIG. 1. Development of *C. difficile* heat-resistant CFU over a 5-day period. Cultures of *C. difficile* 630Δ*erm* (●), *C. difficile* R20291 (▲), and CRG789 (*spo0A*) (▼) were incubated anaerobically at 37°C in BHIS broth. At 24-h intervals, samples were heat treated at 60°C for 25 min before they were plated onto BHIS agar supplemented with 0.1% taurocholate and incubated at 37°C for 24 h. The numbers of heat-resistant CFU per milliliter were determined. The symbols indicate the averages of three independent experiments, and the error bars indicate the standard errors of the means. The detection limit for the assay was 50 CFU/ml.

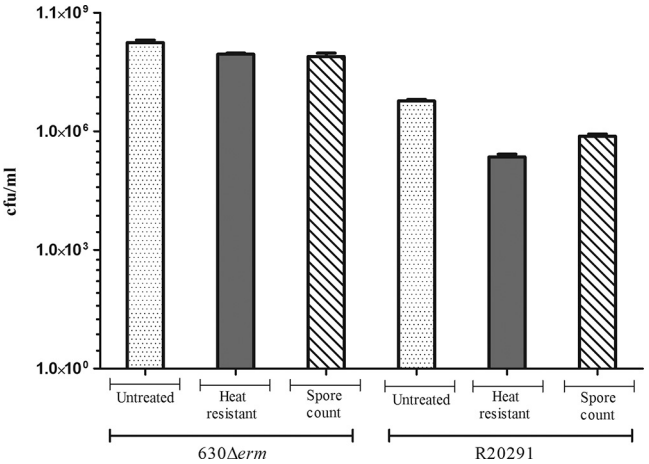

FIG. 2. Numbers of untreated CFU, spores, and heat-resistant CFU of *C. difficile* 630Δ*erm* and *C. difficile* R20291 after 5 days of incubation. Following 5 days of anaerobic incubation at 37°C in BHIS broth, the numbers of heat-resistant CFU were determined. Spore titers were determined by counting spores by phase-contrast microscopy using a Bright-Line hemocytometer (Sigma, United Kingdom). The bars indicate the averages of three independent experiments, and the error bars indicate standard errors of the means. The detection limit for colony counts was 50 CFU/ml, and the detection limit for spore counts was 1.6×10^3 spores.

RESULTS

Comparison of sporulation and germination of *C. difficile* 630Δ*erm* and *C. difficile* R20291. It has been suggested that B1/NAP1/027 "epidemic" strains of *C. difficile* may have different sporulation characteristics than nonepidemic strains (1, 9), although the evidence for this is currently limited. As sporulation is important in the study of spore germination, we decided to study *C. difficile* germination both in a B1/NAP1/027 strain, R20291, and in a nonepidemic strain, 630Δ*erm*. First, we compared the differences in sporulation and germination between these two strains using a series of assays. In this analysis, the development of heat-resistant CFU was observed over a 5-day period (Fig. 1). At different times, samples were heated at 60°C for 25 min before they were plated onto BHIS agar supplemented with 0.1% taurocholate. The resultant colony formation clearly showed that *C. difficile* 630Δ*erm* developed heat-resistant spores more rapidly than R20291. 630Δ*erm* formed countable heat-resistant CFU after 24 h of incubation, while it was only after 48 h that this was the case for R20291. In addition, 630Δ*erm* cultures contained approximately 400-fold more heat-resistant CFU than R20291 cultures after 5 days.

It is possible that the observed difference in heat resistance was not due to sporulation or germination and instead was a result of growth differences between the two strains. The data for non-heat-treated CFU recovered on BHIS agar supplemented with taurocholate after 5 days were also compared, and as the colony formation was found to be 30-fold lower for R20291 than for 630Δ*erm* (Fig. 2), the changes in OD_{600} for both 630Δ*erm* and R20291 were analyzed over a 5-day period. No difference was seen between the two strains (data not shown), which ruled out an elementary growth difference between 630Δ*erm* and R20291 and suggested that the observed difference in the number of non-heat-treated CFU for R20291 was due to the death of nonsporulating cells.

To pinpoint any difference in sporulation between the two strains, the cultures used to measure development of heat resistance were analyzed by phase-contrast microscopy, and the numbers of spores per milliliter were determined. *C. difficile* 630Δ*erm* was found to produce approximately 100-fold more spores per milliliter than R20291 (Fig. 2), suggesting that the previously observed difference in heat resistance between the two strains was due in part to sporulation. However, as 630Δ*erm* produced 400-fold more heat-resistant CFU than R20291, we also hypothesized that fewer R20291 spores than 630Δ*erm* spores germinated under the conditions used for our assay. To test the hypothesized differences in germination between 630Δ*erm* and R20291, the observed spore titers were compared to the numbers of heat-resistant CFU on BHIS agar supplemented with taurocholate. It was found that a greater proportion of 630Δ*erm* spores (97%) than of R20291 spores (30%) formed heat-resistant colonies (Fig. 2). Thus, our data suggest not only that the nonepidemic strain *C. difficile* 630Δ*erm* sporulates earlier and to a greater degree than the B1/NAP1/027 isolate R20291 under the growth conditions employed but also that a higher proportion of 630Δ*erm* spores than of R20291 spores form colonies in association with the bile salt taurocholate.

Heat-resistant colony formation by *C. difficile* mutant cultures supplemented with taurocholate. Previous studies have identified CwlJ and SleB as proteins that are important for cortex hydrolysis in *B. subtilis* (15), while SleC is essential for the same process in *C. perfringens* (26). Homologues are present in *C. difficile* 630 (33); the product of CD0551 (annotated as *sleC*) shows 53% amino acid identity to the previously characterized SleC of *C. perfringens* (17, 26), and the product of CD3563 shows 30% and 45% amino acid identity to *B. subtilis* CwlJ and SleB, respectively. Examination of an incomplete genome sequence available at http://www.sanger.ac.uk

indicated that equivalent homologues are also present in *C. difficile* R20291. To assess the importance of these genes in *C. difficile* germination, the ClosTron system (12) was used to create independent insertional mutants of *C. difficile* 630Δ*erm* in which either the CD3563 or *sleC* gene was inactivated, yielding strains CRG878 and CRG1115, respectively (Table 3 shows intron insertion sites and the frequencies of the desired mutants obtained). These strains were then tested to determine their abilities to form heat-resistant colonies over a 5-day period on BHIS agar supplemented with 0.1% taurocholate (Fig. 3A). Although inactivation of CD3563 had no effect on colony formation, a *sleC* mutant was unable to form heat-resistant CFU at any point over the 5-day period. The data suggest that *sleC* is absolutely required for sporulation and/or taurocholate-induced germination in *C. difficile* 630Δ*erm*, while CD3563 plays no obvious role in either process.

In light of our findings of sporulation and germination differences between *C. difficile* 630Δ*erm* and R20291, we analyzed the effect of *sleC* inactivation in both strains. In this analysis, an equivalent ClosTron-derived *sleC* mutant of *C. difficile* R20291 (CRG1166) was constructed (Table 3 shows the intron insertion site and frequency of mutants obtained), and the effect on colony formation was determined using the conditions employed for strain 630Δ*erm*. This mutant exhibited the same phenotype as its 630Δ*erm* counterpart (Fig. 3B), suggesting that the role of *sleC* in *C. difficile* is the same in both strains.

To ensure that the observed phenotypes of the *sleC* mutants of 630Δ*erm* and R20291 did not result from growth deficiencies, the change in OD_{600} was used to monitor growth over 5 days. The growth of both mutants was indistinguishable from the growth of their parental strains (data not shown), suggesting that there were no obvious growth defects.

Complementation of *sleC* mutant with parental SleC. In order to show that the observed phenotypes of CRG1115 and CRG1166 were a specific consequence of *sleC* inactivation, we performed complementation studies through construction of plasmid pMTL-DB1 carrying the parental *sleC* structural gene and the 244-bp region immediately upstream of the open reading frame presumed to contain its promoter. Strains CRG1555 and CRG1634 were created by introducing this plasmid into *sleC* mutants of *C. difficile* 630Δ*erm* and *C. difficile* R20291, respectively. Empty vector control strains CRG1556 (630Δ*erm*) and CRG1628 (R20291) were also created through introduction of pMTL84151 into the respective *sleC* mutant strains. Successful plasmid transfer was confirmed by recovery of the plasmid, followed by PCR and/or restriction analysis (data not shown). To control for the thiamphenicol selection needed to maintain the plasmids in culture, strains CRG1651 and CRG1652 were created by introducing pMTL84151 into parental strains 630Δ*erm* and R20291, respectively. The development of heat-resistant CFU was then measured, and the data were compared to data obtained previously. The levels of heat-resistant colony formation associated with the taurocholate supplement were fully restored in the *sleC* derivatives of both *C. difficile* 630Δ*erm* (Fig. 3A) and R20291 (Fig. 3B) carrying pMTL-DB1 to the levels in the parental strains containing the control vector pMTL84151. In the case of 630Δ*erm*, there was a difference between the level obtained for the strain with the plasmid and the level obtained for the plasmid-free parental strain, presumably as a result of growth under thiamphenicol selection conditions. The *sleC* mutant controls containing the empty vector performed like the original mutant. Thus, it was possible to complement the heat-resistant CFU defect, indicating that the observed phenotype was due solely to inactivation of *sleC*.

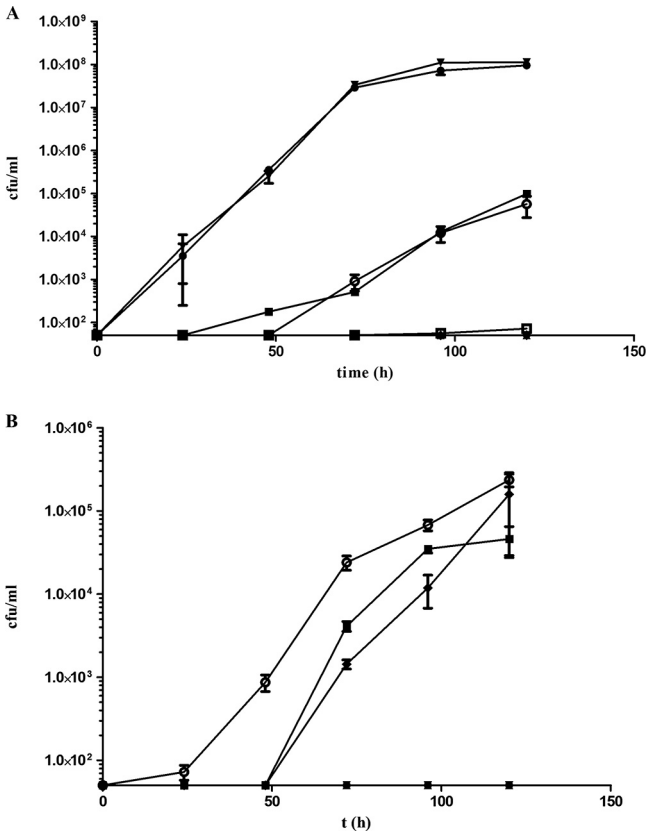

FIG. 3. Development of heat-resistant CFU of *C. difficile* mutants over 5 days. Cultures of *C. difficile* were incubated anaerobically at 37°C in BHIS broth. Every 24 h, samples were heat treated at 60°C for 25 min before they were plated onto BHIS agar supplemented with 0.1% taurocholate and incubated at 37°C for 24 h. The numbers of heat-resistant CFU per milliliter were determined. 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. (A) ●, *C. difficile* 630Δ*erm*; ▲, CRG1115 (*sleC*); ○, CRG1555 (CRG1115 complemented with parental *sleC* gene); □, CRG1556 (CRG1115 harboring pMTL84151 empty vector control); ■, CRG1651 (*C. difficile* 630Δ*erm* harboring pMTL84151); ▼, CRG878 (CD3563); ◆, CRG789 (*spo0A*). (B) (○), *C. difficile* R20291; ●, CRG1166 (*sleC*); ■, CRG1634 (CRG1166 complemented with parental *sleC*); ▼, CRG1628 (CRG1166 harboring pMTL84151); ◆, CRG1652 (*C. difficile* R20291 harboring pMTL84151); ▲, CRG1375 (*spo0A*). All data points for CRG1115 and CRG789 in panel A and for CRG1166, CRG1628, and CRG1375 in panel B are overlaid because there was no heat-resistant colony formation.

Effect of *sleC* mutation on *C. difficile* sporulation. An inability to develop heat-resistant colonies could be a consequence of a number of possible defects. By definition, a heat-resistant colony represents a successfully sporulated vegetative cell that was able to survive heat treatment, germinate, and grow vegetatively. A reduction in the number of observed CFU following heat shock would suggest that a strain is unable to complete one or more of these processes. To examine the sporulation

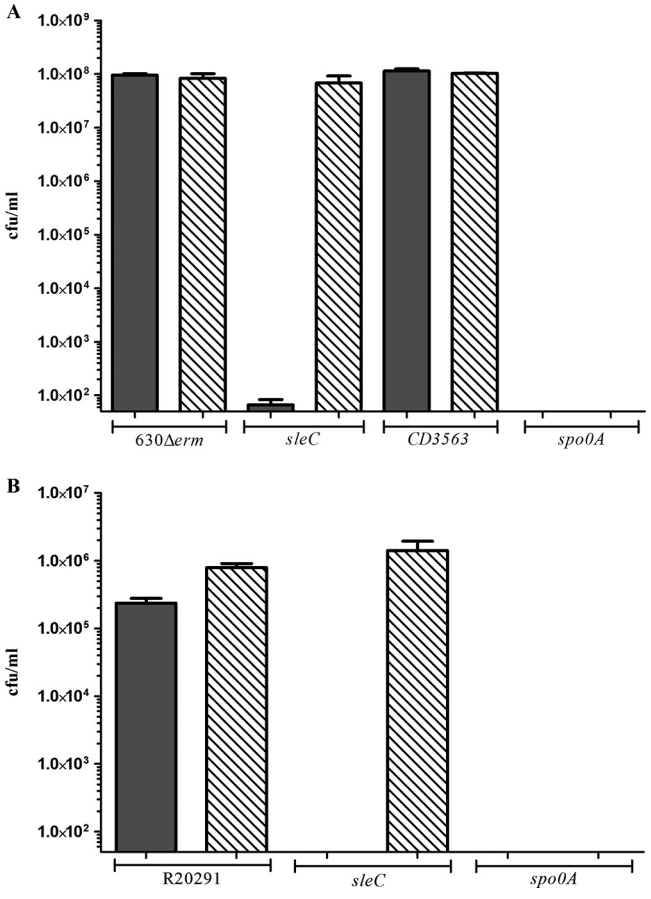

FIG. 4. Numbers of heat-resistant CFU and spore titers after 5 days. Spore counts (bars with diagonal lines) and numbers of heat-resistant CFU (filled bars) were determined for (A) *C. difficile* 630Δ*erm*, CRG1115 (*sleC*), CRG878 (CD3563), and CRG789 (*spo0A*) and (B) *C. difficile* R20291, CRG1166 (*sleC*), and CRG1375 (*spo0A*) after 5 days of anaerobic incubation at 37°C in BHIS broth. Following this incubation, numbers of heat-resistant CFU were determined as described in the legend to Fig. 2. Spore titers were obtained by counting spores by phase-contrast microscopy using a Bright-Line hemocytometer (Sigma, United Kingdom). The bars indicate the averages of three independent experiments, and the error bars indicate standard errors of the means. The detection limit for colony counts was 50 CFU/ml, and the detection limit for spore counts was 1.6×10^3 spores.

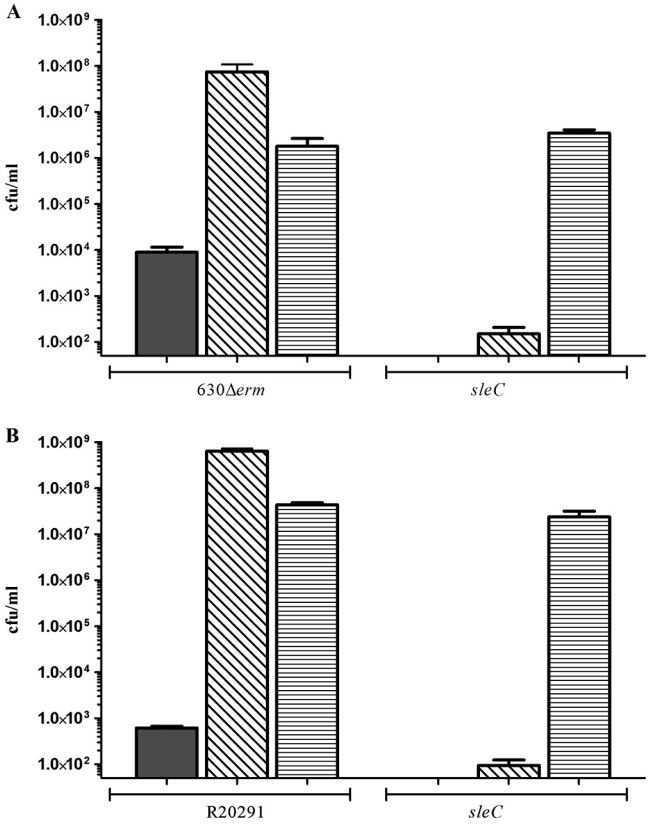

FIG. 5. Viability of *C. difficile sleC* mutant spores. Numbers of heat-resistant CFU were determined for (A) *C. difficile* 630Δ*erm* and CRG1115 (*sleC*) spores and (B) *C. difficile* R20291 and CRG1166 (*C. difficile* R20291 *sleC*) spores. Spore cultures were heated at 60°C for 25 min before they were plated onto BHIS agar (filled bars) or BHIS agar supplemented with 0.1% taurocholate (bars with diagonal lines). Spores were also decoated as described in Materials and Methods before they were plated onto BHIS agar supplemented with 1 μg/ml lysozyme (bars with horizontal lines). The bars 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.

efficiencies of the mutant strains, spores of all *C. difficile* 630Δ*erm* and R20291 strains were counted after 5 days by using phase-contrast microscopy, and the results were compared to the observed development of heat-resistant CFU (Fig. 4). Except for a sporulation-negative control, in which the master regulator of sporulation *spo0A* was insertionally inactivated (12), the sporulation frequencies of all mutant strains were found to be equivalent to those of the corresponding parental strains. This suggests that *sleC* mutants of both *C. difficile* 630Δ*erm* and *C. difficile* R20291 sporulate at parental levels but are unable to form colonies even in the presence of taurocholate.

Effect of *sleC* mutation on *C. difficile* spore viability. Having shown that the rate of sporulation of an *sleC* mutant was not affected compared to the rate of sporulation of the parental strain for both *C. difficile* 630Δ*erm* and R20291, we next demonstrated that the previously noted reduction in heat-resistant colony formation was not a result of altered spore heat resistance properties. Decoating of spores has previously been used to distinguish between spore viability and germination phenotypes (26, 29) as decoating and plating with supplemental lysozyme allow a germination-deficient spore to form heat-resistant colonies. Purified spores of both *C. difficile* 630Δ*erm* and R20291 plated without taurocholate formed few heat-resistant CFU compared to spores plated with taurocholate (Fig. 5). Decoating spores of the parental strains resulted in much higher numbers of heat-resistant CFU even in the absence of taurocholate. Purified spores of *sleC* mutants of both *C. difficile* 630Δ*erm* and *C. difficile* R20291 were unable to form heat-resistant CFU on BHIS agar supplemented with taurocholate (Fig. 4), but decoated *sleC* spores formed parental levels of heat-resistant CFU (Fig. 5), confirming that they were viable.

Finally, we observed *C. difficile* spores by phase-contrast microscopy after heat treatment and incubation in BHIS broth supplemented with taurocholate. Spores of *sleC* mutants of both 630Δ*erm* and R20291 appeared to lose their "phase-

bright" characteristics to the same degree as parental spores after 2 h of incubation. This is consistent with the characterization of *sleC* in *C. perfringens* (26) and suggests that *C. difficile sleC* mutant spores undergo the initial stages of germination in association with taurocholate but do not complete the process and grow as vegetative cells.

DISCUSSION

In this study we identified a homologue of *sleC* from *C. perfringens* that is essential for complete germination (in the presence of taurocholate) of *C. difficile* spores in both the nonepidemic strain 630Δ*erm* and the B1/NAP1/027 isolate R20291. Loss of SleC eliminated the development of heat-resistant CFU over 5 days despite spore formation by the parental strain, and the viability of *sleC* mutant spores was proven as their colony-forming ability after heat shock was restored by decoating. It is interesting that SleC is essential for *C. difficile* spore germination and vegetative cell outgrowth. While *Bacillus* has traditionally been thought of as the model genus for spore germination studies and previous work has identified three SCLEs, the *cwlJ*, *sleB*, and *sleL* products (4, 15, 18), these enzymes are not individually essential for complete cortex lysis (15, 22). On the other hand, recent work with *C. perfringens* suggests that the mechanisms of germination are somewhat different in *Clostridium* (26, 27). The identification of SleC as an essential *C. perfringens* SCLE underlined the difference between this clostridial species and the *Bacillus* paradigm (26), and our findings support the hypothesis that there is a generic difference between the germination of *Bacillus* spores and the germination of *Clostridium* spores. Furthermore, while germination-specific serine proteases have been characterized in *C. perfringens* (24, 37) and homologues have been identified in *C. difficile* 630 (33), these proteases are not known to play any role in *Bacillus* germination.

It has been shown that in *B. subtilis* CwlJ is important for CaDPA-induced germination (22), providing evidence that there is a CaDPA-mediated germination signaling pathway in *Bacillus*. In contrast, we found that a *B. subtilis cwlJ* homologue, CD3563, plays no obvious role in taurocholate-induced *C. difficile* spore germination. Indeed, recent studies have suggested that SCLEs in *C. perfringens* are not triggered by CaDPA (26), although further analysis of CD3563 is required to understand its importance, if any, to *C. difficile* spore germination. As CD3563 shares homology with both *B. subtilis cwlJ* and *sleB*, it is worthwhile to consider the possibility that a variety of functions are possible, although under our assay conditions CD3563 does not seem to play any role. This becomes more important with the knowledge that CD0552 has been annotated as *sleB* in the *C. difficile* 630 genome (33), although the 238-residue protein shares no similarity with any known SCLE.

While we can argue that our characterization of SleC and early studies of a *cwlJ*/*sleB* homologue suggest similarities between the germination of *C. difficile* spores and the germination of *C. perfringens* spores, it must be noted that there are obvious differences when germination characteristics as a whole are compared. Early characterization of *C. perfringens* germinant receptors indicated that monocistronic *gerA* and *gerKB* operons and a bicistronic *gerKA-gerKC* operon play a role in the early stages of germination (25, 27), and similar mechanisms have been described for other spore formers. *B. subtilis* has three tricistronic operons, *gerA*, *gerB*, and *gerK* (36), while a tricistronic *gerA* operon has also been identified and studied in both *Clostridium botulinum* and *Clostridium sporogenes* (2, 5). In contrast, none of these systems are present in *C. difficile*. Spore cortex lysis may proceed in similar ways in *C. difficile* and *C. perfringens*, but the circumstances under which the spores germinate likely differ. As a result, identification of *C. difficile* germinant receptors is crucial to understanding *C. difficile* spore germination.

The emergence of *C. difficile* B1/NAP1/027 strains (8) has strengthened the need to understand *C. difficile* spore germination, and our report is the first report of gene characterization by mutagenesis in a B1/NAP1/027 isolate of *C. difficile*. These strains are associated with more severe disease and greater virulence, and although it is still unclear how sporulation and germination in epidemic and nonepidemic strains compare, it has been suggested that the rate of sporulation is higher in particular B1/NAP1/027 isolates (1, 9). Therefore, the data that we present showing that R20291 sporulates and germinates less efficiently than 630Δ*erm* is perhaps surprising. We showed that *C. difficile* R20291 sporulates slower (Fig. 1) and forms fewer spores than *C. difficile* 630Δ*erm* over a 5-day period (Fig. 2). Furthermore, a lower number of the observed spores of R20291 than of the observed spores of 630Δ*erm* formed heat-resistant colonies, indicating that fewer R20291 spores germinate under our assay conditions. These findings indicate that caution should be used when general conclusions are drawn about the properties of types of *C. difficile* strains without an adequate sample size. Studies encompassing a large number of *C. difficile* isolates are necessary to define what role sporulation and germination play in disease.

The absolute requirement for SleC for *C. difficile* to complete bile-induced spore germination could represent a therapeutic target in the health care environment. Spore germination in the patient could be prevented through inhibition of SleC, and this could reduce the ability of *C. difficile* to cause disease. Conversely, activating SleC could force spores to germinate on surfaces. As vegetative cells are far more susceptible to traditional decontamination procedures, this may allow a second, or combined, disinfection step. Thus, dissemination of *C. difficile* infection may be reduced, and control of disease in the health care environment could be a more realistic target.

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Clostridium difficile spore germination: an update

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Abstract

Endospore production is vital for the spread of *Clostridium difficile* infection. However, in order to cause disease, these spores must germinate and return to vegetative cell growth. Knowledge of germination is therefore important, with potential practical implications for routine cleaning, outbreak management and potentially in the design of new therapeutics. Germination has been well studied in *Bacillus,* but until recently there had been few studies reported in *C. difficile*. The role of bile salts as germinants for *C. difficile* spores has now been described in some detail, which improves our understanding of how *C. difficile* spores interact with their environment following ingestion by susceptible individuals. Furthermore, with the aid of novel genetic tools, it has now become possible to study the germination of *C. difficile* spores using both a forward and reverse genetics approach. Significant progress is beginning to be made in the study of this important aspect of *C. difficile* disease.

Keywords: *Clostridium difficile*; Spore; Germination; Bile; BI/NAP1/027

1. Introduction

Clostridium difficile is the major cause of nosocomial diarrhoea and a major burden to healthcare services worldwide. Outbreaks of *C. difficile*-associated disease (CDAD) can lead to patient isolation, ward closures, and in some cases, hospital closure. In 2008, almost 6000 deaths in England and Wales alone were attributed to CDAD (from the Office of National Statistics, available at http://www.statistics.gov.uk). The principal virulence factors of *C. difficile* have been identified as toxins A and B, which target the Ras superfamily of GTPases for modification via glycosylation, ultimately leading to destruction of intestinal epithelial cells (Voth and Ballard, 2005). Subsequent symptoms can range from mild diarrhoea to a fulminant, relapsing and potentially fatal colitis. In addition, the production of endospores plays a pivotal role in the transmission of disease. Clostridial spores are resistant to all manner of chemical and physical agents (Setlow, 2007), and this provides the mechanism for *C. difficile* persistence on surfaces, leading to infection through inadvertent ingestion of contaminated material (Gerding et al., 2008; Riggs et al., 2007). Spore germination is defined as the irreversible loss of spore-specific properties and it is this process which allows for vegetative cell growth and subsequent toxin production. Consequently, the ability to manipulate *C. difficile* spore germination mechanisms could have direct applications in future disease prevention.

This review will focus on the latest studies which have advanced our understanding of *C. difficile* spore germination, and describe how novel genetic tools now at our disposal will open the way for studies that have not previously been possible in *C. difficile*.

2. Model of bacterial spore germination

Current mechanistic knowledge of germination is based principally on data obtained by studying *Bacillus subtilis*. Germination is initiated when a spore senses specific effectors, termed germinants. Spores of *B. subtilis* can germinate through the binding of germinants, either L-alanine or a mixture of

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asparagine, glucose, fructose and potassium ions, to specific receptors, GerA, GerB and GerK, located in the spore inner membrane (Paidhungat and Setlow, 2000). The spore is then committed to germination and subsequent events include the release of monovalent cations (H^+, Na^+ and K^+) and a large depot (~25% of spore dry weight) of calcium dipicolinic acid (CaDPA) (Setlow, 2003), a process for which SpoVA proteins are required (Vepachedu and Setlow, 2004, 2007a, b). The third major step in germination involves the hydrolysis of the spore peptidoglycan (PG) cortex. It is during this hydrolysis that the previously low water content of the spore is returned to that of a vegetative cell and the core is able to expand, in turn allowing for enzyme activity, metabolism and, ultimately, vegetative cell outgrowth (Setlow, 2003). *B. subtilis* encodes two redundant spore cortex lytic enzymes (SCLEs), CwlJ, required for CaDPA-induced germination (Ishikawa et al., 1998; Paidhungat et al., 2001), and SleB, the mode of activation of which is unknown. The specific proteins YpeB (SleB) and GerQ (CwlJ) are required for enzyme assembly and/or stability in spores (Boland et al., 2000; Ragkousi et al., 2003) and these SCLEs specifically degrade spore PG containing muramic-δ-lactam (Popham et al., 1996), which is not present in normal cell wall PG. This PG modification occurs during sporulation in association with the CwlD protein (Popham et al., 1996; Sekiguchi et al., 1995) and prevents SCLE-associated damage to the vegetative cell on completion of the germination process.

3. Role of bile salts in *C. difficile* spore germination

The specific conditions by which *C. difficile* spores sense a suitable environment for germination remain unclear. Recent work, however, has suggested that bile salts play a pivotal role. Thus following on from the earlier observation that taurocholate improves the recovery of *C. difficile* spores from environmental surfaces (Wilson et al., 1982), a study by Sorg and Sonenshein demonstrated that specific bile salts and glycine act as cogerminants of *C. difficile* spores, while the secondary bile salt deoxycholate prevented vegetative cell growth (Sorg and Sonenshein, 2008). They hypothesise that, in a healthy host, *C. difficile* spores can survive passage into the jejunum where they germinate in response to the high concentrations of cholate derivatives and glycine. However, in such individuals, cholate derivatives that escape enterohepatic circulation are metabolised into the secondary bile salt deoxycholate. Thus, outgrowth of *C. difficile* spores is prevented. Conversely, following disruption of intestinal flora, secondary bile salts such as deoxycholate are not readily produced. *C. difficile* vegetative cell growth is therefore not prevented and disease may occur in susceptible individuals.

A further study by Sorg and Sonenshein (2009) has described the role of the primary bile salt chenodeoxycholate in the inhibition of *C. difficile* spore germination. This adds an interesting element to their model of *C. difficile* colonisation. In equal concentrations, both cholate and chenodeoxycholate derivatives may compete for binding to putative *C. difficile* germinant receptors. However, as the rate of absorption by the colon of chenodeoxycholate is 10 times that of cholate (Mekhjian et al., 1979), spores reaching the large intestine encounter a higher concentration of cholate derivatives. This suggests that germination may be inhibited until *C. difficile* spores reach the anaerobic environment of the large bowel, where conditions are suitable for *C. difficile* vegetative cell growth.

A recent study has further expanded our knowledge of how *C. difficile* spores may interact with bile salts (Giel et al., 2010). The authors described how, using an extract from mouse small intestine, a factor produced in vivo stimulated colony formation of *C. difficile* spores. Treatment of the extract with cholestyramine, a bile salt binding resin, reduced the ability of the extract to stimulate colony formation, suggesting that the compound produced is likely a bile salt. Furthermore, colony formation was stimulated to greater levels in extracts from mice treated with clindamycin, ampicillin or streptomycin, suggesting that disruption of normal intestinal flora plays a role in the germination frequency of *C. difficile* spores in vivo. Indeed, a possible role of antibiotics such as fluoroquinolones and clindamycin in inducing *C. difficile* germination has been described, further supporting this hypothesis (Baines et al., 2009; Saxton et al., 2009).

These studies now suggest that the interaction of *C. difficile* with bile salts and antibiotics, coupled with disruption of normal intestinal flora, plays an important role in *C. difficile* colonisation and subsequent disease. Consequently, it now becomes important to understand the detailed mechanism of such interactions and this requires knowledge of the genetic basis of *C. difficile* spore germination.

4. Studying *C. difficile* spore germination using reverse genetics

The precise mechanisms of germination in *C. difficile* have not been studied in great depth, due in part to a historical absence of genetic tools. However, the ClosTron, a facile directed mutagenesis system, has recently been developed and allows for the creation of stable, insertional mutants in *C. difficile* (Heap et al., 2007; Heap et al., 2010). The result of this is an efficient means to study *C. difficile* spore germination using a reverse genetics approach, by identifying homologues of genes important for germination in other spore formers, inactivating them, and inferring their function from mutant phenotypes.

A direct application of the ClosTron system in *C. difficile* has now been reported with the characterization of an apparent *Clostridium perfringens sleC* homologue in both an erythromycin-sensitive variant of the *C. difficile* 630 genome strain (630Δ*erm*) (Hussain et al., 2005) and a restriction endonuclease type BI, North American pulsed-field type NAP1 and PCR ribotype 027 (BI/NAP1/027) isolate (R20291, isolated from the 2004/2005 Stoke Mandeville outbreak). SleC was found to be essential for colony formation when germination of *C. difficile* spores was induced in nutrient-rich medium supplemented with taurocholate, while CD3563 (an apparent homologue of *B. subtilis cwlJ* and *sleB*) was found to play no obvious role under the same conditions (Burns et al., 2010).

This finding is in line with similar studies in *C. perfringens*, in which *sleC* encodes an enzyme essential for cortex hydrolysis during germination (Paredes-Sabja et al., 2009a, b; Miyata et al., 1995; Shimamoto et al., 2001). It is interesting that SleC is essential for germination and outgrowth of *C. difficile* spores. While *Bacillus* is traditionally thought of as the model organism for studying spore germination and previous work has identified three SCLEs, CwlJ, SleB and SleL, these enzymes are not individually essential for complete cortex lysis. Furthermore, while *B. subtilis* CwlJ is required for CaDPA-mediated cortex lysis, it has been suggested that SCLEs in *C. perfringens* are not triggered by CaDPA (Paredes-Sabja et al., 2009a, b). The evidence to suggest that a CwlJ homologue in *C. difficile* plays no obvious role in germination and outgrowth therefore suggests that the mechanisms of germination in *Clostridium* are somewhat different from *Bacillus*.

With the aid of a directed mutagenesis system such as the ClosTron, reverse genetics studies identifying additional genes required for *C. difficile* spore germination will likely follow in the future. Table 1 lists a number of apparent homologues in *C. difficile* of genes important for spore germination in other organisms, targets which may form a basis for future reverse genetics studies of *C. difficile* germination. Additionally, the 336 spore-associated polypeptides identified by mass spectrometric analysis of *C. difficile* spores represent further key targets for functional analysis, in particular the 88 clostridial spore core proteins and the and 29 proteins identified as *C. difficile* spore specific (Lawley et al., 2009).

5. The future of *C. difficile* germination research

Arguably, the most important aspect of spore germination to understand is the mechanism(s) by which dormant spores sense a suitable environment for germination. As outlined above, this is typically due to germinants binding to receptors located in the spore inner membrane, and such receptors have been identified in a number of other spore formers. Characterization of *C. perfringens* has indicated that monocistronic *gerA* and *gerKB* operons and a bicistronic *gerKA-gerKC* operon play an important role in the initiation of germination, and similar mechanisms have been described in other spore formers. *B. subtilis* has three tricistronic operons, *gerA*, *gerB* and *gerK*, while a tricistronic *gerA* operon has also been identified in *Clostridium botulinum* and *Clostridium sporogenes* (Setlow, 2007). However, *C. difficile* encodes no known homologues of any of these systems, and as a result, the mechanism through which *C. difficile* spores initiate germination remains unknown.

Reverse genetic approaches allow for systematic identification and inactivation of target genes presumed to play a role in *C. difficile* spore germination, based on homology to genes important for germination in other spore formers. However, where no apparent homologues are present, a problem posed in the search for *C. difficile* germinant receptors, it is necessary to use a forward genetics approach. Forward genetics aims to identify the genetic basis of a particular phenotype without assumptions about the genes involved. Based upon this principal, a novel *mariner*-based transposon system for *C. difficile* has recently been developed, which allows for the generation of libraries of random mutants (Cartman and Minton, 2010). Phenotypic screening of a random mutant library in a BI/NAP1/027 strain of *C. difficile* yielded a putative sporulation/germination defective clone with an insertion in a gene showing homology to the germination-specific proteases *cspB* and *cspA* of *C. perfringens* (Paredes-Sabja et al., 2009a, b), showcasing the potential of the system in the detection of genes important for *C. difficile* germination. It is hoped that this system will now form a basis for identification of genes encoding putative *C. difficile* germinant receptors.

6. BI/NAP1/027 strains of *C. difficile*

The recent emergence of BI/NAP1/027 strains of *C. difficile* has strengthened the need to develop effective treatments and/or disinfection measures (O'Connor et al., 2009; Dawson et al., 2009). This type is now the most commonly isolated *C. difficile* type in England and Wales and is associated with outbreaks of increased disease severity (Brazier et al., 2008). Furthermore, some strains of this type have been shown to produce higher levels of toxin in vitro (Warny et al., 2005). Recently, it has been shown that the germination frequency of

Table 1
Known gene targets in *C. difficile* based on homology to genes important for spore germination in other spore formers.

C. difficile target (630Δ*erm*)	Homologues	Hypothesised function
CD0551	*C. perfringens sleC* (Paredes-Sabja et al., 2009a, b)	Spore cortex lytic enzyme
CD3563	*B. subtilis cwlJ/sleB* (Ishikawa et al., 1998; Masayama et al., 2006; Chirakkal et al., 2002)	Spore cortex lytic enzyme
CD2246	*C. perfringens cspA/cspC* (Paredes-Sabja et al., 2009a, b)	Germination-specific protease
CD2247	*C. perfringens cspA/cspB* (Paredes-Sabja et al., 2009a, b)	Germination-specific protease
CD0106	*B. subtilis cwlD* (Popham et al., 1996)	Germination-specific *N*-acetylmuramoyl-L-alanine amidase
CD0773	*B. subtilis spoVAC* (Vepachedu and Setlow, 2004, 2007a, b)	CaDPA release
CD0774	*B. subtilis spoVAD* (Vepachedu and Setlow, 2004, 2007a, b)	CaDPA release
CD0775	*B. subtilis spoVAE* (Vepachedu and Setlow, 2004, 2007a, b)	CaDPA release
CD2578	*B. subtilis prkC* (Shah et al., 2008)	Peptidoglycan-associated germination

the 'epidemic' BI/NAP1/027 isolate R20291 is lower than that of a 'non-epidemic' *C. difficile*, 630Δ*erm* (Burns et al., 2010). This suggests that differences may exist in the germination of epidemic and non-epidemic types of *C. difficile*. As spores represent the infectious stage of *C. difficile*, diversity in the germination characteristics of particular types may contribute to the observed differences in disease severity.

7. Conclusions

It is clear that our knowledge of *C. difficile* spore germination is rudimentary. However, it has now been shown that bile salts play a role in both in vitro and in vivo germination of *C. difficile* spores (Sorg and Sonenshein, 2008, 2009; Giel et al., 2010). In addition, our understanding of the genetic basis of *C. difficile* spore germination has been enhanced through early reverse genetics studies (Burns et al., 2010). As spores represent the principal infectious stage of *C. difficile* (Gerding et al., 2008; Riggs et al., 2007), and as these spores must germinate to allow for vegetative cell growth and toxin production, a detailed understanding of the germination process may have direct applications for disease prevention in the healthcare environment. Germinants could be used to stimulate germination of *C. difficile* spores on surfaces. The resulting vegetative cells are exquisitely sensitive to stresses such as oxygen, traditional decontamination procedures and the gastric barrier. Conversely, inhibition of germination following ingestion of spores may reduce the ability of *C. difficile* to colonise and cause disease. Reports to date have only scratched the surface of *C. difficile* spore germination, but with a range of genetic tools now at our disposal (Heap et al., 2007, 2009, 2010; Cartman and Minton, 2010), the way is open to understand more thoroughly this important aspect of CDAD and thereby develop effective intervention strategies.

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Pathogenesis and Toxins

The diverse sporulation characteristics of *Clostridium difficile* clinical isolates are not associated with type

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ABSTRACT

Clostridium difficile causes diarrhoeal diseases ranging from asymptomatic carriage to a fulminant, relapsing, and potentially fatal colitis. Endospore production plays a vital role in transmission of infection, and in order to cause disease these spores must then germinate and return to vegetative cell growth. Type BI/NAP1/027 strains of *C. difficile* have recently become highly represented among clinical isolates and are associated with increased disease severity. It has also been suggested that these 'epidemic' types generally sporulate more prolifically than 'non-epidemic' strains, although the few existing reports are inconclusive and encompass only a small number of isolates. In order to better understand any differences in sporulation rates between epidemic and non-epidemic *C. difficile* types, we analysed these characteristics using 14 *C. difficile* clinical isolates of a variety of types. Sporulation rates varied greatly between individual BI/NAP1/027 isolates, but this variation did not appear to be type-associated. Furthermore, a number of BI/NAP1/027 spores appeared to form colonies with a lower frequency than specific non-BI/NAP1/027 strains. The data suggest that (i) careful experimental design is required in order to accurately quantify sporulation; and (ii) current evidence cannot link differences in sporulation rates with the disease severity of the BI/NAP1/027 type.

1. Introduction

Clostridium difficile is the major cause of hospital-acquired diarrhoea and a burden to healthcare services worldwide. Endospores, produced during sporulation, are pivotal to disease transmission. The resistance of these spores to all manner of chemical and physical agents [1] provides the mechanism for *C. difficile* persistence on surfaces, leading to infection through inadvertent ingestion of contaminated material [2,3]. Spore germination, defined as the events that lead to the irreversible loss of spore-specific characteristics, is essential for the conversion of spores to vegetative cells, and is absolutely required for subsequent disease. Restriction-endonuclease analysis type BI, North American pulsed-field type 1 (NAP1) and PCR-ribotype 027 (BI/NAP1/027) strains of *C. difficile* have recently been associated with outbreaks of increased disease severity [4,5] and some have been shown to produce higher levels of toxin in the laboratory [6]. It has also been suggested that the *in vitro* rate of sporulation is greater in BI/NAP1/027 strains of *C. difficile* [7–9], although we have recently shown that, in the laboratory, the 'epidemic' BI/NAP1/027 isolate R20291 sporulates and germinates with a lower frequency than the 'non-epidemic' *C. difficile* strain 630Δ*erm* [10]. To-date, the literature contains insufficient data to directly compare the sporulation of different *C. difficile* types. The precise role of sporulation in disease incidence and severity, therefore, remains unclear. In this study, we use an accurate, reproducible assay to measure the sporulation characteristics of a variety of *C. difficile* types, including several BI/NAP1/027 isolates from both North America and Europe (Table 1 lists the strains used in this study).

2. Materials and methods

2.1. Bacterial strains and growth conditions

All *C. difficile* strains were grown at 37 °C in an anaerobic workstation (Don Whitley, United Kingdom) in BHIS (brain heart infusion supplemented with L-cysteine [0.1%, Sigma, United Kingdom] and yeast extract [5 mg/ml, Oxoid]) broth or agar [11], in which *C. difficile* sporulates efficiently.

2.2. Preparation of C. difficile spores

Sporulation of *C. difficile* was achieved by incubating cultures in BHIS broth for 5 days. First, *C. difficile* strains were selected on BHIS

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Table 1
C. difficile strains used in this study.

Strain	Relevant properties	Source/reference
BI/NAP1/027		
R20291	Stoke Mandeville (2004/2005) isolate	Anaerobe Reference Laboratory, Cardiff
CDC 32	Historical USA isolate	[13]
CDC 38	USA isolate	[13]
M13042	Canada isolate	[13]
DH326	Yorkshire and Humberside, Sheffield isolate (2005)	Anaerobe Reference Laboratory, Cardiff
DH1329	West Midlands, Coventry isolate (2007/8)	Anaerobe Reference Laboratory, Cardiff
R12087	Historical EU isolate (1980s)	Institut Pasteur, Paris
Non-BI/NAP1/027		
630Δ*erm*	PCR-ribotype 012 Erythromycin sensitive strain of *C. difficile* 630	[14]
630Δ*erm* spo0A::intron *ermB*	*C. difficile* sporulation-negative control	[15]
GAI 95601	PCR-ribotype 017, Japan	[16]
001-3	PCR-ribotype 001	ECDC – Cardiff collection
Serosubtype A2	PCR-ribotype 002	ECDC – Cardiff collection
Wilcox 078	PCR-ribotype 078	Mark Wilcox
R10459	PCR-ribotype 106	ECDC – Cardiff collection
VPI 10463	PCR-ribotype 003 Toxinotype 0 reference strain	[17]

agar supplemented with cycloserine (250 μg/ml) and cefoxitin (8 μg/ml). To ensure no spores were present on inoculation of the sporulation medium, a starter culture was prepared in BHIS broth with a 1% inoculum of an overnight culture and incubated until an optical density at 600 nm (OD_{600}) of between 0.2 and 0.5 was reached. Finally, the sporulation medium was then inoculated with a 1% inoculum of this exponential starter culture.

2.3. Measurement of *C. difficile* sporulation

In order to measure colony formation, 500 μl samples were removed from the anaerobic chamber and heated at 60 °C for 25 min to kill vegetative cells but not spores. Samples were then returned to the anaerobic chamber, serially diluted in phosphate-buffered saline, and plated onto BHIS agar supplemented with the bile salt taurocholate (0.1%, Sigma, United Kingdom) to induce germination and enhance recovery of *C. difficile* spores [11,12]. The plates were incubated for 24 h before colony forming units (CFU) were enumerated. Total sporulation after 5 days incubation in BHIS broth was assayed by counting spores under phase-contrast microscopy using a Bright-Line™ haemocytometer (Sigma, United Kingdom). All statistical analysis was carried out in GraphPad Prism using Student's *t*-test for the comparison of BI/NAP1/027 with non-BI/NAP1/027, and one-way analysis of variance with Tukey's *post hoc* compensation for multiple comparisons of individual strains.

3. Results and discussion

First we cultivated all of the *C. difficile* strains in BHIS broth for 24 h, monitoring growth by the change in OD_{600}. All strains showed highly similar growth (data not shown), allowing us to exclude growth differences when interpreting subsequent experiments.

In order to understand the rate at which the selected *C. difficile* strains formed spores, we compared the development of heat-resistant CFU over 5 days (Fig. 1). This appeared to show a large degree of variation within, but not necessarily among *C. difficile* types. The CFU observed for each strain after 24 h and 120 h were then plotted and compared separately (Fig. 2A). No obvious difference in heat-resistant CFU was seen between the BI/NAP1/027 and non-BI/NAP1/027 groups after 24 h suggesting that, under the conditions of our assay, there is no association between *C. difficile* type and sporulation over a 24 h period. However, the BI/NAP1/027 group appeared to form lower levels of heat-resistant CFU after 120 h when compared to the non-BI/NAP1/027 group, although this difference was not quite statistically significant ($p = 0.054$). In particular, it was noted that VPI 10463 formed significantly more heat-resistant CFU after 120 h than all tested BI/NAP1/027 isolates ($p < 0.05$). In order to confirm any sporulation differences, we counted spores from all strains by phase-contrast microscopy (Fig. 2B) after 120 h. Spore titres varied significantly among the 14 isolates studied ($p < 0.05$), and although the BI/NAP1/027 group appeared to form fewer spores than the non-BI/NAP1/027 group, this difference was not significant. These data suggest that, while spore titres varied greatly between individual strains, there was no obvious type-associated variation in the spore titres obtained from the *C. difficile* strains in this study.

When analysing the sporulation efficiencies of these *C. difficile* strains, an inconsistency between spore titre and heat-resistant CFU was observed in some cases. Fig. 3 shows the proportion of microscopically-counted spores of each strain which formed heat-resistant CFU at 120 h when germination was induced with taurocholate supplement. This colony formation varied significantly among the 14 *C. difficile* isolates tested ($p < 0.001$), and it was also noted that spores of a number of non-BI/NAP1/027 strains formed more colonies than the spores of certain BI/NAP1/027 strains. In particular, a significantly higher proportion of VPI 10463 and 630Δ*erm* spores formed colonies than the BI/NAP1/027 strains R20291, R12087, CDC 32 and CDC 38 ($p < 0.05$). Furthermore, spores of the non-BI/NAP1/027 isolates Serosubtype A2 and R10459 formed significantly more colonies than R12087 and CDC 38 ($p < 0.05$). However, it was also noted that spores of a PCR-ribotype 078 isolate (Wilcox 078) formed significantly fewer colonies in comparison to VPI 10463 ($p < 0.05$). Not all BI/NAP1/027 strains formed proportionally fewer colonies than microscopically-observed spores, as a significantly higher proportion of DH326 spores formed colonies when compared to R12087 ($p < 0.05$), while not showing any significant differences to VPI 10463, R10459, Serosubtype A2 or 630Δ*erm* (non-BI/NAP1/027). Additionally, when the BI/NAP1/027 and non-BI/NAP1/027 groups were directly compared, there was no significant variation observed in the proportion of spores which formed colonies ($p = 0.14$). Taken together, these data indicate that following heat treatment, spores of a number of *C. difficile* BI/NAP1/027 isolates appear to form colonies at a lower frequency than certain non-BI/NAP1/027 isolates. However, a large degree of variation was observed between individual *C. difficile* strains, and the smaller proportion of microscopically-observed spores forming colonies was not specific to the BI/NAP1/027 type. As spores must complete germination in order to

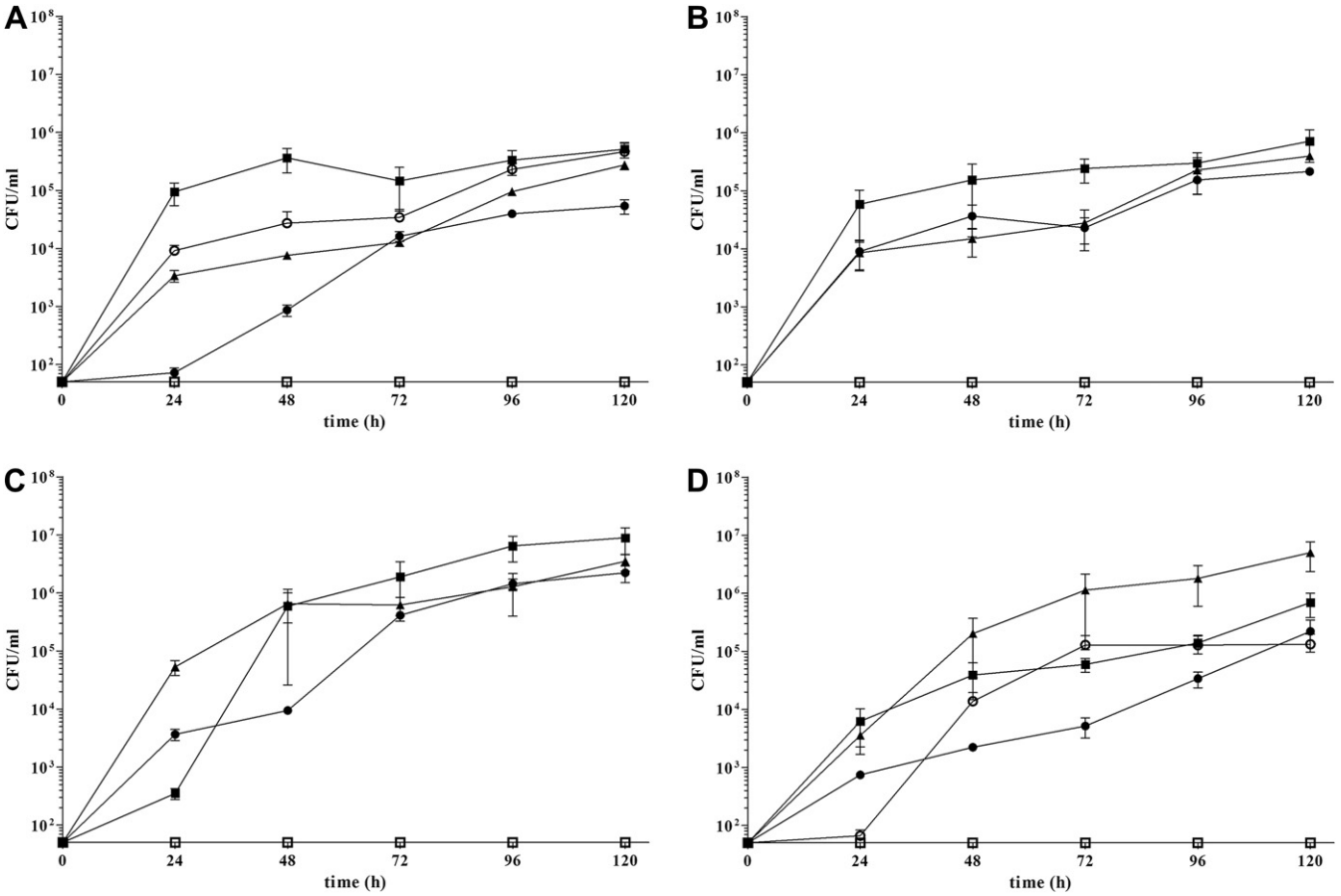

Fig. 1. The development of heat-resistant CFU of *C. difficile* strains over five days. (A) ○, CDC 32; ■, M13042; ▲, R12087; ●, R20291; □*C. difficile* 630Δ*erm spo0A*; (B) ●, CDC 38; ■, DH326; ▲, DH1329;□, *C. difficile* 630Δ*erm spo0A*; (C) ▲, R10459; ●, 630Δ*erm*; ■, VPI 10463; □, *C. difficile* 630Δ*erm spo0A*; (D) ■, 001-3; ▲, Serosubtype A2; ●, GAI 95601; ○, Wilcox 078; □ *C. difficile* 630Δ*erm spo0A*. The data represent the average of three independent experiments and error bars indicate standard errors of the means. The detection limit for colony counts was 50 CFU/ml.

form colonies, we hypothesize that the inconsistency between spore titre and colony formation among these *C. difficile* strains may be caused by a difference in the frequency of germination and spore outgrowth.

Given the recent suggestion that BI/NAP1/027 strains of *C. difficile* sporulate at a greater rate than strains of other types [7–9], the data we present here is perhaps surprising. A particularly interesting difference between our study and previous work was noted with *C. difficile* VPI 10463. It has been suggested by Akerlund et al. that VPI 10463 sporulates poorly over a 48 h period [7], but our data show the opposite. While few heat-resistant CFU were seen after 24 h (Fig. 2A), we found VPI 10463 to sporulate at least as well as any other strain tested from 48 h onwards (Figs. 1 and 2B). Two notable differences between our study and the one by Akerlund et al. are the growth conditions employed and the methods of assaying sporulation. Akerlund et al. used a broth containing peptone-yeast and without cysteine and glucose [7], while our strains were cultivated in BHIS broth. Furthermore, Akerlund et al. expressed sporulation rate as a ratio of spores to vegetative cells within the population after 48 h, a method previously used to compare sporulation of *C. difficile* isolates [8]. However, this relative measure can be affected by growth differences between strains and also by the survival of non-sporulating vegetative cells. Additionally, without an actual spore titre it is difficult to accurately quantify sporulation. We feel it is more appropriate to (i) measure the change in OD_{600} to evaluate growth differences; (ii) observe the development of heat-resistant CFU to assess the rate of sporulation; and (iii) enumerate spore titres independently of colony formation, by microscopy, to allow for an accurate measurement of *C. difficile* sporulation.

Previous studies of *C. difficile* sporulation rates have been limited to a time-course of 48 h [7,9] or 72 h [8]. Interestingly, we observed that a low level of heat-resistant CFU after 24 h in comparison to other isolates, such was the case with *C. difficile* VPI 10463, did not necessarily correlate to a low level of heat-resistant CFU after 120 h. To the contrary, it was noted that while VPI 10463 formed few heat-resistant CFU after 24 h, significantly more heat-resistant CFU were observed after 120 h than all tested BI/NAP1/027 isolates ($p < 0.05$). Conversely, when compared to the other individual strains, a high level of colony formation observed after 24 h, such was the case with the BI/NAP1/027 isolate *C. difficile* M13042, did not always result in high levels of colony formation after 120 h when compared to other strains. This suggests that in a case where a strain initiates sporulation particularly early, such a strain may not necessarily form a higher total number of spores than a strain which initiates sporulation at a later stage. Consequently, by studying *C. difficile* sporulation characteristics until a plateau of spores is observed, the relationship between sporulation rate and total sporulation can be better described.

The 'hypervirulence' associated with BI/NAP1/027 strains of *C. difficile* has been largely attributed to an increase in toxin production [6], although recent studies have argued that BI/NAP1/027 strains are more prolific in terms of sporulation *in vitro*, suggesting that sporulation characteristics play a role in disease

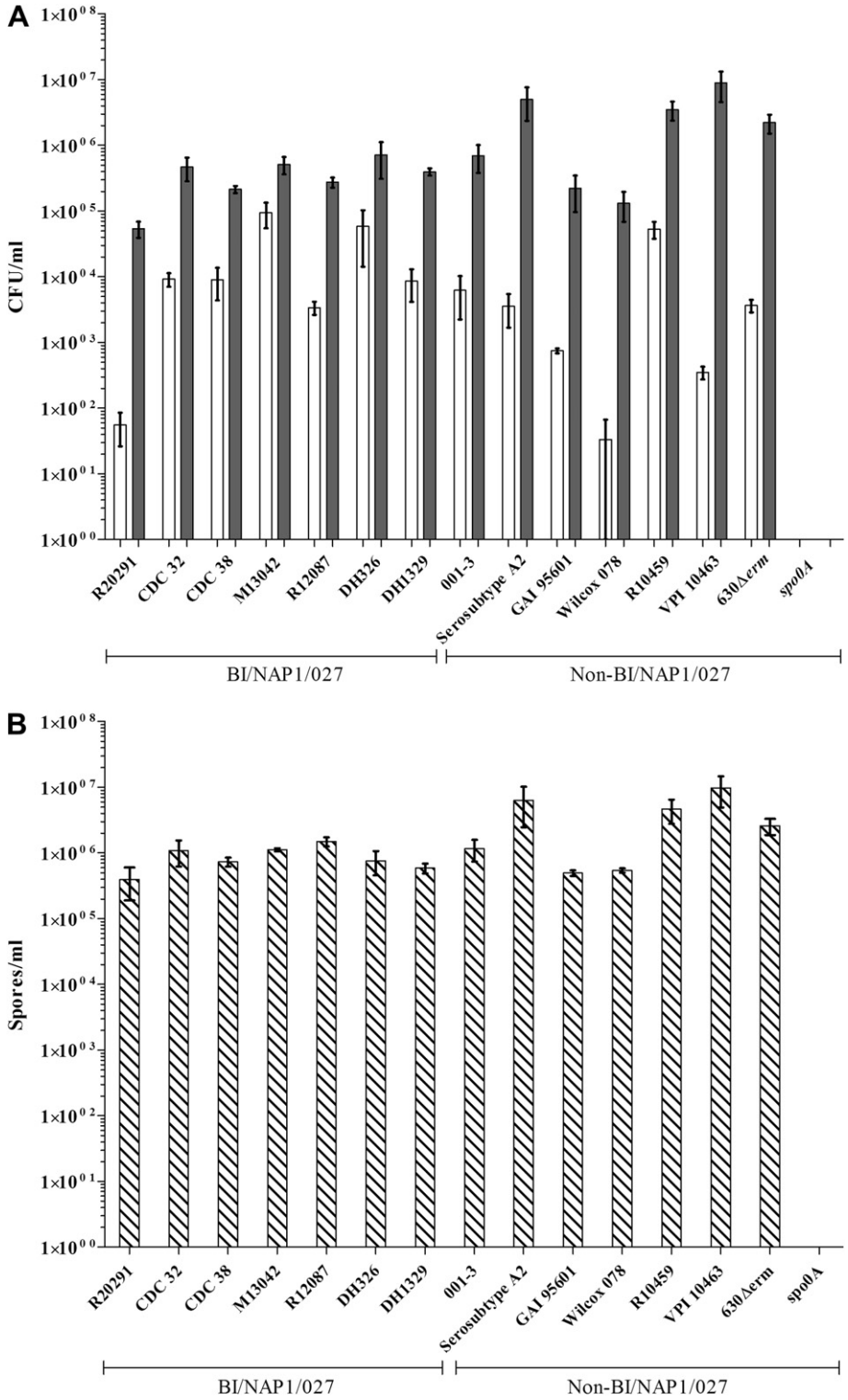

Fig. 2. (A) CFU after heat treatment of *C. difficile* strains after 24 h (open bars) and 120 h (filled bars), enumerated from the same sporulation cultures as described in Fig. 1; (B) microscopically-counted spore titres after 120 h. The data represent the average of three independent experiments and error bars indicate the standard errors of the means. The detection limit for colony counts was 50 CFU/ml and for spore counts 5×10^3 spores.

incidence and severity [7–9]. On the other hand, both this study and our previous work [10] would suggest that the *in vitro* sporulation rate of *C. difficile* BI/NAP1/027 strains is not significantly higher than non-BI/NAP1/027 strains, while in some cases the sporulation rate is lower than other types. The present study also provides evidence of further BI/NAP1/027 strains whose spores may germinate to form colonies, in the presence of taurocholate, at a lower frequency than a number of non-BI/NAP1/027 strains. Overall, our findings to date show that the variation in *C. difficile* sporulation frequency *in vitro* is far greater among individual strains than among types. The obvious

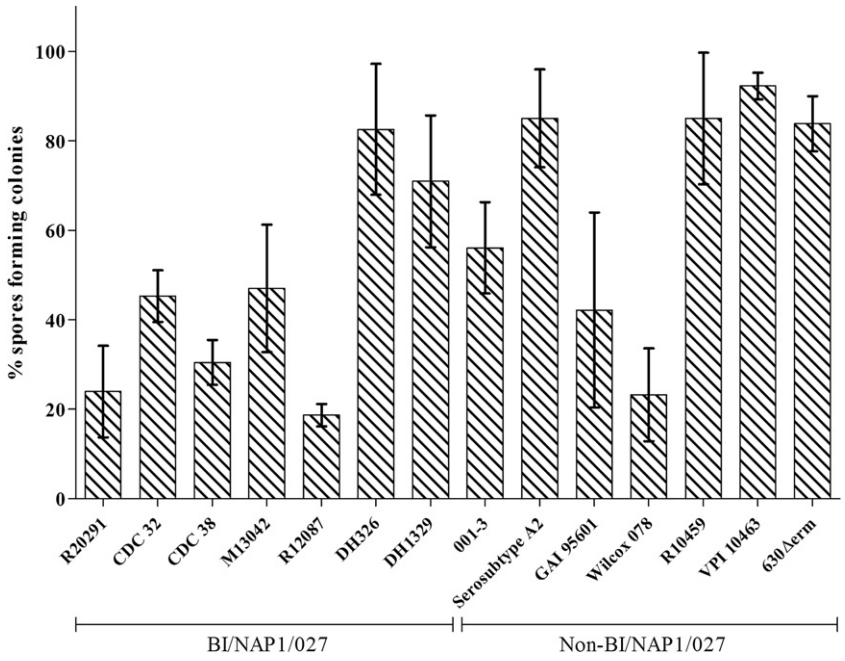

Fig. 3. The proportion of *C. difficile* spores, counted by phase-contrast microscopy, that formed colonies after heat treatment. The data represent the average of three independent experiments and error bars indicate standard errors of the means.

question remains of how the *in vitro* sporulation characteristics described in this study relate to sporulation proficiency *in vivo*. Consequently, until our understanding of *in vivo C. difficile* sporulation improves, it will be difficult to associate the sporulation characteristics of *C. difficile* BI/NAP1/027 with the severity of disease caused by this type. Furthermore, the observed inconsistency between spore counts and heat-resistant CFU of a number of *C. difficile* strains in this study also raises questions about the germination rates of different *C. difficile* strains. If differences do exist in the germination rates of different *C. difficile* types, future studies analysing these characteristics may provide a greater understanding of what factors contribute to the incidence and severity of *C. difficile* disease.

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