Use of *Bacillus subtilis* spores in treatments for *Clostridium difficile* infection

A thesis submitted for the degree of Doctor of Philosophy

by

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DECLARATION OF AUTHORSHIP

I, Claire Colenutt, hereby declare that this thesis and the work presented in it is entirely my own. Where I have consulted the work of others, this is always clearly stated.

Signed: _____ (Claire Colenutt)

Date: _____

ABSTRACT

Clostridium difficile infection is the primary cause of nosocomial diarrhoea in developed countries and the cost to healthcare providers is substantial. *C. difficile* is an opportunistic pathogen, with disease occurring when the normal colonic flora is disrupted. Dysbiosis of the intestinal microbiota, usually associated with antibiotic use, allows ingested spores of *C. difficile* to germinate and proliferate. Clinical symptoms are mediated by production of two major exotoxins, toxin A and toxin B. Clinical manifestations of infection can range from a non-symptomatic carrier state to diarrhoea and inflammation of the gut. Severe infections have potential to be fatal. Current treatment strategies for this infection rely heavily on just a few available antibiotics, as strains display multiple antibiotic resistances.

This thesis investigates the use of alternative treatment strategies, using *Bacillus subtilis* spores as a basis. One aspect of this assesses the use of *B. subtilis* spores as a mucosal vaccine delivery system. Delivery of a spore based vaccine by both oral and sublingual routes demonstrates these mucosal routes as promising strategies for generating protection against CDI. This thesis also uses *B. subtilis* spores as an oral probiotic treatment. The application of *B. subtilis* probiotics in a murine model of CDI is investigated and it is shown through optimisation of dose regimens that suppression of CDI symptoms can be achieved. Both use of vaccines and alternative treatments such as probiotics have the potential to reduce reliance on antibiotic treatment methods.

The role of *C. difficile* spores in infection was also investigated. Genetic manipulation was used to produce specific mutations in the spore coat and the resulting mutant phenotypes were analysed. This work demonstrated that the spore coat protein BclA1 plays a key role in colonisation of the host. This finding contributes to understanding how *C. difficile* can establish infection.

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Abbreviations

AAD – Antibiotic Associated Diarrhoea

BHI – Brain Heart Infusion

BSA – Bovine Serum Albumin

CAI – Community Associated Infection

CDC- Centres for Disease Control and Prevention

CDI – *Clostridium difficile* infection

CDTA – C- terminus Domain of Toxin A

CFU – Colony Forming Units

CLSI – Clinical Laboratory Standards Institute

CNS – Central Nervous System

Cwp – Cell wall protein

DDT - Dithioreitol

DMEM – Dulbecco's Modified Eagle Medium

DSM – Difco Sporulation Media

EDTA – Ethylenediaminetetracetic Acid

EFSA – European Food Safety Authority

ELISA – Enzyme Linked Immunosorbant Assay

ERM – Erythromycin

FAO – Food and Agriculture Organization of the United Nations

FBS – Foetal Bovine Serum

GI – Gastrointestinal

GM – Genetically Modified

GRAS – Generally Regarded As Safe

HAI – Healthcare Associated Infection

H&E – Hemotoxylin and Eosin

IBD – Inflammatory Bowel Disease

IgA – Immunoglobulin isotype A

IgG – Immunoglobulin isotype G

IgG1 – Immunoglobulin isotype G subtype 1

IgG2a – Immunoglobulin isotype G subtype 2a

IL – Interleukin

kDa - Kilodalton

IM – Intra muscular

IN – Intra nasal

IVC – Individually ventilated cage

LCT – Large Clostridial Toxin

NCIMB – National Collection of Industrial and Marine Bacteria

NIH – National Institutes of Health

Mab – Monoclonal Antibody

MIC – Minimum Inhibitory Concentration

OD – Optical density

OG – Orogastric

ONS – Office for National Statistics

ORF – Open Reading Frame

PaLoc – Pathogenicity Locus

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PFGE – Pulsed Field Gel Electrophoresis

PPI – Proton Pump Inhibitor

PP108 – *B. subtilis* spore vaccine

REA – Restriction Endonuclease Analysis

RNA – Ribonucleic Acid

RT – Room Temperature

RT-PCR – Reverse Transcriptase Polymerase Chain Reaction

SATH – Spore Adhesion to Hydrocarbon

SDS-PAGE – Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SLP – Surface Layer Protein

SMC – Sporulation Medium supplemented with L-cysteine

TBS – Tris Buffered Saline

TEM – Transmission Electron Microscopy

TGY – Tryptic-Glucose-Yeast Extract Medium

T_H1 – T Helper Lymphocyte Type 1

T_H2 – T Helper Lymphocyte Type 2

TLR – Toll Like Receptor

TNF – Tumour Necrosis Factor

TMB – Tetramethyl Benzidine

UV – Ultraviolet (radiation)

v/v – volume per volume

w/v – weight per volume

Chapter 1

Introduction

1.1. *Bacillus subtilis*

Bacillus subtilis belongs to the genus *Bacillus,* a diverse group of endospore forming, Gram positive organisms. The group also contains *B. anthracis*, the causative agent of anthrax and is also considered a potential biological weapon. *B. cereus* is also a member of the group and is linked to occurrences of food poisoning. *B. subtilis* has been reported occasionally to cause opportunistic infections in immunocompromised patients (Oggioni *et al*. 1998), but on the whole is not considered pathogenic. Other species of *Bacillus* have important roles in industrial production of both antibiotics and enzymes (Schallmey *et al*. 2004). Examples of members of the *Bacillus* group include *B. amyloliquefaciens H*, which produces the BamH.1 restriction endonuclease commonly used in molecular biology. *B. thuringiensis* also has significant importance to the agricultural industry, producing crystal proteins known as delta endotoxins that have insecticidal action. *B. thuringiensis* is widely used as a natural method of pest control. Genes from this species have been used to develop several varieties of successful pest resistant GM crops (Tu *et al*. 2000). The group of *Bacillus* is well studied, with several members having genome sequences published (Takami *et al*. 2000; Read *et al*. 2003; Rey *et al*. 2004).

A wealth of information is available regarding *B. subtilis*, as it has been studied extensively as a model for cell differentiation and for applications in the biotechnology industry. The

genome sequence for this species was published in 1997 (Kunst *et al*. 1997). Current understanding of sporulation in Gram positive spore formers is due to studies on *B. subtilis,* the use of genetic tools allowing in depth study of the role of specific genes and proteins. *B. subtilis* is used as a model organism for Gram positive spore formers, enabling parallels to be drawn between this and other species. Much of the information known about the pathogen *B. anthracis* also comes from studies based on knowledge from the study of *B. subtilis*, highlighting its importance as model organism.

1.1.1. Spore formation

Spores are metabolically dormant, partially dehydrated structures produced to enable survival of bacteria when adverse conditions prevent vegetative growth. In more favourable conditions, spores germinate and vegetative growth can occur. The length of time that spores can lie dormant and then be revived is difficult to put an upper limit on. There are reports of spores being recovered from rocks millions of years old (Kennedy *et al*. 1994) although the potential for these spores being more recent contaminants from the environment is high in such cases. Describing accurate ages of dormant spores is difficult in such studies, but the scale of protection the spore structures give to the bacterium is highlighted. A study using soil from tree root samples stored since 1640 also demonstrated recovery of spores (Sneath 1962), which due to specific storing of the samples gives a more credible time scale to spore recovery. Spore formation is beneficial to the bacterium in terms of long term survival, but also represents a risk as there is no guarantee that the spore will ever be exposed to conditions that allow germination and regrowth.

The Bacillaceae and Clostridia diverged 2.3 billion years ago (Paredes *et al.* 2005), but produce spores via an evolutionarily conserved mechanism. These two groups are not the only bacterial spore forming genera, but the process is less well understood in other species.

In laboratory cultures of *B. subtilis*, sporulation (**Figure 1.1**) starts at the beginning of stationary phase, when nutrient depletion occurs. The process of sporulation begins with asymmetric cell division, creating a structure called the sporangium which provides a vessel for the formation of spores. This structure contains two compartments, a large mother cell and a forespore, which will eventually become the spore. A single spore is produced from each cell and contains genetic material from the cell division. The mother cell engulfs the forespore, in a process similar to phagocytosis, forming a double membrane around the forespore. In coordination with the formation of this double membrane, two external protective structures are formed. The cortex is made of peptidoglycan (Popham 2002) and assembles between the inner and outer spore membranes. At the same time as the cortex assembles, the proteinaceous coat that comprises the outermost layer of the spore accumulates (Driks *et al.* 1994). The spore structure is a series of concentric layers, first visualised using high powered electron microscopy (Bulla *et al.* 1969). At the centre is the partially dehydrated core, where most of the water is replaced by Ca^{2+}-dipicolinic acid, which contains the genetic material. The core is surrounded by the inner forespore membrane and then a germ cell wall. The next layer is the peptidoglycan cortex, which is covered by the outer forespore membrane and contributes to the resistance of the spore. The outer layers of the spore are the spore coat, and a recently identified layer known as the spore 'crust' (McKenney *et al.* 2010). In *B. subtilis*, the spore coat is made of two layers,

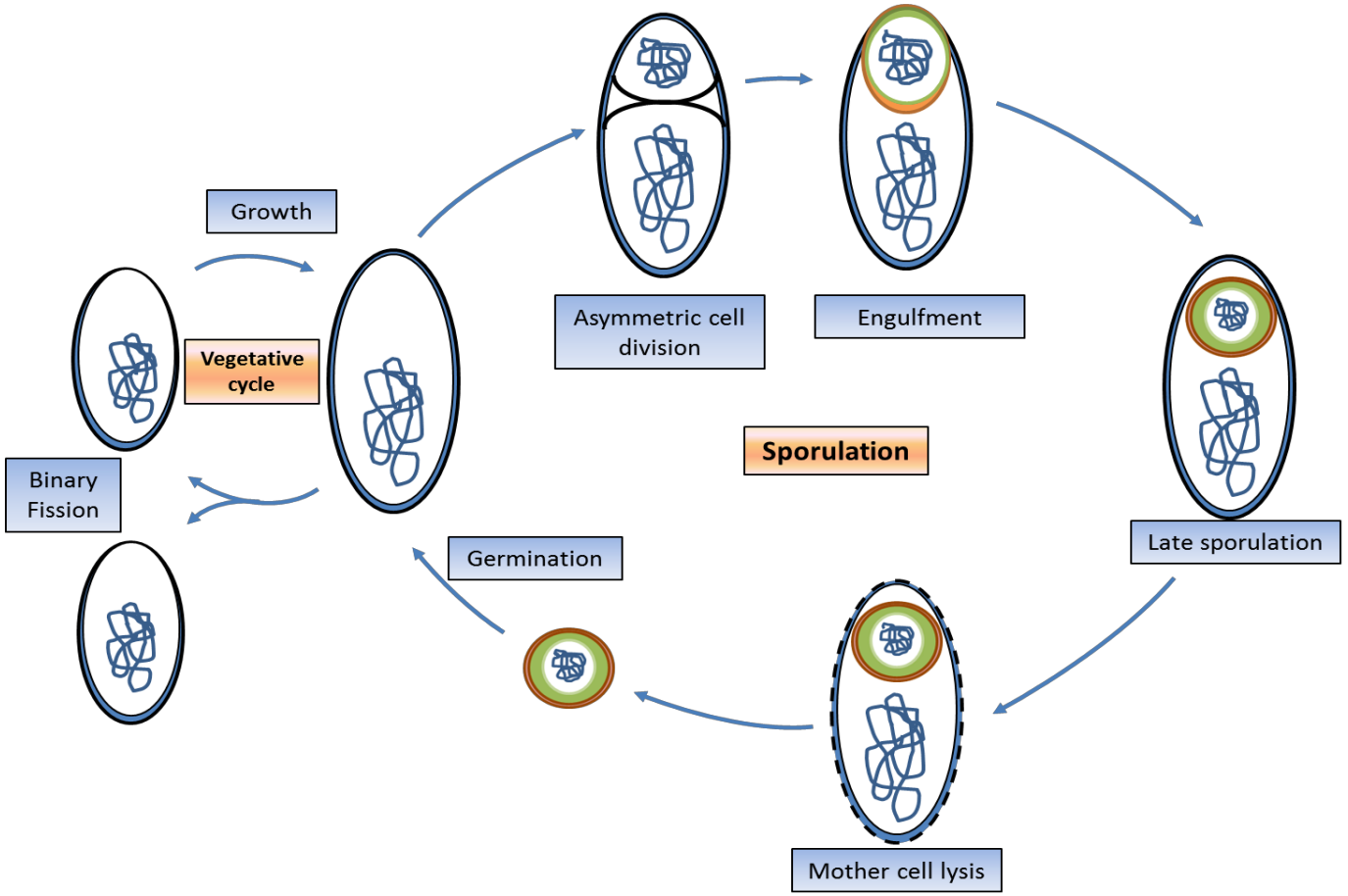


Figure 1.1. The sporulation and germination cycle of *B. subtilis*. The sporulation process begins with asymmetric cell division in a sporangium to produce two compartments: the mother cell and the forespore. The forespore is engulfed by the mother cell and following membrane fission at the opposite pole of the sporangium, a double membrane bound forespore is formed. Spore coat assembly begins after the initiation of engulfment and continues throughout sporulation. During late sporulation, the peptidoglycan cortex is assembled between the inner and outer forespore membranes, building up the layers of protection around the cortex of the spore. in the final stage, the mother cell lyses to release the mature spore. On contact with environmental signals and specific molecules, spores are capable of germinating rapidly and continuing vegetative growth cycles.
Adapted from McKenney *et al.* 2013

the lamellar-like inner coat and an electron dense coat. In some species of spore formers, a sac like layer known as the exosporium is the final layer of the structure, although this is not present in *B. subtilis*.

The spore coat can vary greatly between species, with little conservation of proteins present in the spore coat between species (Abhyankar *et al*. 2013). Spore forming bacteria inhabit a diverse range of habitats, and this is reflected by the variation in proteins forming the spore coats. The spore coat is the bacterium's only contact with the environment while in the dormant form, so this structure needs to be adapted to the specific species and the ecological niche it occupies. A generic protein coat with a similar composition in all spore formers would not provide specific interactions required for survival of the bacterium. For example, the spore coat can contain enzymes used in detoxification of the surrounding environment, or for roles in germination (Driks 2002). In *C. difficile* spores, the spore coat protein CotE has been identified as a bifunctional enzyme with peroxiredoxin and chitinase activity (Permpoonpattana *et al*. 2013), proposed to aid access to nutrients for newly germinated cells. Proteins with enzymatic activity could contribute to virulence by causing inflammatory responses within the GI tract, or aid survival by making nutrients available to vegetative cells. Variation in the content of the spore coat means that proteins from this layer can also serve as important tools in the detection of specific species of spores, especially important for spore formers that could potentially be used as bioweapons such as *B. anthracis* (Swiecki *et al.* 2006).

B. subtilis spores have the ability to resist extreme conditions, including ultraviolet (UV) radiation, chemicals, heat and other environmental stresses (Setlow 2006), with the goal of protecting the genetic material of the bacterium. Use of genetics, biochemistry studies and molecular biology has led to greater understanding of mechanisms behind such resistance

(Nicholson *et al*. 2000). Such work includes investigating the role of α/β-type small acid soluble proteins (SASPs) in protection of spore DNA and the heat resistance provided by thick layers of peptidoglycan (Henriques & Moran 2007). Survival of spores also occurs through the mammalian GI-tract, with spores displaying resistance to digestive enzymes and extremes of pH (Hoa *et al*. 2001). *B. subtilis* is generally regarded as a soil dwelling organism, although it has been shown to have a reduced metabolic level when grown at low temperatures (15°C) (Budde *et al.* 2006). This decreased activity indicates that the soil is not the primary environment that the vegetative stage of the *B. subtilis* life cycle is adapted to. The presence of spores in soil suggests that the environment acts as a reservoir for spores, but their detection in both animal and human GI tracts (Hong *et al*. 2009; Schyns *et al*. 2013) implies successful colonisation of alternative environments. The high number of spores found in the soil could accumulate over time as spores are excreted in animal faeces, rather than soil being the predominant habitat of *B. subtilis*.

1.1.2. Applications of *B. subtilis*

Extensive study of *B. subtilis* as a model organism and the resulting wealth of information now available have led to the exploitation of this bacterium.

In the Cutting laboratory, *B. subtilis* spores have been used as antigen delivery systems in vaccine design. Two methods are used for the display of antigens, one using the physical properties of the spore coat to absorb antigens and an alternative approach using genetic manipulation to express antigens fused to spore coat proteins (Mauriello *et al*. 2004; Duc *et al*. 2007). *B. subtilis* spores have been shown to have immunogenic properties (Duc *et al.* 2004; Huang *et al.* 2010) and can therefore act as an adjuvant in the vaccine formulation.

The intrinsic resistance properties of the spores make them excellent candidates for use in oral delivery. In an approach similar to use of spores as vaccines, *B. subtilis* spores are also being experimented with as drug delivery agents. A recent study successfully expressed streptavidin as a fusion protein with the spore coat protein CotB and used these recombinant spores to target cancer cells (Nguyen *et al*. 2013). The findings of this study suggest that spores of *B. subtilis* show great potential as universal drug carriers to target specific biomarkers on cells.

B. subtilis spores are commonly found in soil, often in association with the roots of a variety of plant types (Pandey & Palni, 1997; Cazorla *et al.* 2007). These interactions have been investigated, and properties of the strains involved have been exploited for use in commercial biocontrol agents (Nagórska *et al.* 2007; Ongena & Jacques 2008). Specific mechanisms behind *B. subtilis* biocontrol were studied using a model of tomato wilt caused by *Ralstonia solanacearum* (Chen *et al*. 2012, 2013). Results of these studies showed that biocontrol efficacy was strongly linked to biofilm formation. Identification of genes involved in biofilm formation allowed the generation of relevant mutations in *B. subtilis* that were deficient at biofilm formation. These mutant strains also demonstrated weak biocontrol efficacy, linking the two factors.

1.1.3. *B. subtilis* summary

The ability for simple genetic manipulation of *B. subtilis* has significantly contributed to understanding how this species functions. Increased understanding of the species has enabled exploitation of useful properties associated with *B. subtilis* such as in the biocontrol of plant pathogens and potential drug delivery agents. Parallels from studies can

also be drawn with other species that are less amiable to genetic manipulation, benefiting the study of further species.

1.2. *Clostridium difficile*

Clostridium species are a group of anaerobic, Gram positive bacteria with the ability to form endospores. They are ancient in evolutionary terms, occupying and well adapted to a range of environmental niches. Several species within this genus are well known human pathogens, whilst others have industrial applications in processes such as solvent production. *Clostridium acetobutylicum* and *C. beijerinckii* are both used in acetone, butanol and ethanol (ABE) fermentation processes (Jones & Woods 1986; Qureshi & Blaschek 2001; Lee *et al.* 2012). Use of these species contributes towards commercial production of solvents and also development of biofuel production.

Pathogenic members of this group are the causative agents of several well-known diseases. *C. botulinum* produces a neurotoxin that is one of the most lethal naturally occurring toxins. Production of toxins by this species causes botulism, a paralytic disease that can be fatal in humans. Disease occurrence is rare, but has severe economic implications as it is most commonly associated with foodborne poisoning (Cherington 1998). Toxins from *C. botulinum* are also used to decrease muscle movement for both therapeutic and cosmetic practices (Arnon 2001). *C. perfringens* produces many toxins with five toxinotypes (A, B, C, D and E) described based on the production of four major toxins (Petit *et al*. 1999). Types A and C are associated with human disease, with a range of clinical manifestations depending on infection site. For example, Type A strains produce the alpha (α) toxin and are

associated with gangrene and gastrointestinal disease. Ingested spores can cause gastrointestinal diseases and are linked with food poisoning from contaminated foods (Brynestad & Granum 2002). Wound infections also occur and the production of toxins in the wound site causes gangrene. *C. perfringens* is also a significant zoonotic pathogen, causing enterotoxaemias in a range of animals. *C. tetani* is another member of the group that mediates disease through production of toxins. Infections occur through open wounds where *C. tetani* can then proliferate and produce toxin. The neurotoxin interferes with the central nervous system (CNS) causing muscle contractions and spasms that are classic symptoms of tetanus, also known as 'Lockjaw' (Bruggemann *et al*. 2003).

Clostridial pathogens do not generally display host specificity, causing disease in both humans and animals. Preventing disease from these infections represents a key area of interest within animal husbandry and farming in order to reduce economic losses (Songer *et al.* 2006; Songer 2010).

1.2.1. *Clostridium difficile* disease

Clostridium difficile is a further example of a clostridial pathogen and will be the topic of this thesis. *C. difficile* is the primary cause of nosocomial antibiotic associated diarrhoea (AAD) in developed countries and is the etiological agent of pseudomembranous colitis. Ingested spores are able to germinate and colonise the human gastrointestinal (GI) tract, taking advantage of dysbiosis of the gut flora normally caused by antibiotic treatment to proliferate and induce a toxin mediated disease (**Figure 1.2**). Symptoms of infection range from asymptomatic colonisation to mild diarrhoea, progressing to more severe infections causing colitis, inflammation and in extreme cases, toxic megacolon (Rupnik *et al*. 2009).

Severe infections can result in death. Spores are shed in the faeces of infected individuals facilitating further spread of the disease. Infections are not just limited to humans, but also occur in animals, demonstrating that this bacterium is capable of inflicting costs far beyond the human disease burden. *C. difficile* presents a significant economic cost to the farming industry, with infections in both cattle and swine reducing output (Songer & Uzal 2005; Songer 2010)

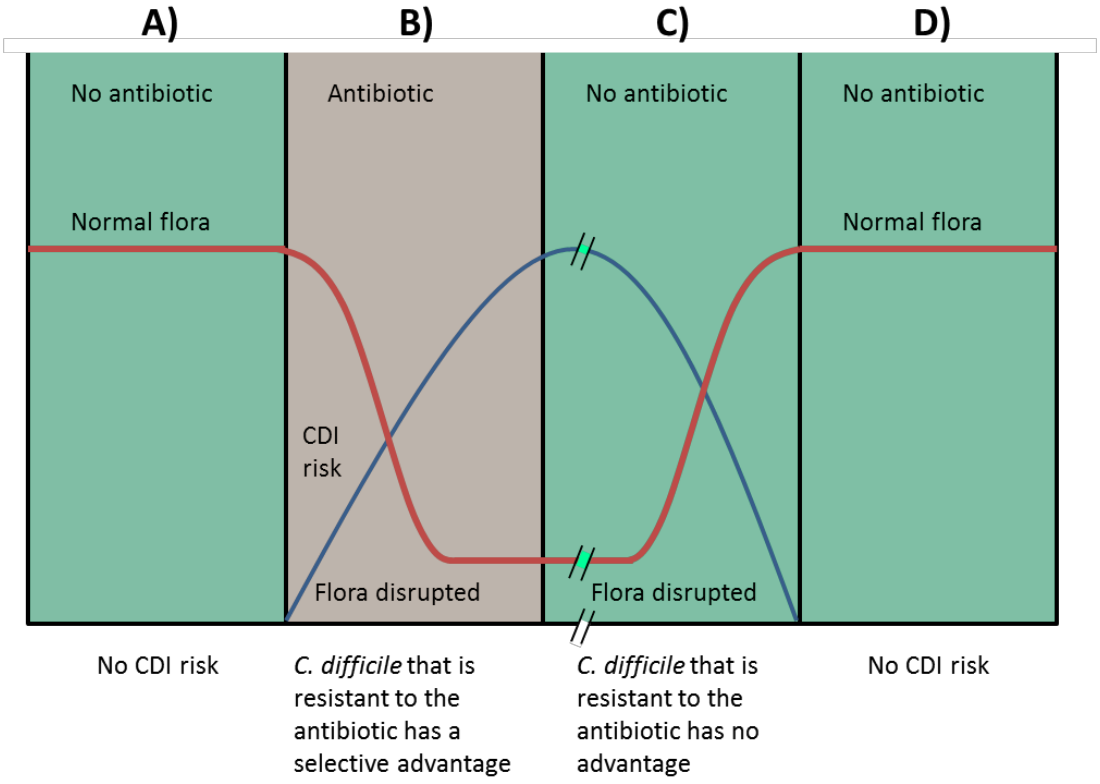


Figure 1.2. CDI in response to antibiotic treatment. The effects of antibiotic usage on the human microflora resident in the GI-tract. In the presence of antibiotics (B) the gut flora declines and *C. difficile* present in the gut that is resistant to the antibiotic can proliferate. When antibiotic treatment stops (C), the normal gut microflora recovers but a certain delay exists depending upon the antibiotic regimen used. It is at this point (C) that there is a risk of CDI, whether by resistant or susceptible strains of *C. difficile* (modified from Rupnik *et al*. 2009).

C. difficile was first reported as *Bacillus difficilis* by Hall and O'Toole (Hall & O'Toole 1935) as a component of neonatal microflora. In the 1960's a study of extra-intestinal *C. difficile* infections concluded that this species did not represent a hazard to human health, but was more likely to be an opportunistic resident of the infection sites investigated by the study (Smith & King 1962). It was not until the 1970's however, that this bacterium was associated with the gastrointestinal pathogenicity that it is well known for today. Studies found *C. difficile* to be the principle causative agent for pseudomembranous colitis and also noted it's frequent isolation in patients undergoing antibiotic treatment involving clindamycin (Tedesco *et al*. 1974; Gurwith *et al.* 1977; Lusk *et al*. 1977). A strong association was also observed between colitis and the depletion of the normal microflora during antibiotic treatment (Marr *et al.* 1975), introducing the link between antibiotic use and GI tract disorders. Information from studies in the late 1970's and early 1980's contributed to increased diagnosis and development of treatments for *C. difficile* infection. Key risk factors such as hospitalisation, old age and antibiotic use were also identified (Bartlett 2008). Observations of a correlation between the use of specific antibiotics and the prevalence of *C. difficile* infections prompted the identification of antibiotic use as a key risk factor. Clindamycin was the first antibiotic associated with risk of *C. difficile* infection (CDI), but was quickly followed by cephalosporins and broad spectrum penicillins as use of these classes of drugs increased. Wide scale use of fluoroquinolones has now been linked with occurrence of CDI, particularly with the emergence of the 027 ribotype, which displays resistance to this class of antibiotic through a mutation in the *gyrA* gene (Drudy *et al*. 2007). Increasing reliance on antibiotics and aging populations in developed countries have contributed to the current prevalence of this pathogen. In order to manage the incidence and development of this pathogen, better antimicrobial stewardship must be employed by

healthcare providers. As an example, development of fluoroquinolone resistance in *C. difficile* has been observed to be associated with several different fluoroquinolone antibiotics. This suggests that rather than switching between different drugs within a class, use of that entire class should be restricted to control development of resistance (Drudy *et al*. 2007). Strict measures such as this are necessary as rates of antibiotic resistance increase.

The predominance of this nosocomial pathogen has made it the subject of popular media and news, not just the focus of scientific reporting. In 2007, mandatory reporting of cases was introduced, and the Health Protection Authority (HPA, now Public Heath England, PHE) has collated data on number of instances of infection since that point. In 2007, there were over 50,000 cases reported (HPA) (**Figure 1.3A**). Numbers of cases have declined since, due to increased awareness and implementation of better cleaning and disinfection regimens. However, reduction in the number of cases is now plateauing, as new treatments are required to achieve superior rates for eradication of infection. In addition to the health burden caused by this infection, monetary cost to the healthcare industry is estimated at over $1 billion in the USA annually (Kyne *et al*. 2002). This cost is due to additional treatment and prolonged hospital stays required once patients develop the infection. Implementation of disinfection and isolation regimens is also costly. Traditionally, *C. difficile* is associated with nosocomial infection, spread between patients in hospitals and healthcare centres. However, recently there has been an increasing trend in community associated infections (CAIs) (Wilcox *et al.* 2008; Khanna *et al.* 2012), with individuals who have no recent history of hospital stays or antibiotic usage developing infections. This is reflected in epidemiology data collected by the HPA, showing a decrease in the proportion of cases that are attributed to specific NHS Trust hospitals, implying that the disease is

becoming increasingly prevalent in the community (**Figure 1.3B**). This change in epidemiology highlights the need for further study and new treatments.

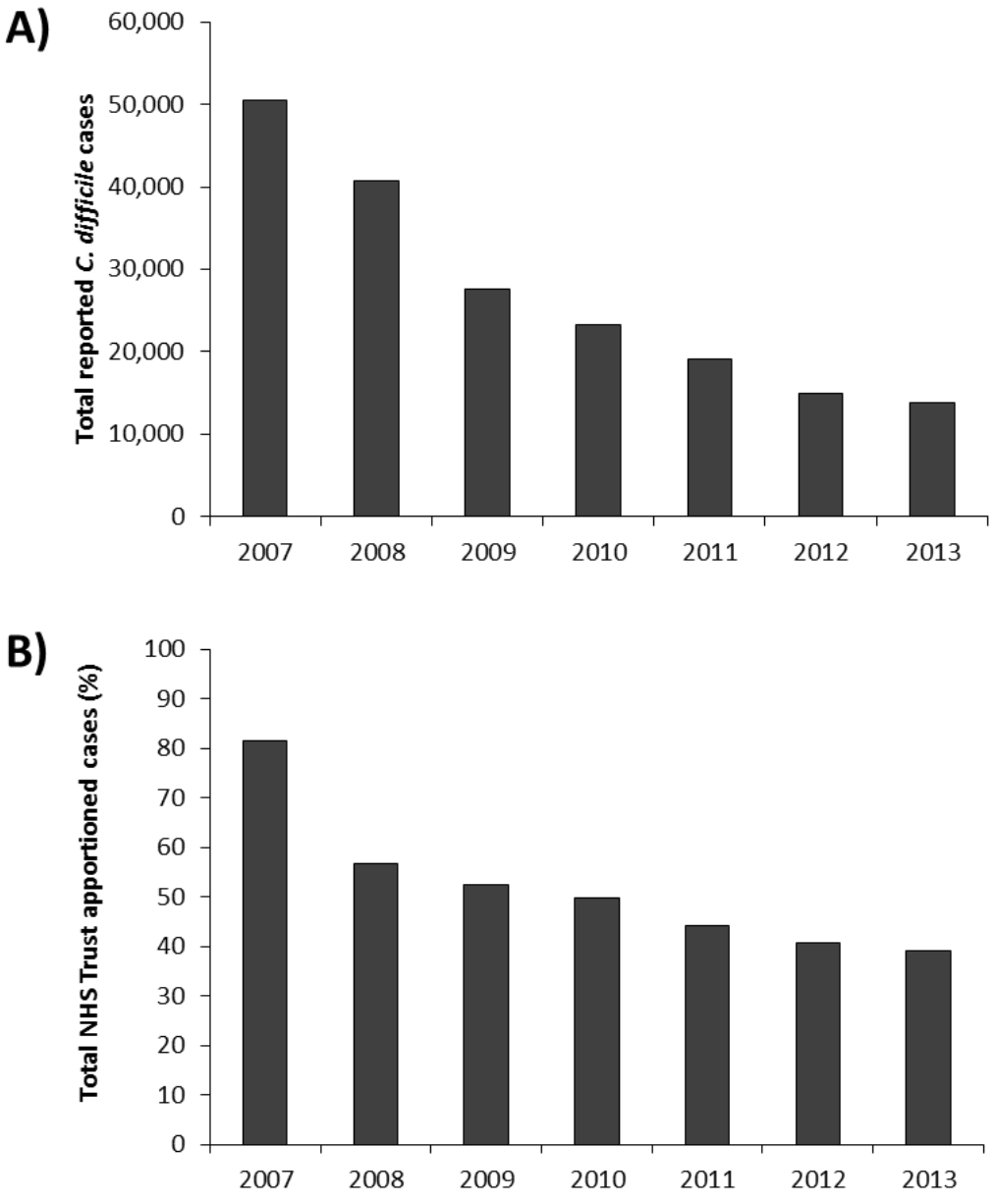


Figure 1.3. HPA *C. difficile* epidemiological data from the mandatory reporting scheme introduced in 2007. A) Total reported cases of CDI over the last 5 years. B) % of total reported cases that are categorised specifically as Trust apportioned. 'Trust apportioned' is defined as a positive case of *C. difficile* infection in a patient at an Acute NHS Trust where the infection occurs on the fourth day or later post admission.

Managing *C. difficile* infection is a difficult task, especially given the persistent and resistant nature of the spores produced by this bacterium. Colonisation with *C. difficile* can result in asymptomatic carriage, rates of which appear to depend on the population being studied. Screening of long term care patients found 51% of subjects were asymptomatically colonised with *C. difficile* (Riggs *et al*. 2007), with 37% of the isolates identified as epidemic strains. In a different population, screening of healthy adults over a nine month period showed transient colonisation also occurred in this group, with persistent colonisation by the same PCR ribotype found only in several individuals (Ozaki *et al*. 2004). While detectable colonisation will vary depending on the health status of the host, it has been reported that around 60% of the population has antibodies present against *C. difficile* toxins (Viscidi *et al*. 1983). This figure supports the hypothesis that a larger number of the population experiences some level of transient colonisation. Alternatively, the presence of antibodies could be a legacy of exposure to *C. difficile* in infancy, with much higher colonisation rates reported in children under 2 years old (Brazier 1998). Asymptomatic carriers of *C. difficile* act as potential reservoirs of infection, enabling the pathogen to spread to further hosts. Shedding of *C. difficile* spores also occurs in patients that have recovered from infection, even after resolution of symptoms. Spores shed by individuals can be transmitted by direct contact, but evidence also suggests that spores can be aerosolised (Best *et al.* 2010), increasing potential for wider dissemination and spread of spores into the environment.

Additionally, farm animals have been reported to be a potential reservoir of infection, further increasing potential for exposure to this pathogen. Investigation into prevalence of particular ribotypes (Rodriguez-Palacios *et al*. 2006) and toxinotypes (Jhung *et al*. 2008)

found that similar strains of *C. difficile* are present in both human and animal infections, with clades showing no specificity to a particular host (Stabler *et al*. 2006).

1.2.2. Virulence factors

C. difficile produces two main toxins; TcdA and TcdB, which belong to the large clostridial cytotoxin (LCT) family. Other toxins in this family include *C. sordellii* haemorrhagic and lethal toxins as well as *C. novyi* alpha toxin (Voth & Ballard 2005). Toxins of the LCT family display a number of common characteristics including high molecular weight and similar protein domain structure (von Eichel-Streiber *et al.* 1996). Domains of the toxins include an amino terminal enzymatic domain, a central hydrophobic region and a carboxy terminal domain with carbohydrate recognition sequence repeats. TcdA and TcdB fulfil the high molecular weight requirement, being 308kDa and 270kDa respectively. These toxins are encoded by a region of the genome known as the pathogenicity locus (PaLoc), a 19.6kb section that contains five open reading frames (**Figure 1.4**). TcdA and TcdB are expressed in late log and stationary phases of growth (Hundsberger *et al*. 1997). A distinct homology exists between the two toxins, suggesting that they may have been the subject of a duplication event. Like other members of the LCT family, TcdA and TcdB are organised as modular domains, each domain specific to a function (Davies *et al.* 2011) (**Figure 1.4A**). The N-terminus is enzymatic, the glucosyltransferase domain. This domain is the section translocated into host cells causing damage through intracellular processes. The hydrophobic domain in the centre of the toxin structure is thought to be involved in translocation of the N terminus, but this mechanism is not well understood. The C terminus contains polypeptide repeats that facilitate binding to the target host cells.

The two *C. difficile* toxins behave similarly to others in the LCT family, causing damage by modulation of cell physiology and altering the host environment (Aronoff 2013). The host environment is altered by targeting the Ras superfamily of small GTPases for modification by glycosylation. This action inactivates these small regulatory proteins, causing the disruption of signalling within the target cell. In seminal studies by Just and colleagues in the mid 1990's, the mechanistic action of these toxins was described (Just *et al*. 1995*a*; Just *et al*. 1995*b*). These studies demonstrated the ability of TcdB to glycosylate RhoA via transfer of a sugar moiety to Thr-37 of the GTPase, using UDP-glucose as a substrate. Rac and Cdc42 were also shown to be glycosylated by TcdB. Rho proteins are primary regulators of the actin cytoskeleton and are found ubiquitously in mammalian cells (Hall, 1990). Inactivation of these proteins within a cell leads to actin condensation, cell rounding and membrane blebbing, finally leading to apoptosis and cell death. Effects of TcdA include neutrophil infiltration (Castagliuolo *et al*. 1998), cytokine and chemokine production (Pothoulakis & Lamont 2001). TcdA and TcdB both contribute to disruption of tight junctions and apoptosis (Hecht *et al*. 1988). One or more of these effects leads to fluid accumulation within the host environment and inflammatory responses, which causes the signature diarrhoea and inflammatory symptoms of CDI.

In order to affect damage, the toxins need to be able to gain access to the cells, and a potential receptor has been described for TcdA. Galβ1-4GlcNac has been reported to enable translocation of TcdA (Tucker & Wilkins 1991), although it should be noted that this receptor is not present in all cells that TcdA has cytotoxic effects in. TcdB has toxic effects on a broad range of cells, but as yet no receptor has been described, suggesting a ubiquitous receptor for this toxin.

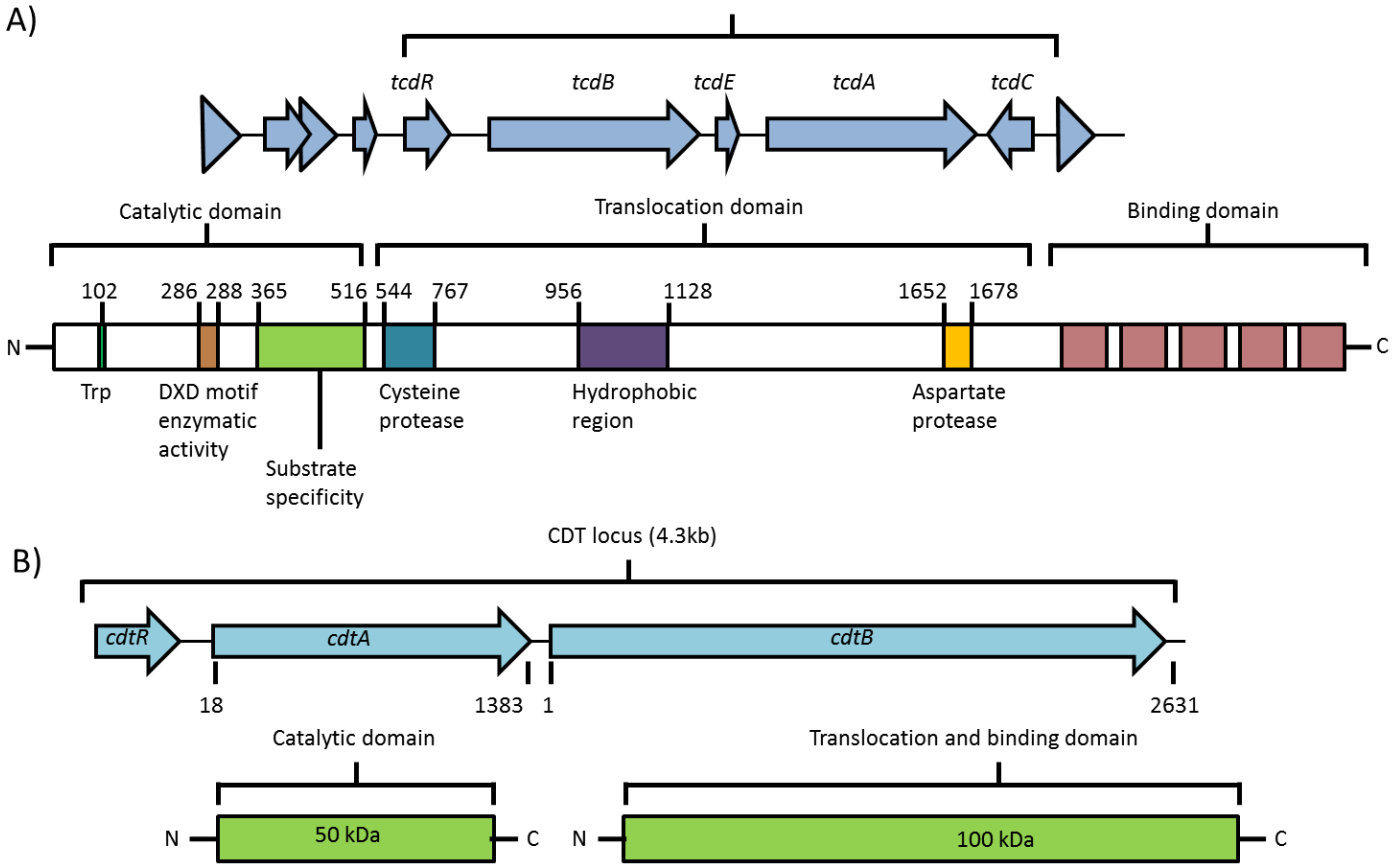


Figure 1.4. *C. difficile* toxins A) The PaLoc chromosomal locus encodes toxin A (308 kDa) and toxin B (269 kDa) in pathogenic (ToxA+ ToxB+) strains. The PaLoc comprises five genes and in non-toxigenic strains this region is replaced by a short 115 bp sequence. Both toxins are single-chain proteins with distinct functional domains and motifs. TcdB is shown in detail below the PaLoc with catalytic, translocation and binding domains indicated. B) A separate region of the chromosome codes for a third toxin, the binary toxin or CDT. This CdtLoc comprises three genes, with the binary toxin composed of two unlinked proteins, CdtB and CdtA. CdtB has a binding function and CdtA is the enzymatic component. (Adapted from Rupnik *et al.* 2009)

The importance of the two toxins has been the cause of some debate. $TcdA^-$ $TcdB^+$ strains have been isolated from clinical cases (Drudy *et al*. 2007), but no naturally occurring $TcdA^+$ $TcdB^-$ strains have been reported. This suggests that TcdA is not necessary for causing the disease, although this does not rule out its importance. Development of genetic tools has allowed further study of the toxins, through generation of strains with disrupted toxin genes (Lyras *et al.* 2009; Kuehne *et al*. 2010). These studies showed that fatal disease could be induced in a hamster model of infection with strains producing just one of the toxins. Complexity of toxin involvement in disease is highlighted by a recent study (Buckley *et al*. 2013) in which damage to the intestinal tract of hamsters was seen after infection with a non-toxigenic strain. Clearly, this is a complex disease with multiple mechanisms for causing damage to the host that are still to be fully understood.

As mentioned above, the PaLoc has five ORFs (**Figure 1.4A**), only two of which have been addressed. The remaining reading frames encode proteins that have regulatory roles. TcdC acts as a modulator in toxin production, and deletions in this gene have been identified in 027 strains (Spigaglia & Mastrantonio 2002), suggesting that regulation of toxin production is altered in 'hypervirulent' strains. TcdR (also named as TcdD) is the activator for toxin production. TcdE bears structural resemblance to holins, so has a predicted role in release of toxin from the bacterial cell. A comparison of toxin production between different ribotypes found increased expression of TcdE in 027 strains (Vohra & Poxton 2011). This suggests a role in increased release of toxin from cells, linking to reported increased toxin production in the 027 ribotype (Warny *et al*. 2005). It should be noted that not all studies have found an increase in toxin production in the 027 ribotype, so variation is likely to exist even within ribotypes (Merrigan *et al*. 2010).

The presence of the binary ADP-ribosyltransferase toxin, CDT (*Clostridium difficile* transferase) (Popoff *et al.* 1988), has also been reported in 027 ribotype strains. This is not encoded within the PaLoc (**Figure 1.4B**). The binary actin-ADP-ribosylating family of toxins are produced by pathogenic members of the Clostridia and Bacillus, including the *C. perfringens* iota toxin and the *B. cereus* vegetative insecticidal proteins. The CDT toxin from *C. difficile* acts on microtubules, causing disruption of the actin cytoskeleton resulting in microtubule based protrusions in epithelial cells (Schwan *et al*. 2009). It is proposed that these protrusions aid adherence of *C. difficile* in the GI tract, enabling the toxin to have a two-fold contribution to pathogenicity, facilitating increased damage to tissues lining the GI tract and allowing increased persistence of the infection.

The toxins of *C. difficile* are the predominant virulence factors in *C. difficile* disease, but other factors also contribute to pathogenicity. Various proteins associated with the vegetative cell act as accessory virulence factors, facilitating the initial colonisation phase of infection through interactions with host tissues. Adhesins, including surface layer proteins (SLPs), have been shown to bind to gastrointestinal tissues (Calabi *et al.* 2002) and also generate immune responses (Wright *et al.* 2008). Specifically, proteins such as cell surface protein Cwp66, flagellal proteins FliC and FliD and fibronectin binding protein Fbp68 have all been defined as having adhesin-like properties. Adhesins enable binding interactions between the *C. difficile* cell and epithelial cells in the host (Péchiné *et al*. 2005). Proteolytic enzymes such as the cysteine protease Cwp84 have also been identified as having roles in initial colonisation of the infection (Denève *et al.* 2009; Bien *et al.* 2013).

Spores of *C. difficile* also contribute to the spread and persistence of disease. ClosTron technology (Heap *et al.* 2007) has been used to inactivate the *spo0A* gene which acts as a transcriptional master regulator in the sporulation process of both *Bacillus* and *Clostridium*

species (Deakin *et al*. 2012). With this gene inactivated spores were not formed, which severely affected the ability of the strain to persist in infection and spread between individuals. Spore coat proteins should also be considered as potential virulence factors, as specific proteins enable interaction of the spore with its environment. Analysis of the spore coat has identified 54 proteins (Abhyankar *et al*. 2013) that make up the *C. difficile* spore coat. The protein make-up of spore coats varies between species, as it is adapted for specific functions. Study of specific spore coat proteins in *C. difficile* has demonstrated enzymatic properties present on the spore, suggesting a contribution to inflammatory responses during infection (Permpoonpattana *et al*. 2013). Further interest in spore proteins has led to an interest in the exosporial BclA protein (Pizarro-Guajardo *et al*. 2013), previously identified as a key protein in the *B. anthracis* exosporium. Studies identified the BclA protein as an immunodominant component of the exosporium (Steichen *et al.* 2003; Brahmbhatt *et al*. 2007*a*). While this protein is not required for virulence in *B. anthracis*, it does have some involvement in binding interactions (Bozue *et al*. 2007*a*). Comparisons drawn between the two spore forming species make this a potentially important protein in *C. difficile* interactions.

1.2.3. Hypervirulence

The appearance of the 027 ribotype led to an observed increase in severity of associated infections (Pépin *et al*. 2004), resulting in use of the term 'hypervirulent strains' to describe strains causing outbreaks in hospitals. There has been some debate about the definition of this term and the properties and characteristics of a strain that contribute to an increase in virulence. Many studies have generalised the 027 ribotype as presenting similar properties,

but variation exists within ribotypes, so specific properties of 'hypervirulence' are difficult to define. Separate studies have shown 027 strains to produce higher levels of toxin (Vohra & Poxton 2011) and also have a higher sporulation rate (Merrigan *et al*. 2010) both of which could contribute to an increase in virulence and incidence. Using a large number of isolates to assess sporulation rates however, a further study has demonstrated that differences exist within the 027 ribotype (Burns *et al.* 2011). This highlights the case for not attributing general characteristics to certain ribotypes.

Detailed study of 027 ribotype *C. difficile* strains has led to variations in toxin structure being identified. 027 strains, which belong to toxinotype III, show variation in the 3' end of the TcdB sequence in comparison to the reference sequence in toxinotype 0. This variation has been implicated in increasing severity by altering the binding ability of the toxin and potentiating cytotoxic ability (Denève *et al*. 2009). TcdB from 'hypervirulent' strains has also been demonstrated to have increased toxicity based on the ability to enter cells more rapidly than non 'hypervirulent' toxins (Lanis *et al.* 2010). Genetic analysis of the PaLoc has shown that 027 strains can be characterised and detected by a specific mutation in the *tcdC* gene (Wolff *et al.* 2009). An in frame, non-specific, 18bp deletion and a specific point mutation at position 117 of the PaLoc results in a frame shift mutation. This results in a truncated, inactive TcdC protein in 027 strains. TcdC is a negative regulator of toxin production in *C. difficile* (Dupuy *et al.* 2008) therefore an inactive version of this protein could contribute to the reported increase in toxin production from 027 strains (Curry *et al*. 2007). Mutations in other genes may also contribute to hypervirulence. Mutations have been identified in the *gyrA* and *gyrB* genes of 027 strains that result in antibiotic resistance against fluoroquinolones and erythromycin. Increased antibiotic resistance increases the

risk factor associated with antibiotic usage, giving the 027 strains a significant advantage in causing outbreaks of disease.

1.2.4. Analysis of strains and genetic tools

Molecular biology provides several methods for identifying and distinguishing between strains of *C. difficile* (Janezic & Rupnik 2010), including PCR ribotyping, restriction endonuclease analysis (REA) and pulsed field gel electrophoresis (PFGE). These methods work on the principle of creating a genetic profile or fingerprint of a strain. This allows for grouping of isolates with varying degrees of sensitivity. Strains involved in outbreaks can then be identified and spread of particular groups can be tracked. Use of microarray data has also contributed to understanding of the pathogen through increasing the power of genetic studies (Stabler *et al*. 2006).

The toxins of *C. difficile* serve not just as virulence factors but also provide a means of detection and typing of strains. Positive cases of CDI are confirmed by the presence of toxins through a cell culture rounding assay and culture of *C. difficile* from a stool sample. These methods represent the gold standard for detection and confirmation of infection. ELISA detection kits are used more frequently for rapid detection although this results in a compromise between speed and reliability. Typing of strains is achieved by genetic analysis of the PaLoc, analysing variations that occur within this sequence. A reference strain, VPI10463, is classed as toxinotype 0 and strains displaying variability from this are grouped into toxinotypes denoted I to XXVII. 27 toxinotypes have been described at present (Rupnik *et al.* 1998; Rupnik 2010).

The advent of a method of genetic manipulation of the Clostridia has greatly improved the ability to work with and understand *C. difficile* (Heap *et al*. 2007). ClosTron is a method of creating stable mutations in the genome of Clostridium species. The system uses group II intron based technology to allow knockouts of specific genes to be created, broadening the scope of research possible with this group of bacteria. *B. subtilis* is used as a model organism for Gram positive spore formers, due to the ease with which genetic studies could be conducted. This has led to a wealth of information being available on the species which can be applied to similar organisms. The use of genetic tools could potentially achieve the same for the study of *C. difficile*, with the information all the more valuable given its standing as a human pathogen.

1.3. *C. difficile* as a human pathogen

C. difficile infection can present at a variety of clinical levels, ranging in severity from mild diarrhoea to serious inflammatory conditions such as pseudomembranous colitis. The most severe cases can result in toxic megacolon and perforation of the gut, which can lead to fatalities. Data collated by the Office for National Statistics (ONS) shows that *C. difficile* was listed as a cause or contribution to death in 0.8% of all hospital deaths between 2010 and 2012 (ONS 2013), with 1646 deaths in 2012 involving *C. difficile* infection.

The disease caused by *C. difficile* is primarily mediated by the release of toxins from vegetative cells that are able to proliferate in the gut following disruption of the microflora. As this organism is a strict anaerobe, transmission of disease is facilitated by the spread of highly resistant spores, which generally enter the body through ingestion and then can

reside in the GI tract. Bile salts secreted by the liver pass through the GI-tract and are reabsorbed in transit. Levels of the bile salt taurocholate are optimum to serve as germination agents for the spores in the colon, allowing germination, outgrowth and proliferation of *C. difficile* (Sorg & Sonenshein 2008). The bile salt chenodeoxycholate acts as a germination inhibitor (Sorg & Sonenshein 2009), but is only present in low levels in the colon. Studies using 027 strains have also suggested that the presence of antibiotics in the gut could promote germination of spores (Saxton *et al.* 2009).

Various risk factors have been identified in the study of *C. difficile*, with the clearest correlations for disease shown between old age and antibiotic usage (Carroll & Bartlett 2011). The elderly are more at risk from infection for a number of reasons; immunosenescence means a reduced ability to mount an effective immune response, and advanced age also leads to changes in the microflora. Use of treatments such as antibiotics and proton pump inhibitors (PPIs) affect the microflora of the gut in patients, creating niches that allow colonisation of *C. difficile* (Garey *et al.* 2008; Loo *et al.* 2011). Immunosuppressant treatment and other under-lying conditions also contribute to increased susceptibility to *C. difficile* infection. There is also a suggestion of genetic susceptibility to the disease, with the identification of a polymorphism in the human IL-8 gene that renders individuals more susceptible to infection (Jiang *et al*. 2006). With the evolution of virulence in strains, prevalence of *C. difficile* in groups previously thought to be at low risk is increasing. This includes an increase in cases of CDI in peripartum women, a group that previously had a low association with *C. difficile* (Garey *et al.* 2008). Cases of community associated infections are also increasing (Wilcox *et al.* 2008; Chitnis *et al.* 2013; Heslop *et al.* 2013), creating a more complex field of disease for monitoring.

Adding to the complexity of this disease, around 20% of CDI patients will experience a relapse of infection (Barbut *et al*. 2000; Johnson 2009). Recurrent infections are attributed to either residual spores in the GI tract, reinfection from original source, or infection with a new strain. Higher relapse rates have been reported for infections involving 027 strains (Figueroa *et al*. 2012). Once patients have experienced a relapse of infection, continued recurrent disease is much more likely to occur. Recurrent episodes of disease tend to occur in those who have mounted ineffective immune responses during in primary infection (Garey *et al.* 2008). Failure of the gut microflora to recolonise post antibiotic treatment or illness is also a common factor in relapse. The costs of relapsing infection are not only financial but are also substantial in terms of personal health. Preventing recurrent infection is a key issue in the control of *C. difficile*.

1.3.1. Transmission of infection

Current control methods for preventing the spread of *C. difficile* are based on transmission occurring between infected patients, normally on hospital wards. Infected individuals are isolated and strict cleaning regimens are implemented to avoid further spread of infection (Vonberg *et al*. 2008). A key issue related to the control of *C. difficile* is the highly resistant spores that remain in the environment, and can be transmitted on inanimate objects or even by airborne transmission (Best *et al*. 2010). However, genetic tools and molecular typing systems are now being used to track the spread of individual infections. In a hospital based study, cases of symptomatic *C. difficile* infection were analysed using molecular typing. Using these data, it was shown that around 20% of cases in hospitals have obvious sources within the hospital itself, a figure much lower than expected. The study did not

screen all patients and visitors, so does not account for asymptomatic carriers being sources of infection within hospitals. Data from the study also suggests that a minority of symptomatic patients (<10%) could remain infectious for up to 8 weeks, far beyond the infectious period anticipated by control measures employed in hospitals. This study could be improved by screening asymptomatic patients for potential sources of infection, but it does address an important issue for *C. difficile* infection in that not all cases can be accounted for by transmission from an obviously infectious source. A more recent study also addressing the issue of ward based transmission found that isolates from 45% of those cases studied were genetically diverse from existing cases in a hospital (Eyre *et al*. 2013). This highlights how *C. difficile* infection is not just transmitted patient to patient, but infection can come from diverse sources. The complex epidemiology of CDI means that control measures need to be extended beyond the hospital setting. Nosocomial infections have been reduced by increasing awareness of *C. difficile* so it is plausible that increasing awareness outside of the hospital setting could aid control.

Use of genomics to track the spread of *C. difficile* strains (Stabler *et al*. 2006) shows how easily infections and virulent strains can spread in our 'hyper-connected' world. Global transmission highlights the need for widely used control measures, for example, improved antimicrobial stewardship should be global, not simply a localised effort.

1.3.2. Epidemiology

Classically, *C. difficile* is associated with nosocomial infection and is limited to vulnerable individuals who are exposed to risk factors for the infection. However, the prevalence of community associated (CA) *C. difficile* infection is increasing and reported cases of

community acquired infections are increasing in severity (Heslop *et al*. 2013). Warny and colleagues (Warny *et al*. 2005) have also reported isolating strains of the 'hypervirulent' 027 ribotype from community associated cases. Studies on CDI tend to focus on hospital cases and populations, so a bias exists in location reports of CDI. More recent studies are starting to address this imbalance in information however. In an epidemiological study (Khanna *et al*. 2012), a cohort population displayed a significant number of cases (41%) occurring in the community, rather than being associated with a hospital stay. These cases occurred in a younger age group than normally associated with nosocomial *C. difficile* and were generally less severe. This implies that the burden of disease caused by this pathogen is likely to be underestimated, although more severe cases remain associated with hospital infections. Attention needs to be focused on the reservoir of infection in the community that has potential to increase in severity, just as has occurred with hospital associated cases of infection.

It is difficult at some points to distinguish between community associated and health care associated infections, especially when this requires investigating the medical history of patients for exposure to risk factors for *C. difficile* infection. Reports on the risk of community acquired CDI state that it is an issue to be considered alongside hospital acquired cases (Wilcox *et al.* 2008; Brown *et al.* 2013; Chitnis *et al.* 2013). The increased geographical distribution of cases makes monitoring the overall occurrence of disease more complex, but such monitoring would enable better awareness of where the risk of CDI is greatest. This information would allow more comprehensive control measures to be implemented, not just in hospitals.

1.4. Treatment of *Clostridium difficile* infection

1.4.1. Antibiotics

Antibiotic use is a key risk factor for CDI but despite this, antibiotics are still the main treatment for this infection. Vancomycin and metronidazole are the two main antibiotics used in treatment. However, approximately 20% of initial cases will experience a relapse following this treatment (Johnson 2009), highlighting the need for more effective antibiotic agents or alternative treatments. Fidaxomycin is a recently developed antibiotic that is reported to have increased efficacy against *C. difficile* (Goldstein *et al.* 2012). It is a narrow spectrum antibiotic, having little effect against other groups in the microflora such as Gram negative aerobes, anaerobes and yeasts so causes less disruption to the microflora than broader spectrum antibiotics. This could be a factor that contributes to the lower relapse rate reported with use of fidaxomycin compared to vancomycin (Louie *et al*. 2011). Reducing relapse rate is an important step in the treatment of CDI, but it is dangerous to rely on a single antibiotic. Further development of new antibiotics is required in order to avoid issues with antibiotic resistance developing. This is especially relevant with *C. difficile* where increased virulence has been associated with development of resistance to antibiotics.

1.4.2. Toxin binding

A diverse range of alternative treatments have been investigated in an attempt to reduce reliance on antibiotics. One such treatment is the development of 'Tolevamer', a novel toxin binding polymer. This works on the basis of neutralising toxins by binding to them in

order to prevent toxin mediated damage to the host in disease (Braunlin *et al.* 2004; Hinkson *et al.* 2008). When tested in a human gut model however, the polymer did not perform well (Baines *et al.* 2009), suggesting that this method would need to be refined for successful use in a clinical setting.

1.4.3. Probiotics

Antibiotic use is key risk factor in development of CDI as it can cause dysbiosis of the gut microflora. Several treatment strategies address the depletion of the microflora as a factor in disease. An example of this is the use of probiotics; studies have been carried out using various species and strains of bacteria to assess the potential for treatment of *C. difficile* with some success (McFarland 2006; Islam *et al*. 2012). The current state of available information contributes to a lack of clear conclusions regarding probiotics as a potential treatment. Studies are often carried out using small sample sizes or are unclear as to the regimen used in treatment, making results difficult to replicate. As probiotic effects are likely to be species or even strain specific, clear identification of the probiotic agent is also a necessity. Improved design and communication of results is needed if probiotics are going to be considered seriously, especially when doubts exist within the scientific community (Miller 2009).

1.4.4. Faecal therapy

Building on the idea of probiotics supplementing the gut flora leads to one of the most successful alternative treatments to antibiotics. Faecal transplants use stool samples from a

healthy 'donor' which are then implanted into the colon of a *C. difficile* patient. The microbiota in the donor stool recolonises the gut and enables clearance of the infection, restoring the gut environment to a normal state This treatment is reported to have excellent efficacy in resolving infection (Gough *et al.* 2011), especially in cases where relapse of disease has been a significant issue. This treatment is now being refined, with the development of 'synthetic stools'. This involves identifying and isolating key species within the faecal microflora and using these to create a treatment that delivers a restorative microflora to the infected gut. This eliminates the risk of transplanting further pathogens into the patient and results in a more attractive treatment option (Lawley *et al*. 2012; Petrof *et al*. 2013).

Increasing knowledge regarding how *C. difficile* causes and behaves during progression of disease is useful in the design of new treatments. For example, a recent paper reported the identification of a germination receptor that interacts with bile salts present in the gut to initiate germination (Francis *et al.* 2013). This receptor could be used in drug design in order to prevent germination of ingested spores, eliminating the threat of disease, or as a potential antigen in vaccine design.

1.4.5. Vaccines

There is as yet no available vaccine for prevention of *C. difficile* infection. Development of a preventative vaccine would reduce the use of antibiotics in treatment of this infection, addressing a key issue in finding alternative treatments. The majority of potential *C. difficile* vaccines use parenteral delivery of toxin based antigens to generate systemic immune responses against *C. difficile* toxins. This method produces antibodies that have neutralising

activity against the toxins produced in disease, so protects against the damage caused during infection. The correlation between antibody response against toxins and protection from disease is well established (Torres *et al.* 1995). This response can be achieved by immunising subjects with inactivated toxins, or toxin fragments. Several studies have used chimeric proteins which use fusions of toxin A and toxin B in vaccine formulations (Tian *et al*. 2012; Wang *et al*. 2012). Sanofi Pasteur currently is the most advanced company in trials for a candidate vaccine, having announced entry into Phase III of testing for their vaccine candidate in the summer of 2013. This vaccine consists of formalin inactivated toxins A and B from *C. difficile* reference strain VPI 10463, delivered in an intra-muscular (IM) injection (Alkan *et al*. 2012).

Vaccine strategies focus on production of systemic responses to *C. difficile*. However it is possible that alternative approaches need to be considered. Study of interactions between *C. difficile* and the host has indicated the involvement of a mucosal response to infection (Johnson *et al.* 1992), which may represent an alternative option for vaccination strategies. Previous studies have addressed the use of an alternative route for immunisations, including use of cellular antigens delivered mucosally in an attempt to prevent colonisation of *C. difficile* (Pechine *et al*. 2007). The development of a *B. subtilis* spore based vaccine for CDI was based around mucosal delivery of toxin antigens (Permpoonpattana *et al*. 2011*a*). This approach used oral immunisation of spores expressing a toxin A antigen to produce protection against *C. difficile* infection in the hamster model. The route of dosing and use of the bacterial spore effectively as an adjuvant for the vaccine are important factors in this study. The delivery strategy of an antigen can alter the patterns of immune response produced (Lima *et al.* 2004) so the choice of adjuvant and delivery strategy can be vital to identifying a successful candidate vaccine. In the generation of mucosal responses, an

entity that interacts with the innate immune system to boost responses could be important (Kaisho & Akira 2002; Rhee *et al.* 2012; Woodrow *et al.* 2012). *B. subtilis* spores have been shown to interact with aspects of the innate immune system (Huang *et al*. 2010) and results from use of the *B. subtilis* vaccine show that it is capable of inducing both cellular and humoral responses to support protection against symptoms (Permpoonpattana *et al*. 2011*a*). Manipulation of multiple aspects of the immune system to evoke a more complete response is presented as an avenue worth investigation. Understanding the immune response required for protection will aid the development of more efficacious vaccines. For example, a connection between the mounting of an initial immune response to infection and protection from relapse has been established (Kink & Williams 1998). Analysis of immune responses in relapsing and non-relapsing patients could provide important information about what is required to protect against an initial infection.

1.4.6. Passive immunotherapy

Passive immunotherapy involves a similar idea to vaccination for treatment of *C. difficile*, using antibodies against toxins directly as a treatment. This avoids relying on a vaccine to induce production of antibodies within the host to neutralise toxins (Salcedo *et al*. 1997; Wilcox 2004). High production costs of antibodies make this treatment less commercially feasible than others however.

1.4.7. *C. difficile* summary

C. difficile is a relatively new pathogen and represents a significant challenge to healthcare providers for several reasons. Firstly, control of the disease is difficult with highly resistant spores contributing to spread and persistence of infections. Secondly, the ability of the pathogen to evolve has resulted in altered virulence properties such as increased antibiotic resistances and more robust toxin production. This leaves both research and treatment options playing catch up with the pathogen. Thirdly, the epidemiology of this infection is also evolving. Previously *C. difficile* infections were associated with hospitals and elderly patients, but it is now becoming more prevalent in younger individuals and in community settings away from associations with common risk factors.

Research investigating all facets of the infection is useful to understand further how this pathogen causes disease. As more information becomes available, it can be exploited in the generation of novel treatments to aid control of this pathogen.

1.5. The Gastrointestinal tract

The gastrointestinal (GI) tract is essentially a tube, split into specialised sections enabling digestion of ingested food, to obtain nutrients required by the body and then dispose of the remaining waste. The histology throughout the digestive system varies, reflecting the differences in speciality of functional anatomy. For example, villi in the small intestine increase surface area for absorption of nutrients. Concentric layers of tissue form the gut, the mucosa being the innermost of these layers. This layer is important as it creates a barrier between the contents of the gut and the tissue layers. The mucosa is itself in three

layers; the epithelium which is responsible for most of the digestive, absorptive and secretory processes. The lamina propria provides a layer of connective tissue, and this is encased by the thin muscular layer of the muscularis mucosae. In the lumen of the GI tract, especially in the lower colonic regions, a large number of microbial inhabitants are present. Relevant to this thesis, the colon is also where *C. difficile* infection is localised to.

1.5.1. Microbiome of the gut

A commonly cited figure is that humans are 10% human cells and 90% microbial cells, demonstrating the large number of microbes that colonise the human body. The majority of these colonising microbes inhabit the gastrointestinal tract (Savage 1977). The microflora of the gut is of vast importance to human health and our ability to digest many foods. In 2007, the Human Microbiome Project was initiated with funding from the NIH. This mammoth project built on the success of the Human Genome Project, using advancing sequencing technology to screen the microbial flora of over 200 individuals (Yatsunenko *et al*. 2012). This included samples from 15 sites, including skin, mouth, nose and stools. The aim was to develop a set of reference microbial genome sequences and perform a preliminary characterisation of the human microbiome. This information would be used then as a base to understand the role of microbes from the human body in health and disease. The project confirmed that the number of microbial cells in our bodies far outnumbers the amount of human cells, by over a magnitude. The human genome contains over 22,000 protein coding genes, but the microbiome contributes a further 8 million unique protein coding genes (Human Microbiome Project Consortium, 2012; Li *et al.* 2012). The presence of the microflora provides access to a vast wealth of genes that are not

available within just the human genome. In the GI tract, the microflora aid digestion, help regulate homeostasis and synthesise vitamins and enzymes otherwise absent from the environment. The metabolic activity performed by the microflora has led to it being termed the 'forgotten organ' (O'Hara & Shanahan 2006). Much of the capability of the gut is due to its microbial residents and when the complex composition of this flora is altered, it can have significant effects.

A wealth of information is now available surrounding the composition of the gut microflora and developments in genetic tools enable huge amounts of data to be analysed. A study in 2011 proposed three main 'enterotypes' of gut flora, based on analysis of gut microbial communities, that were independent of sex, nationality, age and body mass index (BMI) (Arumugam *et al*. 2011). Despite not attributing specific factors to definitions which would describe each enterotype, this study opens up the possibility of using biomarkers from the flora to identify microbial signatures of factors such as age, or disease susceptibility. Studies comparing the composition of healthy or diseased subjects can demonstrate clear differences in the microflora of individuals. For example, microbial signatures of irritable bowel syndrome (IBS) have been identified (Saulnier *et al*. 2011). Significant differences between the flora of obese and lean individuals can also be seen (Turnbaugh *et al*. 2009) in a comparison of microflora.

The microbiota of the GI-tract is of key importance to *C. difficile* infection, as dysbiosis in this environment is a key risk factor for development of disease. In a healthy individual, with a robust microflora, *C. difficile* carriage presents little problem and is normally asymptomatic. However, when dysbiosis of the gut microflora occurs, the shift in microbial composition allows *C. difficile* to proliferate and disease mediated by production of toxins occurs. Dysbiosis is commonly caused by antibiotic treatment, but other factors can also

have a disruptive effect. The state of the microflora of an individual is therefore very important to study of CDI. As the disease is a product of dysbiosis, the obvious solution is to counter this, leading to the use of microbial therapy in treatment of the disease (Petrof *et al*. 2013). The effect of treatments on the existing microflora is also a key factor in development of new drugs. Fidaxomycin, a recently licenced antibiotic for treatment of CDI, has been successful as it reduces the rate of relapse of infection. This is due to its narrow spectrum antimicrobial action, reducing the effect on the remaining microflora and encouraging return of the microflora and the GI tract environment to a healthy state. Having identified the significant role that the microflora plays in the human body, it is important that this is taken into consideration when developing new medical treatments.

1.5.2. The Gut Associated Lymphoid Tissue (GALT)

The microbiota of the GI tract also plays a key role in development of the immune system. The intestine contains the largest mass of mucosa associated lymphoid tissue (MALT) in the body and commensal microbiota significantly contributes to the development of the gut associated lymphoid tissue (GALT). Lymphoid tissue plays a role in protecting the body from invasion by pathogens, by facilitating recognition of pathogens and initiating responses to clear infections. In animal studies, germfree mice showed low densities of lymphoid tissue (Guarner & Malagelada 2003) and whilst colonisation with specific strains of commensal flora helped reinstate expected immune responses, administration of complete normal flora is required to restore the full spectrum of immune responses (Cebra 1999). In humans, colonisation of the GI tract begins just hours after birth, with studies showing that the method of birth and subsequent feeding method affects the composition of an infant's

microflora (Grönlund *et al.* 1999; Jarvis 1996). In a study from Finland, researchers investigated if the effect linked with the method of birth was evident in the long term. The composition of microflora from seven year olds who had been delivered either naturally or by caesarean section was compared. This found that bacterial composition levels were similar between groups apart from the Clostridia*,* which were higher in the group of children that had been naturally delivered. Interestingly, all children included in the study that had a history of asthma had lower levels of Clostridia present (Salminen *et al.* 2004).

The link between microflora composition and allergy related illness is interesting. The development of the intestinal immune system is due to exposure to antigens presented through the colonisation of the GI tract. A popular theory connecting increased cleanliness with increase in allergic conditions such as eczema and asthma is known as the 'Hygiene hypothesis' (Strachan 1989). The hypothesis implies that with less exposure to microbial antigens, the immune system develops differently, with a T-cell response (T_H2) bias that is associated with allergic reactions. Several studies have provided supporting evidence to this; in the comparison of infants from Sweden and Estonia, lower levels of enterococci and bifidobacteria were found in children with allergic conditions. The differences in the composition of flora were seen from the first month of life, highlighting that colonisation from the very beginning of life is important (Björkstén *et al.* 2001). This study also noted that bifidobacteria, common in probiotic applications, are known to elicit T_H1 responses, which would counter development of allergy associated T_H2 responses. The development of the microflora from the neonatal to the adult composition is important with respect to the development of the intestinal mucosal immune system. The mature immune system should have the ability to discriminate between pathogenic microorganisms and the vast array of antigens to which it is exposed over a lifetime. Where such development has not

occurred to the proper extent, such discriminations cannot be made effectively, leading to conditions such as IBS and colitis.

The link between the microbiome and the GALT has a two-fold importance. Firstly, the development and presence of the gut microflora stimulates the development of the lymphoid tissue. This interaction from an early age contributes to development of a mature and protective immune system. Secondly, the location of the lymphoid tissue allows the microflora to interact with immune receptors in the gut, enabling maintenance of homeostasis within this system. When this regulation is disrupted, a range of effects can follow, from acute infections by gastrointestinal pathogens to chronic inflammatory conditions. When the structure or composition of the microflora is altered, so is the ability to suppress potential pathogens.

The link between the GI tract microflora and the development of a mature immune system highlights the importance of the intestinal immune system. When investigating infections that occur within this environment, interactions with the local immune tissues should be a consideration. This is especially important when developing a vaccine or treatment for such an infection. In the case of *C. difficile*, which as yet does not have a commercially available vaccine, antibiotics are relied on for control of infections. Perhaps an approach including consideration of interactions within the gut would bring a more effective treatment.

1.6. Thesis objectives

The work of this thesis centres on strategies for treatment of CDI that avoid the use of antibiotics. Part of developing new treatments requires information about how infections work and behave in order to find novel targets for treatments. Based on this, the thesis falls into two sections; Firstly, the investigation of how *B. subtilis* spores can be incorporated into alternative treatments that can be employed in the management of *C. difficile* infections. Secondly, identification of spore coat specific functions during infection, focusing particularly on the role of the BclA proteins on the *C. difficile* spore coat.

1.6.1. *B. subtilis* based treatments

A *B. subtilis* spore based vaccine candidate will be evaluated using oral and sublingual delivery routes. Activation of a mucosal immune response by this potential vaccine rather than parenteral delivery of antigens has previously been shown to be successful in preventing disease (Permpoonpattana *et al.* 2011*a*). Evaluating alternative delivery routes for this vaccine will aid understanding of the role of the mucosal response in protecting against CDI.

B. subtilis spores also display great potential for their use as probiotics, with a history of safe human consumption. The use of spores provides a treatment that is simple to produce and store, which can be delivered through simple oral doses. Spores of *B. subtilis* will be trialled as a probiotic treatment for CDI using animal models to replicate the *in vivo* human infection.

1.6.2. BclA proteins on the *C. difficile* spore coat

The spore of *C. difficile* is the infectious agent, so plays a pivotal role in development of disease. By use of strains with mutations in *bclA* genes, spores lacking these proteins can be investigated for any anomalies in infectious behaviour. Both *in vitro* and *in vivo* characteristics of the *bclA*$^-$ spores will be investigated in order to ascertain whether these proteins contribute to significant processes associated with infection.

Chapter 2
Materials and Methods

2.1. General Methods

2.1.1. *Bacillus subtilis*

General methods for working with *B. subtilis* are described elsewhere (Harwood & Cutting 1990).

2.1.2. *Clostridium difficile* strains

630 is a toxigenic (*tcdA*$^+$ *tcdB*$^+$) strain of *C. difficile* isolated from a patient with pseudomembranous colitis during an outbreak of *C. difficile* infection (CDI). For ClosTron mutagenesis and mutant analysis an erythromycin-sensitive derivative 630Δ*erm* was used (provided by N. Minton, Univ. Nottingham, UK). R20291 is an epidemic strain of ribotype 027 isolated from Stoke Mandeville Hospital in 2006 and was obtained from T. Lawley (Wellcome Trust Sanger Institute, UK). VPI10463 (provided by B. Wren, London School of Hygiene and Tropical Medicine, UK) was originally isolated in the USA in 1980 and has since been used as a reference strain.

2.1.3. Growth of *C. difficile* and preparation of spores

C. difficile was routinely grown in vegetative culture by overnight growth in TGY- medium. Spores of *C. difficile* were prepared by growth on SMC agar plates using an anaerobic incubator (Don Whitley, UK) as described previously (Permpoonpattana *et al*. 2011*b*). After growth for seven days at 37^{o}C spores were harvested from agar plates and purified using HistoDenz as follows. Crude spore suspensions were washed five times with ice-cold sterile water, then aliquots were re-suspended in 500µl of 20% HistoDenz (Sigma, MO, USA) and layered over 1ml of 50% HistoDenz in a 1.5ml tube. Tubes were centrifuged at 10,000 x g for 15 min. The spore pellet was recovered and washed three times with ice-cold sterile water. Spore purity was assessed by phase contrast microscopy and spore yields in individual preparations were estimated by counting colony forming units (CFU) of heat-treated (60°C, 20min) aliquots on BHIS agar plates (Brain heart infusion supplemented with 0.1% L-cysteine and 5 mg ml^{-1} yeast extract) supplemented with 0.1% sodium taurocholate (BHISS).

2.1.4. Toxin detection

Faecal samples were collected fresh and kept at -20^{o}C until assay. Caecal samples were treated in the same manner as faecal samples for extraction of toxins. Toxins were extracted using a protease inhibitor buffer as described previously (Permpoonpattana *et al*. 2011*a*) and detected by a capture ELISA method as follows. ELISA plates (Greiner, high binding) were coated with rabbit polyclonal antibodies against toxin A or toxin B (Meridian Life Science; 1µg ml^{-1} in PBS buffer). After blocking plates with 2% BSA (1h at 30^{o}C), samples (10µl) and reference toxin A or toxin B (AbD Serotec) were added to plates.

Incubation was made at 30°C for 3h. For detection, monoclonal antibodies (AbD Serotec) against toxin A (1/500) and toxin B (1/500) were used. Plates were incubated for 1h at 30°C. After washing, the secondary antibody, HRP-conjugated anti-mouse IgG was added with incubation at RT for 1h. Colour was developed with the TMB substrate and measurement at $OD450_{nm}$. The titres of toxin in samples were calculated against a serial dilution of commercial reference toxins A or B. The sensitivity of the assays for toxin A and B were 7ng ml^{-1} for both toxins.

2.1.5. Spore enumeration

Post treatment for toxin extraction, faecal and caecal samples were resuspended in ethanol (100%) and then incubated for 20 minutes at RT to kill all vegetative forms in samples. Samples were pelleted by centrifugation at 10,000 x g for 5 minutes and ethanol was removed from the sample. Pellets of faecal/caecal matter were resuspended in 1ml sterile H_2O, and serially diluted. Dilutions were plated on BHIS agar supplemented with 0.1% sodium taurocholate, before incubation in anaerobic conditions at 37°C for 48hrs. Colonies were counted and number of spores/g calculated based on the original weight of the sample. A detection limit of 10^2 spores/g was applied to prevent over estimation of spore content.

2.1.6. Animal studies

All animal work was carried out under Home Office Project License 70/7025.

2.2. Chapter 3 – PP108 sublingual immunisation

2.2.1. PP108 spore preparation

PP108 is derived from *B. subtilis* PY79 and expresses a *C. difficile* toxin A antigen on spore coat proteins CotB and CotC. The original construction is described elsewhere (Permpoonpattana *et al*. 2011*a*). Growth and sporulation protocols for *B. subtilis* are described in detail elsewhere (Harwood & Cutting 1990). Briefly, spores were produced as follows; single colonies grown on antibiotic resistance selective plates were used to inoculate DSM broth liquid cultures. Once cultures had reached OD_{600} 0.5-0.8, each culture was diluted to an OD_{600} of 0.1 and spread on DSM agar. Trays of agar were incubated at 30°C for 48 hours. Spores were harvested using ice cold sterile H_2O washed over agar and sterile loops to mix bacterial overlay into a suspension. This suspension was removed from trays of agar and stored in falcon tubes at 4°C. Following harvesting, spores were purified as follows. Spores were washed three times in sterile H_2O prior to suspension in lysozyme buffer (Tris HCl 1.5M, EDTA 0.5M, lysozyme 50mg/ml). Spores were incubated with lysozyme buffer for 1hr at 37 °C with agitation. Lysozyme buffer was removed by washing three times with sterile H_2O. This was followed by salt washes (1x 1M NaCl, 1x 1M KCl). The spore pellet was recovered and washed three times with ice-cold sterile water. Spore purity was assessed by phase contrast microscopy and spore yields in individual preparations were estimated by counting colony forming units (CFU) from serial dilutions made on DSM agar plates.

2.2.2. Formaldehyde inactivation of spores

Formaldehyde solution (1% and 4%) was made by dilution of 37% formaldehyde (Sigma, MO, USA) with sterile H_2O. Spore pellets were suspended in formaldehyde solutions and incubated at 37°C for between 2 and 24 hours in Protein LoBind tubes (Eppendorf) for preliminary assessment of inactivation. Spore suspensions were washed three times to remove traces of formaldehyde and then checked for residual levels using a titration based formaldehyde quantification kit (Hach, Düsseldorf, Germany). Efficacy of inactivation was tested by plating spore suspensions on DSM agar to ascertain if colony formation would occur. Inactivated spores were stored in sterile H_2O at 4°C over 7 days to assess longevity of inactivation. Spores used for immunisations were incubated with 4% formaldehyde for 4hours at 37°C. After washing, these spores were used immediately for immunisation.

2.2.3. Immunisation schedule

Groups of 6-8 week C57/Bl6 mice (Charles River) received oral or sublingual immunisations on days 1, 15, 36, 64 and 85. Oral doses of spores were $4x10^{10}$ /dose and were delivered by oral gavage with mice under light sedation. To receive sublingual doses, mice were briefly immobilised using a 0.1µl intraperitoneal (i.p.) dose of hypnorm/hypnovel mix (1:1:2 hypnorm:hypnovel:H_2O). 10µl containing 2×10^9 spores was placed under the tongue of individual mice. Due to volume restrictions of this dosing method, the total sublingual dose was delivered over three days, with mice receiving 2×10^9 spores per day. To account for this disparity in dose regimen, control oral dosed groups were included that received the oral dose over three days, with one dose of 1.3×10^{10} given per day. Groups received live or formaldehyde inactivated PP108 spores, with the wild type *B. subtilis* PY79 strain used as

control for immunised groups. Mice were weighed regularly to ensure immunisation doses or use of anaesthesia had no ill effects.

2.2.4. Determination of mouse antibody titres by indirect ELISA

For analysis of responses, serum was collected on days -1, 20, 34, 63, 79 and 108, and stored at -20°C. Faeces were collected on days -1, 10, 35, 43, 71 and 99 and kept at -20°C. Faecal sample extractions were made at 1/5 (weight/vol) dilution in extraction buffer (2% foetal bovine serum (FBS) in Dulbecco's modified eagles medium (DMEM) plus protease inhibitor cocktails; trypsin 0.1mg/ml, leupeptin 1µg/ml, benzamide 1µg/ml, aprotinin 10 µg/ml, phenylmethylsulphonyl fluoride 1mM and ethylenediaminetetraacetic acid (EDTA) 0.05 mg/ml). Samples were gently shaken for 30 min at 4°C to disrupt solid material and then centrifuged (14,500 g 15 min). Supernatants were filtered (0.45 µM) before analysis. Antibodies from serum (IgG) and faeces (IgA) were determined by indirect ELISA. Greiner 96 well plates (Maxisorp) were coated with purified 1µg/ml rA26-39 (CDTA14) proteins (50µl/well) in PBS buffer, overnight at RT. After blocking for 1h at 30°C with 2% bovine serum albumin (BSA) two-fold serially diluted samples were added, starting at 1/50 (IgG) or 1/5 (IgA) in diluent buffer (0.01 M PBS [pH7.4], 0.5% (w/v) BSA, %5 (v/v) FBS, 0.1% (v/v) triton X-100, 0.5% (v/v) tween 20). Replicate samples were used together with a negative control (pre-immune serum or faecal extraction). Plates were incubated for 2 h at RT before addition of appropriate horseradish peroxidase conjugated anti-mouse antibodies in conjugate buffer (5% FBS (v/v), 1% BSA (w/v) 0.05% tween-20 in 0.01 M PBS). Plates were incubated for 1 h at RT and then developed using tetramethyl benzidine (TMB) substrate (0.1 mg/ml 3.3', 5.5'-tetramethylbenzidine in 0.1 M sodium acetate buffer (pH 5.5) in

distilled water). Reactions were stopped using 2 M H_2SO_4 and absorbance was measured at 450nm. Dilution curves were created for each sample and end-point titres for each specific antibody were estimated at the maximum dilution of serum giving an absorbance reading of 0.1 units over the absorbance of naïve samples.

2.2.5. Cytokine flow cytometry

Immunised mice were culled 14 days post the last immunisation and splenocytes were recovered. $5x10^5$ splenocytes were seeded per well in 96 well plates (Corning) and stimulated with the following antigens: rA26-39 (CDTA14) protein 1µg/ml with ConA 1µg/ml as a positive control for stimulation. Supernatants were removed after 96h of incubation (37°C, 5% CO_2) and stored at -20°C. A Cytometric Bead Array (CBA) mouse $T_H1/T_H2/T_H17$ cytokine kit (BD Bioscience, Oxford, UK) was used to evaluate cytokine levels in supernatant samples according to manufacturer's instructions. Briefly, 50µl of sample was added to mixed capture beads. PE detection reagent (BD) was added to all tubes and then incubated for 2h at RT in the dark. After incubation, 1ml of wash buffer was added to each reaction tube then tubes were centrifuged to pellet beads. The supernatant was removed and pellets suspended in 300µl of wash buffer. Fluorescence characteristics of samples were acquired and recorded using a BD Accuri C6 flow cytometer. Levels of each cytokine (IL-2, IL-4, IL-6, IFN-γ, TNF, IL-17A, and IL-10) were evaluated based on production of a standard curve for each cytokine included in the assay.

2.2.6. Challenge studies

After the final immunisation, mice were given oral antibiotic doses as described elsewhere (Chen *et al*. 2008) but with minor alterations. Mice received antibiotic doses via orogastric (o.g.) gavage rather than in drinking water. Doses on days 1, 2 and 3 were antibiotic cocktails (kanamycin, gentamycin, colistin, metronidazole and vancomycin). A single oral dose of clindamycin (30mg/kg) was given on day 5, followed by orogastric challenge with 10^4 *C. difficile* R20291 spores. Animals were then monitored for the appearance of symptoms including diarrhoea, lethargy, pilo-erection and weight loss. Symptoms were scored on a numeric scale, where 0 represented no symptoms (no diarrhoea, active and no weight loss), and 3 represented severe disease (diarrhoea, weight loss, reduced activity and obvious lethargy). Animals were caged in IV-C units (Tecniplast UK), with sterilised bedding and *ad libitum* access to food and water. All handling of infected animals was carried out in a biosafety cabinet.

2.3. Chapter 4 – *B. subtilis* probiotics

2.3.1. Strain preparation

B. subtilis PXN21 is one of 14 strains in the commercial product BioKult (Probiotics International Ltd). The strain was obtained from NCIMB (www.ncimb.com) where it is registered as NCIMB strain 30223. Spores of this strain were prepared as described previously (Harwood & Cutting 1990). Pure suspensions of spores were obtained using lysozyme treatment to remove vegetative cells. Phase bright microscopy was then used to visually assess spore suspension for presence of vegetative cells or debris. If debris

remained in suspension, spores were washed in sterile H_2O until suspension only contained spores as seen under the microscope. Counts of spores were made using serial dilutions on solid DSM agar. Spore stocks were stored at -20°C until use.

2.3.2. Probiotic dose regimen

Mice: Assessment of probiotic treatment in animals used female C57Bl/6 mice (Charles River, UK) aged 6-8 weeks. Probiotic doses were delivered via orogastric gavage as stipulated by treatment group (**Table 2.1**). One dose was delivered per animal per day.

Table 2.1. Study groups for probiotic treatments

Study	Regimen
Pre vs. post infection treatment	Daily dose of $1x10^9$ spores. Pre infection treatment for 7 days prior to infection. Post infection treatment for 7 days post infection.
Dose response	Daily dose of either $1x10^7$ or $1x10^9$ spores for 7 days post infection.
Viability	Daily dose of either $1x10^9$ live spores, heat killed spores or vegetative cells for 7 days post infection.

Susceptibility to infection was induced by antibiotic treatment. In colonisation experiments, a single o.g. dose of clindamycin (30mg/kg) was used one day prior to o.g. infection with 10^4 R20291 spores. To achieve required severity of infection, *C. difficile* strain VPI10463 was used in probiotic treatment studies. Antibiotic treatment as described by Chen and colleagues (Chen *et al*. 2008) was delivered via o.g. dosing followed by infection with 10^4 VPI 10463 spores. Animals were monitored for appearance of symptoms, with faecal

samples and weights recorded daily. Animals were culled at the clinical end point of disease, which was considered when animals lost 20% of original body weight.

2.3.3. Hamster challenge

Golden Syrian hamsters weighing approximately 100g were obtained from Charles River. Animals were housed individually in IVC units with sterilised bedding and had *ad libitum* access to sterilised food and water. A single oral dose of clindamycin (30mg/kg) was used to induce susceptibility to *C. difficile* infection. 24 hours after clindamycin treatment, animals were infected using 100 spores of *C. difficile* 630. Pre-treatment with *B. subtilis* PXN spores consisted of 5x oral daily doses of 1×10^9. Post treatment doses were delivered every 12 hours after infection. Animals were monitored for appearance of symptoms, and culled as clinical end point of infection was reached, based on severity of symptoms.

2.3.4. Cytokine ELISAs

Murine macrophage cell line RAW264.7 were grown to 70% confluence in 12 well plates (Corning, NY, USA) in DMEM (Sigma, MO, USA) growth medium supplemented with 10% foetal calf serum (FCS), incubated at 37°C, 5% CO_2. Wells were washed twice with fresh growth medium and then medium containing 10^7 spores per ml was added to cell monolayers. Cells were incubated with spores for 24hrs before growth medium was removed for use in assays. Detection of cytokines in the medium was carried out using commercial ELISA kits (eBioscience) for IL-6 and TNFα according to manufacturer's instructions.

2.3.5. Histology

C57Bl/6 mice (Charles River) were culled three days post infection with *C. difficile*. Colon and caecum were removed from animals that had received probiotic treatment either pre or post infection, using non-treated animals as control for apparent tissue damage. Tissue was also taken from non-infected mice. Contents of the tissue were carefully washed out and tissues were fixed using 4% paraformaldehyde (PFA). Tissues were then sectioned, fixed on slides and stained with hemotoxylin and eosin (H&E) (TUPI manufacturing, Woodbridge, UK). Appearance of tissue was assessed under the microscope once stained and images were taken using associated camera and software (GT Vision, Haverhill, UK).

2.3.6. Toxin binding assay

$1x10^9$ *B. subtilis* PXN21 spores were washed with 0.1M PBS (pH7) and then pelleted by centrifugation in Protein LoBind tubes (Eppendorf). The supernatant was removed and pellet was resuspended with PBS containing commercially purified *C. difficile* toxins (AbD Serotec) to a total volume of 500µl. The spore suspension was incubated with gentle agitation for 12hrs at 4°C. Spores were pelleted by centrifugation and washed three times in PBS to remove unbound toxin. Spore coats were then extracted using an SDS-DTT buffer described elsewhere (Harwood & Cutting 1990). Spore coat extracts were run on a 7% SDS-PAGE gel then were transferred to a nitrocellulose membrane for detection of toxins by western blot.

2.3.7. DotBlot assay

Female C57Bl/6 mice were infected with 10^4 *C. difficile* VPI spores following treatment with an antibiotic cocktail as used previously. Daily o.g. doses of $1x10^9$ *B. subtilis* PXN spores were given to mice post infection, totalling two doses before tissue was harvested. Mice were culled 48hrs after infection and the caecum removed intact with contents. The caecum was then split open longitudinally and the contents washed out carefully with 1ml PBS. The contents were placed in a Protein Lobind tube (Eppendorf), pelleted by centrifugation and washed 3x in PBS to remove unbound toxin. The resuspended pellet was washed through a cell strainer (Corning, NY, USA) to remove solid caecal matter but allow spores to remain in suspension. Spores were pelleted and spore coats were extracted as described elsewhere (Harwood & Cutting 1990). BioDot apparatus (BioRad, Hemel Hempstead, UK) was then used to transfer proteins in samples to a nitrocellulose membrane as per equipment instructions. Briefly, 3 layers of Whatman filter paper and the membrane were pre-soaked in TBS and layered onto the BioDot base. The equipment was screwed together and a vacuum applied. Wells were washed with 100µl TBS before samples were added to wells. Once samples had run through the membrane, wells were washed 2x using TBS. The membrane was then removed from the apparatus and toxins were detected using western blot.

2.3.8. Detection of toxins by western blotting

The membrane was blocked in 5% skim milk TBS for 1hr at RT. Monoclonal α-mouse primary antibody (AdB Serotec) for toxin detection was added, either α-toxin A or α-toxin B at 1/500 dilution for 1 hr. The membrane was then washed 3x in TBS + 0.05% tween. The

secondary antibody, HRP labelled α-mouse IgG was incubated for 1hr at RT. Following incubation with antibodies, the membrane was washed 3x in TBS + 0.05% Tween and developed using a chemiluminesence developer.

2.3.9. TLR2 expression

RAW264.7 macrophage cells were seeded to 6 well plates (3 x 10^5) in antibiotic free media (DMEM supplemented with 10% foetal bovine serum) and incubated for two days until around 70% confluent. Cells were washed twice with fresh growth medium, then media containing infecting agent (spores or vegetative cells of *B. subtilis* PXN21 1 x 10^7/ml) were added to the wells. Cells were incubated with these media for 4 hours before washing two times more with sterile PBS. Macrophages were then lysed *in situ* and homogenised by passing cell lysate through a 20 –gauge needle five times. Total RNAs were then extracted using RNeasy kit (Qiagen, Netherlands) as per manufacturer's instructions. Purified RNA samples were stored at -80°C.

RNA samples from RAW264.7 macrophages were reversed transcribed to cDNA using Precision qScript Reverse Transcriptase kit (PrimerDesign, Southampton, UK) using reaction conditions as follows: annealing step 65°C for 5mins, extension step 55°C for 20mins, 75°C for 15 mins and stop at 4°C. qPCR was carried out using primers targeted at the TLR2 gene (Table 2), with β-actin used as a reference gene. A three step PCR cycle was used with following conditions: 95°C for 15secs, 55°C for 30 secs, 72°C for 10 secs. Cycle was repeated 50 times. Melt curves were produced at the end of each PCR to check primer efficiency. Rotor-Gene 6000 series software was used for analysis of real time data, and

relative gene expression was calculated using relative standard curve method, with β-actin as a reference gene.

Table 2.2. Primer sequences

Gene	Forward 5'-3'	Reverse 5'-3'
TLR2	AAGAGGAAGCCCAAGAAAGC	CAATGGGAATCCTGCTCACT
β-actin	AGAGGCAAATCGTGCCTGAC	CAATAGTGATCATCACCTGGCCCT

2.3.10. Antibiotic resistance

Six hour cultures of *Bacillus* strains were diluted in Muller-Hinton broth to an OD_{600} of 0.15, which gives a concentration of approximately 10^7 cells/ml. This was used as inoculating culture for the assay. In a 96 well plate (Corning, NY, USA) 150µl of Muller-Hinton broth was added to each well. Antibiotics were diluted in the plate, in 2 fold dilutions from 64µg/ml to 0.03µg/ml. Control wells contained 150µl Muller-Hinton broth without antibiotics. 10µl of inoculating culture was added to each well and plates were then incubated overnight on a shaking platform at 37°C. After overnight incubation, OD_{600} of each well was read using a spectrophotometer (Molecular Devices). Wells that showed an increase in OD_{600} were considered positive for bacterial growth. Wells with no increase in OD_{600} were considered negative for bacterial growth, and so were recorded as inhibitory concentrations. Assay was carried out with duplicates, and was run three times in total. Values for minimum inhibitory concentration (MIC) were compared with those published in guidelines from the European Food Safety Authority (EFSA) and the Clinical Laboratory Standards Institute (CLSI).

2.4. Chapter 5 – BclA spore coat proteins

2.4.1. Growth and sporulation curves

BHI broth was inoculated with a single colony from an overnight streak plate. For growth curves, a 1ml aliquot was taken from the culture every hour. OD_{600} was recorded using a spectrophotometer. Sporulation samples were taken every 24 hours. Total bacterial counts were produced using serial dilutions of culture plated on BHIS agar supplemented with 0.1% sodium taurocholate. For spore counts, samples were heated at 60°C for 30 minutes to kill vegetative forms before plating on sodium taurocholate supplemented BHIS agar.

2.4.2. Germination Assays

Spore germination was carried out in a 96-well plate (Greiner Bio-One) and germination of spores was measured by the percentage change in OD_{600}. HistoDenz-purified spores at an OD_{600} of ~0.8–1.0 (~1 X 10^8 ml^{-1}) were pelleted by centrifugation (10,000g, 1min) and suspended in 1ml of BHIS supplemented with 0.1% sodium taurocholate (germinant) or 0.1% sodium chenodeoxycholate (inhibitor). The initial OD_{600} was recorded and then measured at 1 minute intervals over 30 minutes using a microplate reader (Molecular Devices, Spectramax plus). Plates were shaken in between readings to keep spores in suspension. % fall in OD was determined as (recorded OD_{600} at time interval/initial OD_{600}) X 100. The experiment was performed three times. For preparations of sonicated spores ten cycles of sonication were used as described elsewhere (Permpoonpattana *et al* 2011*b*). Using phase contrast microscopy a plateau in optical density readings was confirmed to

correspond to 100% of spores losing phase brightness after incubation with the germinant. Loss of phase brightness indicates initiation of the germination process.

2.4.3. Spore adhesion to hydrocarbon (SATH) assay

This method is described elsewhere (Huang *et al*. 2010), with minor alterations. HistoDenz-purified spores were washed in 1M NaCl and then suspended in 0.1M NaCl for assay. 500µl of spore suspension was added to 800µl *n*-hexadecane (Sigma, MO, USA) and vortexed for 1min. Samples were then incubated for 10 min at 37°C with mild agitation, vortexed (30s) and optical density (OD) (OD_{600nm}) recorded. % hydrophobicity was determined from the OD of the original spore suspension (A_1) and the OD of the aqueous phase after incubation with hydrocarbon (A_2) using the equation: $\%H = [(A_1 - A_2)/A_1]$.

2.4.4. Mouse colonisation experiments

Groups of C57LB/6 mice (6-8 weeks old; female) were given a single o.g. dose of clindamycin (30mg/kg in 0.1ml of sterile water). Groups of animals were kept in IV-C units (with sterilized food, bedding and water). Animals were infected with different doses of R20291, 630Δ*erm* or *bclA1*$^-$ mutant spores five days later since the 630Δ*erm* and *bclA1* mutant strains are sensitive to clindamycin. Spore counts in freshly voided faeces were determined by ethanol treatment (20 min incubation of homogenate in 100% ethanol) and plating on BHISS supplemented with supplemented with cefoxitin and cycloserine (Bioconnections, Knypersley, UK).

2.4.5. Hamster infections

Golden Syrian Hamsters (female, ~100g; Charles River) were given doses of clindamycin (30mg/kg) by oral gavage, then infected 5 days later with *C. difficile* spores. Histodenz-purified spores of wild type (630Δ*erm*) and the *bclA1* mutant were given by oral gavage, at doses of either 10^2, 10^3 or 10^4. Hamsters were then monitored for signs of disease progression and culled as individuals reached the clinical end point, based on severity of symptoms. Time to the clinical end point was recorded for each individual. Statistical significance between groups was calculated using a student's t-test. Caecum samples were examined for toxin B by ELISA as described in general methods. To assess spore counts caeca were removed from euthanized animals and added to protease inhibitor buffer (Thermo Scientific). Tissue was roughly homogenized; aliquots ethanol treated (100%, 20 minutes) and serially diluted on BHISS agar plates. Plates were incubated anaerobically for 48 hours before counting colonies. Number of spores/gram was calculated based on the weight of the original sample.

2.4.6. Resistances

Spores purified using Histodenz (Sigma, MO, USA) were tested for resistance to heat, ethanol and lysozyme.

Heat: 10^7 spores suspended in sterile water were incubated in a heat block at 60°C for 1hr and 24hrs. After incubation spores were placed on ice before serial dilutions were made.

Ethanol: 10^7 spores were suspended in 70% ethanol and incubated at room temperature with agitation for 1 or 24 hours. After incubation period, spores were washed once with sterile water and serially diluted for enumeration of surviving spores.

Lysozyme: 10^7 spores were suspended in a lysozyme buffer (20mM Tris HCl (pH8.0) and 300mM NaCl) and incubated with agitation at 37°C. One set of spores was incubated in a buffer containing 250µg/ml lysozyme for 10 minutes and a second set was incubated in a buffer containing 1mg/ml lysozyme for 20 minutes. Spores were washed once in ice cold sterile buffer before serial dilutions were made.

Serial dilutions for enumeration of surviving spores were plated on BHI agar supplemented with 0.1% sodium taurocholate. Plates were incubated in anaerobic conditions at 37°C for 48 hours before colonies were counted.

Chapter 3

Mucosal Immunisation with a Spore Vaccine

3.1. Introduction

3.1.1. *Clostridium difficile* vaccines

Current vaccine research is focused on the use of toxoids and recombinant proteins to elicit an immune response against *C. difficile*, via production of a systemic response. Injection of these proteins will initiate immune responses and in many reported cases results in production of toxin neutralising antibodies (Sougioultzis *et al*. 2005; Tian *et al*. 2012; Wang *et al*. 2012). Protection in animal models has been reported and there are several potential vaccine candidates at various stages of development. The most advanced of these is a toxoid based vaccine from Sanofi Pasteur (Alkan *et al*. 2012), which entered in to Phase III clinical trials in the summer of 2013 having been granted fast track designation by the American FDA in 2010. Currently, no product has yet come to market for human use.

The appearance of so called hypervirulent strains (Pépin *et al*. 2004) highlights the complexity of treatment and control of *C. difficile.* Strain evolution (Stabler *et al*. 2009) and development of antibiotic resistance (He *et al*. 2012) have contributed to CDI becoming increasingly difficult to treat. Multiple virulence factors for the pathogen have been described (Voth & Ballard 2005; Janoir *et al.* 2007; Rupnik *et al.* 2009), including toxins and

cell associated proteins. The best described virulence factors are Toxins A and B, two major exo-toxins produced by *C. difficile* during vegetative growth. However, the importance of these two toxins has been a point of contention between some researchers (Lyras *et al*. 2009; Kuehne *et al*. 2010). It should be noted that not all strains produce the two major toxins, as naturally occurring virulent strains of A^-B^+ *C. difficile* have been reported (Johnson *et al*. 2003; Drudy *et al*. 2007*b*). Understanding of CDI cases is further complicated as incidences independent of the hospital setting, including community associated CDI, are now more commonly reported (Warny *et al*. 2005; Khanna *et al*. 2012). It is necessary to find treatments that will resolve infections of the most tenacious nature, beyond the scope of the few antibiotics that are used currently. Development of a successful vaccine would significantly help in reducing the burden of disease caused by *C. difficile* and reduce the pressure of reliance on antibiotics.

Previous work from the Cutting Laboratory resulted in the development of a vaccine candidate that focuses on production of a local mucosal response (Permpoonpattana *et al.* 2011*a*). This approach differs from the majority of current studies that use parentally delivered toxoids and focus on production of systemic immune responses (Giannasca & Warny 2004). Mucosal vaccination will be the focus of this thesis chapter, as the generation of immune responses and potential methods for use of a *B. subtilis* spore based vaccine are evaluated. The spore based vaccine candidate, referred to as PP108 (Permpoonpattana *et al.* 2011*a*), confers a 75% protection rate in a hamster model of infection. Furthermore, PP108 produces both mucosal and systemic neutralising antibodies against toxins. In the same study, it was demonstrated that antibodies against a *C. difficile* toxin A fragment also recognised toxin B and that antibodies against toxin A were able to provide protection against an A^+B^+ strain of *C. difficile* in challenge studies.

3.1.2. Stimulation of mucosal immune responses

Classical vaccination using parenteral delivery of antigens aims to protect against *C. difficile* infection by systemic generation of toxin neutralising antibodies. The primary site of infection is the colon, where the pathogen can rapidly multiply and cause damage to the lining of the gut through production of toxins. The mucosal surface of the gastrointestinal tract is therefore the main site of contact with the developing infection. In order to produce an immune response local to the site of infection, alternative vaccination strategies need to be investigated. Use of mucosal routes of immunisation, for example, to produce localised immune responses could harness the powerful potential of the immune tissue in the G. I. tract – the Gut Associated Lymphoid Tissue (GALT). Specific to *C. difficile*, a localised immune response could increase protection of the gut during infection through neutralisation of toxins at the surface where damage is most likely to occur.

While an important factor of vaccine development includes identifying immunogenic antigens, a significant component involves finding a safe and efficient way to deliver that antigen (Levine & Sztein 2004). By demonstrating the potential of a particular delivery approach with one successful vaccine, this could be applied in development of further vaccines. Specific to this, development of vaccines that exclude the use of needles represents an important step in producing vaccines for use in the developing world. Spread of infectious disease through misuse of needles could be prevented by development of an alternative delivery system for vaccinations. A novel vaccine must also be relatively cheap if it is to be widely distributed and used. With *C. difficile*, groups with increased risk of developing the infection have been identified such as the elderly and patients receiving antimicrobial therapy (Loo *et al*. 2011), representing a considerable target market for a vaccine protecting against *C. difficile*.

Immunisation can result in stimulation of different responses – systemic and mucosal. Protection through systemic responses relies on production of circulating antibodies against the pathogen in question. Mucosal responses involve production of secreted antibodies at mucosal surfaces. Localised mucosal immunisation can lead to antigen specific IgA production at remote mucosal sites (Kunkel & Butcher 2003), allowing immunisations to be delivered at easily accessible surfaces to produce a response at an alternative site. This property of mucosal immunisation is limited however, in that specific routes will not produce responses at all mucosal sites. For example, oral dosing can produce immune responses in the gut, but the effect is limited when considering the mucosa of the respiratory system (Holmgren & Czerkinsky 2005).

Mucosal surfaces form a physical barrier against invading pathogens and IgA is the predominant immunoglobulin isotype in mucosal secretions (Fagarasan & Honjo 2003). When pathogens penetrate this layer it is potentially the first point of contact with the host's immune system. Therefore a response local to the infection could aid in preventing colonisation by potential pathogens, rather than relying on the systemic response to be initiated once an infection has proliferated. If a vaccine can stimulate both mucosal and systemic responses the host is provided with two levels of protection, increasing the potential for success of the immunisation.

The focus on mucosal immune responses stems from CDI occurring in proximity to the mucosal surfaces of the G.I. tract (Goulding *et al*. 2009). The mucosal surface is a prime target for stimulating a local immune response, with mucosal responses classically achieved through intra nasal (IN) or oral dosing (Neutra & Kozlowski 2006).

Oral dosing is a relatively simple process, requiring little equipment or specialist knowledge for delivery of doses, unless a patient is unable to swallow independently. This route does however require large amounts of the antigen in question to initiate a response, as dilution of the antigen can occur before contact with the appropriate site. Oral dosing is also often linked to tolerance mechanisms (Weiner *et al.* 1994; Azizi *et al.* 2010). Additionally, the oral route requires passage through the stomach and G.I. tract, which contain various extremes of pH plus exposure to enzymes that could cause degradation to antigens before reaching the target mucosal surface.

Nasal dosing requires smaller amounts of antigen as delivery to mucosal surface is more direct, but use of this route has associated safety concerns. Reports of antigen and adjuvant trafficking to the central nervous system (CNS) have caused issues with regard to use of IN dosing as a delivery route for immunisations. Some adjuvants used in vaccines are able to bind to olfactory nerve fibres and can potentially reach the olfactory lobes of the brain (van Ginkel *et al.* 2000; van Ginkel *et al.* 2005). A review of an intranasal influenza vaccine used in Switzerland reports on occurrences of Bell palsy cases associated with use of the vaccine (Mutsch *et al*. 2004). Currently an intranasal vaccine for influenza is licensed in the USA, but it is recommended only for individuals aged 2-49 years and it is advised that individuals with underlying health conditions do not receive the vaccine (CDC 2013c). Intranasal routes do, however, provide a reliable route for generation of mucosal immune responses in the respiratory tract (Kweon *et al*. 2002).

A disadvantage of mucosal immunisation is the higher quantity of antigen required when compared to parenteral immunisation. (Holmgren 1991; Neutra & Kozlowski 2006). The potential cost of a vaccine is a significant consideration in vaccine development; increasing the amount of antigen used will escalate costs (Levine & Sztein 2004). Quantification of

antigen necessary for parental vaccination is relatively simple, but when using mucosal pathways to deliver antigens the process is more complex (Ogra *et al.* 2001). Increased amounts of antigen are required in mucosal immunisations for several reasons. Firstly, mucosal routes of immunisation can cause degradation to the antigen through harsh pH or presence of enzymes, the effects of which is difficult to quantify. Secondly, methods of antigen delivery for mucosal immunisation such as orogastric dosing do not deliver the antigen directly to the mucosal surface. The lack of direct delivery results in dilution of the antigen, so a higher dose of antigen is required to counteract the reduced amount of antigen reaching the final targeted mucosal site.

3.1.3. Sublingual antigen delivery

Sublingual (SL) comes from Latin – literally 'under the tongue'. Sublingual immunotherapy (SLIT) has been used previously in allergy therapy as it has been shown to successfully modulate immune responses (Durham *et al.* 2006; Moingeon *et al.* 2006). Sublingual based therapies have also been reported in delivery of pain relief drugs and vitamin supplements (Zhang *et al.* 2002). Recently SLIT has been applied to use in vaccines as an alternative route of delivery for antigens. A group in Korea (Song *et al.* 2008) have developed a successful influenza vaccine based on sublingual delivery of antigens and induction of a mucosal immune response through this route. More recently, the same group have used *B. subtilis* spores as a mucosal adjuvant in a H5N1 vaccine study (Song *et al*. 2012), demonstrating the successful use of *B. subtilis* for invoking protective immune responses.

Delivery of antigens to mucosal surfaces using orogastric and intranasal routes can result in both local and systemic responses (Permpoonpattana *et al*. 2011*a*; Song *et al.* 2012).

Sublingual (SL) dosing of antigens has also been demonstrated to produce both mucosal and systemic responses (Cuburu *et al*. 2007) and has several benefits over the orogastric and intranasal routes. SL dosing of antigens allows delivery straight to the mucosa in the oral cavity, bypassing the stomach and G.I tract where the antigen could be destroyed or damaged. SL dosing also enables a reduction in the amount of antigen used as the delivery of the dose to the mucosal surface is more precise. As yet, no safety concerns have been reported with this method of antigen delivery (Song *et al.* 2008). With regard to the mechanism behind sublingual immunisation, studies investigating the immune responses associated with sublingual delivery of antigens suggest the involvement of Langerhans cells and submucosal dendritic cells in uptake of the antigen (Noirey *et al.* 2000; Kweon 2011). The cervical draining lymph node has been identified in several studies as the primary site of antigen presentation in sublingual immunisations (Song *et al*. 2009; Kweon 2011).

3.1.4. *Bacillus* as vaccines

Bacillus subtilis spores have been used in studies by the Cutting group as vehicles for delivery of antigens to mucosal surfaces in order to elicit immune responses. Display of an antigen on the surface of these spores can be accomplished by either of two methods: the antigen can be absorbed on to the spore surface (Huang *et al*. 2010), or the strain can be genetically engineered to express the antigen fused to existing spore coat proteins (Isticato *et al.* 2001; Mauriello *et al.* 2004; Duc *et al.* 2007). Successful generation of systemic and local immune responses has been shown using several different spore bound or expressed antigens, including *C. perfringens* alpha toxin and the TTFC (tetanus toxin fragment C) antigen from *C. tetani* (Huang *et al*. 2010).

One of the most recently developed *B. subtilis* based vaccines is the PP108 spore vaccine for CDI (Permpoonpattana *et al.* 2011*a*). PP108 was produced using genetic modification to express a fragment of the *C. difficile* toxin A protein fused to coat proteins (cotB-$A_{26\text{-}39}$ and cotC-$A_{26\text{-}39}$), facilitating expression on the spore coat. The fragment A26-39 is taken from the carboxy-terminal repeat domain of toxin A that is involved in cell binding (**Figure 3.1B**). The spore vaccine candidate has previously been demonstrated to confer a 75% protection rate in a hamster model of infection (Permpoonpattana *et al.* 2011*a*). Furthermore, the study demonstrated that PP108 produces both mucosal and systemic neutralising antibodies against toxins.

The advantages of using *B. subtilis* spores are that the spores are relatively simple to produce in large quantities and are also heat stable (Nicholson *et al.* 2000). The intrinsic heat stable nature of *B. subtilis* spores has been shown to extend to antigens expressed on the spore coat. Specifically, tetanus toxin fragment C (TTFC) expressed on the spore surface was shown to be effective at producing protection even after spores were lyophilised and stored for 12 months (Lee *et al*. 2010). *B. subtilis* also demonstrates a history of safe human consumption through use as a probiotic supplement (Hong *et al.* 2005).

This thesis will investigate use of the PP108 vaccine in a murine model of infection. More detailed study of immune responses can be carried out using a murine model, as reagents for murine studies are more readily available than those for hamster studies. Results from previous work with the PP108 vaccine candidate have been used to argue for the importance of a mucosal response in protection against CDI (Permpoonpattana *et al.* 2011*a*). Continuing on this track, investigation of alternative routes for stimulation of mucosal responses is of interest as the established oral route can be associated with development of tolerance to the antigen (Song *et al.* 2008). The work in this thesis will

compare two mucosal routes for immunisation: oral and sublingual. Nasal delivery of antigens will be avoided to rule out complications from potential infections that could occur with this method of delivery and the use of live bacteria.

3.1.5. Experimental Objectives

As yet, no vaccine is available to protect against *C. difficile* infection. Interactions of *C. difficile* with the immune system have yet to be fully elucidated; this makes understanding what is required for a successful vaccine candidate difficult to define. This study will use mucosal delivery of a spore based vaccine, with the aim of investigating the role that mucosal responses play in protection from infection. This will address the hypothesis that a localised mucosal response is key in preventing the symptoms of *C. difficile* infection.

The PP108 strain is genetically modified (GM) to express a *C. difficile* antigen. Use of GM organisms introduces a host of issues, including ethical and regulatory concerns. Therefore, the efficacy of the vaccine strain will be assessed when spores are inactivated with formaldehyde. Use of inactivated viruses and antigens is common practice in production of vaccines and also helps avoid the issue of using live GM organisms. Use of the inactivated spores will contribute to two aspects of this study. Firstly, non-viable spores serve as a control for viable spores that have potential to germinate during dosing and transit through animals. PP108 expresses the *C. difficile* antigen on its spore coat so when the spore germinates and enters a vegetative phase it will no longer display the antigen. This introduces the possibility that a potential response from the animal would be reduced if vaccinated with live spores. Germination of *B. subtilis* spores has been shown to occur *in vivo* (Hoa *et al*. 2001), but in this study did not attempt to quantify levels of germination

linked to the different immunisation routes. Secondly, excluding the possibility of germination allows responses to be attributed solely to the spore as a vaccine rather than implying some probiotic effect by use of live organisms. Inactivation of spores also enables the comparison of immunogenicity between live and inactivated spores.

A murine model of infection will be used to quantify immune responses generated by oral and sublingual delivery of the PP108 spore vaccine. Mucosal IgA and systemic IgG titres will be measured alongside analysis of responses based on cytokine profiles from vaccinated animals. Levels of protection generated by immunisations will be assessed in a challenge experiment.

3.2. Results

3.2.1 Production of PP108 spores

PP108 spores for use in immunisations were prepared on solid media in batches. Each batch was tested for consistent expression of the antigen by extracting the spore coat and probing with αA26-39 after SDS-PAGE separation for presence of the antigen (**Figure 3.1A**).

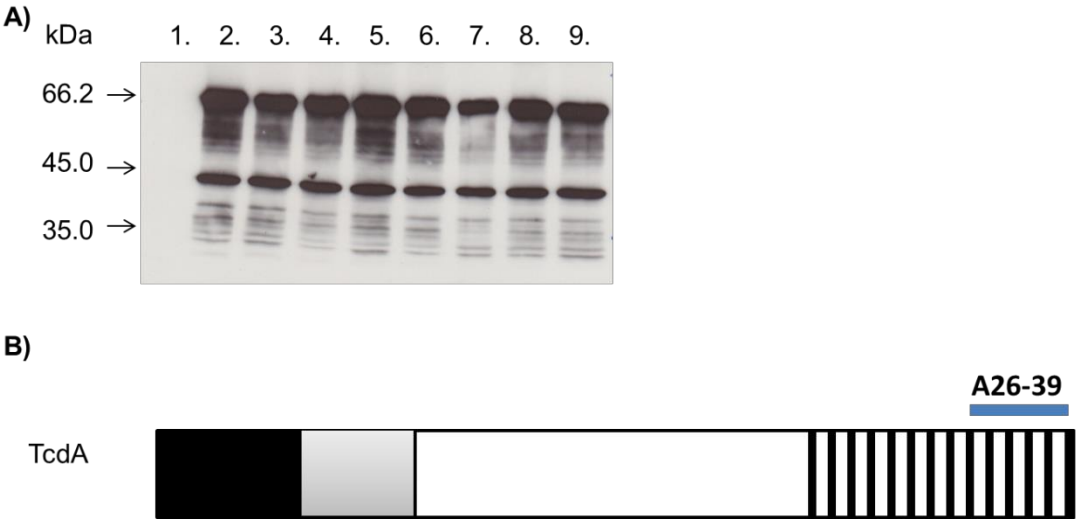


Figure 3.1. Display of antigen on spore coat: A) Western blot showing expression of A26-39 on spores of PP108 (CotB-A_{26-39} and CotC-A_{26-39}). Extracted spore coat proteins from PP108 were probed with antibodies to rA26-39. Lane1 is proteins extracted from PY79 spore coat. Lanes 2-9 are spore coat proteins extracted from individual batches of *B. subtilis* PP108 spores. B) Functional domain of toxin A, including the glycotransferase (black), cysteine protease (grey), translocation (white) domains and repetitive sequences involved in cell binding. The A26-39 region expressed on PP108 spores is shown in blue. (Figure adapted from Permpoonpattana *et al*. 2011)

Inactivation of spores in suspensions of 10^9 or 10^{10}/ml was achieved with use of 4% formaldehyde solution at 37° (**Figure 3.2**). Viability of formaldehyde treated samples was tested by plating on DSM agar to produce colonies from any remaining viable spores. This inactivation is not 100% effective or permanent. When samples were tested after storage at 4°C for one week, a slight increase in spore recovery from inactivated samples was observed. In preliminary work two incubation periods were tested for inactivation efficiency (**Figure 3.2A and 3.2B**). The two hour spore incubation was more successful than the 24 hour time period. Considering success of inactivation and practicality of the protocol, a four hour inactivation period was used for preparation of the spores for immunisation doses (**Figure 3.2C**). Spores for use in immunisations were always used immediately after inactivation, but levels of viable spores were tested after seven days to assess the long term success of the protocol (**Figure 3.2C**).

Groups of Balb/C mice were used to compare immunogenicity of PP108 spores in the live and inactivated state. Mice received two doses of the spores delivered via IP injection and then serum samples were tested for specific IgG responses (**Figure 3.3**). Elevated levels of α-A26-39 specific IgG show that when PP108 spores were delivered via the I.P. route live spores produce the highest systemic response against the antigen. Formaldehyde inactivated spores produce a lower response after two doses compared with live spores. Gluteraldehyde inactivated spores (Russell 1990) were the least immunogenic although a low antigen specific response was detected. Formaldehyde inactivation is classically associated with vaccine production for antigen stabilisation (Rappuoli 1994) and spores inactivated by this method generated a detectable immune response so use of this method was continued in this study.

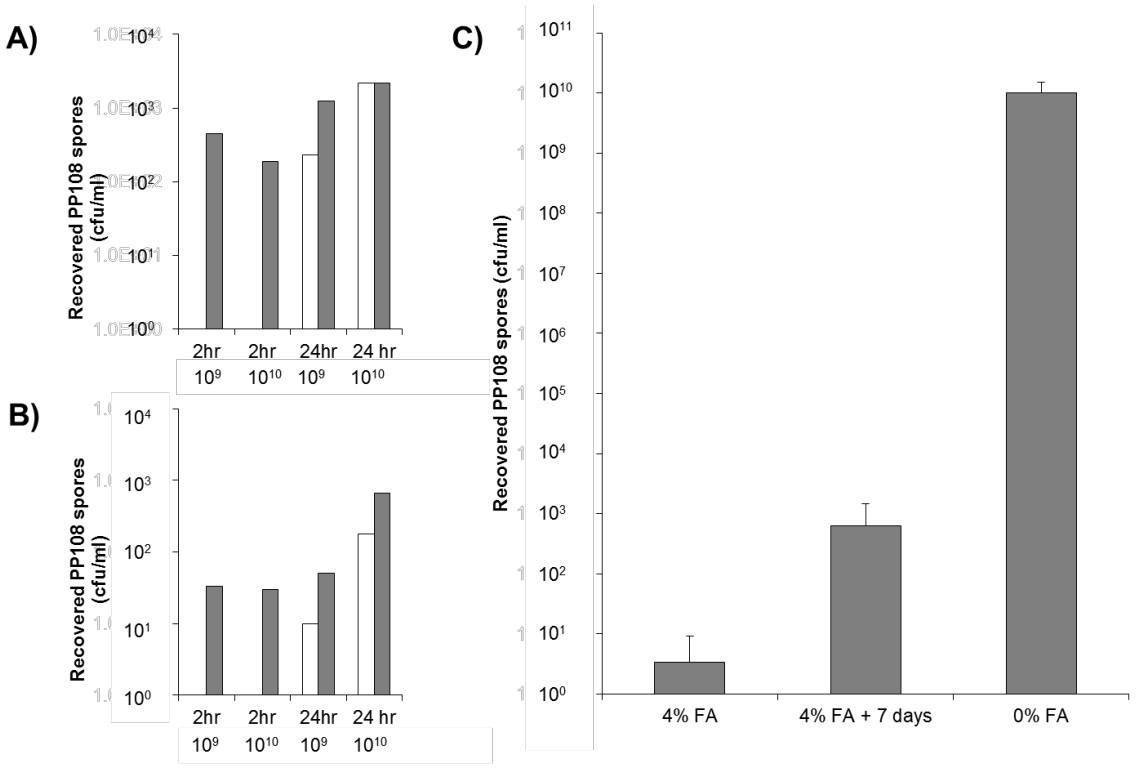


Figure 3.2. Recovery of PP108 spores following inactivation with A) 1% formaldehyde and B) 4% formaldehyde. In preliminary inactivation studies, spores were incubated at 37°C for either 2 or 24 hours, then washed 3x in cold sterile H_2O then serial dilutions were plated on DSM agar to enumerate surviving spores (white bars). After 7 days of storage in H_2O at 4°C, surviving spores were enumerated again on DSM agar (grey bars). PP108 spores used in immunisations were inactivated using C) 4% formaldehdye and 4 hours incubation at 37°C.

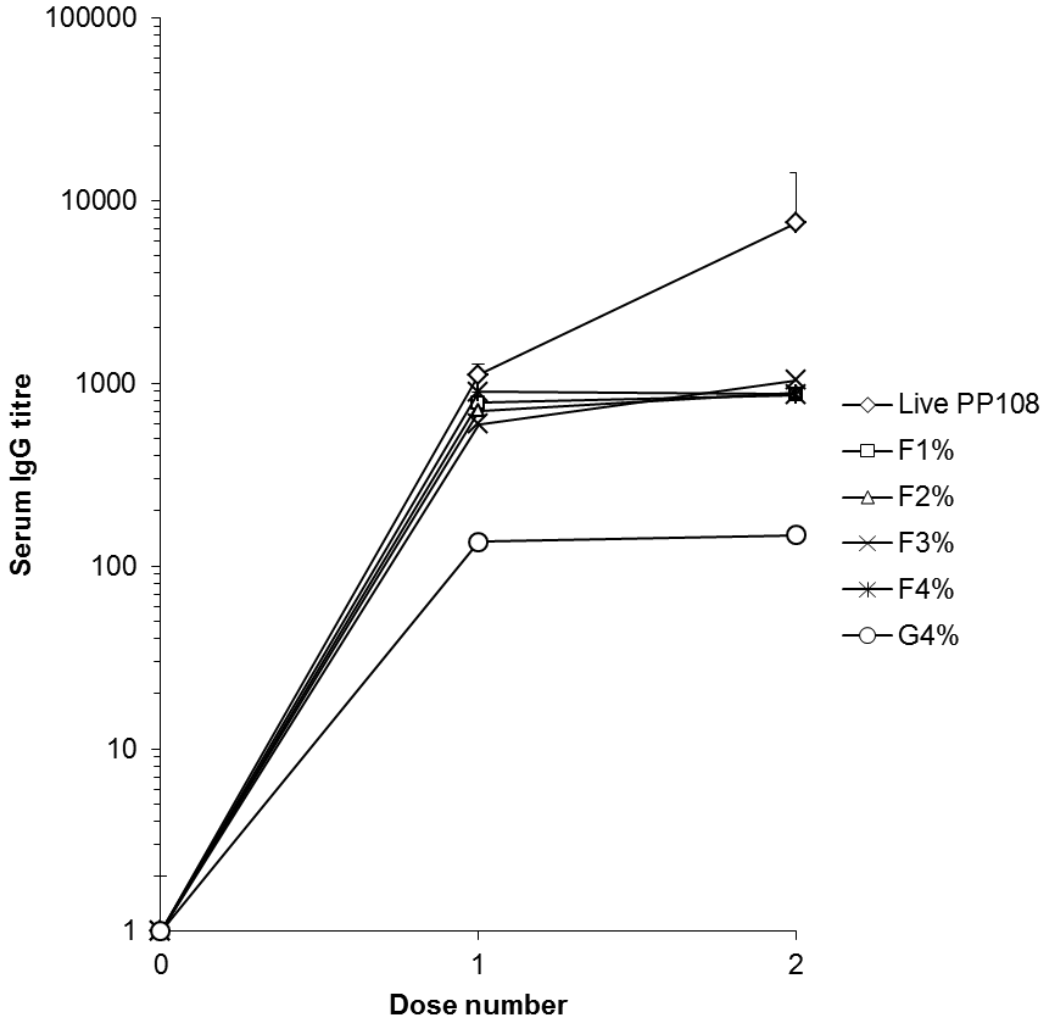


Figure 3.3. **Immune response to PP108 spores** in Balb/C mice, with spore doses ($2x10^9$ PP108 spores/dose) delivered via I.P injection. Doses were given at intervals of two weeks with αA26-39 specific IgG response measured by ELISA from serum samples taken ten days after delivery of each dose. F denotes inactivation of spores with a solution of formaldehyde, G denotes inactivation with gluteraldehyde.

3.2.2 Protection from CDI in immunised mice

Groups of mice received doses of live or inactivated PP108 spores for assessment of oral and sublingual immunisation. After the final vaccine dose, mice were infected with *C. difficile* R20291 to evaluate levels of protection. Symptoms during infection were assessed (**Figure 3.4**) by ranking on a numerical scale (1-3) where a score of three represents the most severe symptoms, and one represents no symptoms. Weights were also recorded to monitor loss of body mass during infection (**Figure 3.5**).

Mice immunised sublingually with inactivated spores were the best protected based on symptoms displayed during infection (**Figure 3.4B**). No severe symptoms were recorded over the seven day monitoring period of infection (**Table 3.1**). In terms of total symptoms (1 observation per day over 6 days) 14% were recorded as mild (symptoms recorded as two on the numerical scale) compared with a protection rate of 86% (**Table 3.1**) demonstrating that sublingual immunisation with inactivated spores was the most successful within the groups tested. Mice receiving PP108 live spores via the sublingual route also showed some protection against development of symptoms (**Figure 3.4A**) although not to the same level as mice receiving inactivated spores via the same route. The occurrence of symptoms scored as severe was 2.8%, and 38.9% of observed symptoms were recorded as mild giving a protection rate of 58.3% (**Table 3.1**).

Live spores delivered orally demonstrated protection against CDI symptoms although not to the same extent as inactivated spores delivered via SL route (**Figure 3.4C**). Protection rate was similar to the sublingual live spore immunisation group at 55.7%, with 41.5% mild symptoms and 2.8% severe symptoms recorded (**Table 3.1**). Oral delivery of inactivated spores gave the lowest level of protection (**Figure 3.4D**) with 47.2% of recorded symptoms

at 1 on the numerical scale. Mild symptoms were recorded at 50.5% and severe symptoms 2.8% (**Table 3.1**).

In PY79 control groups (**Figure 3.4E**), 22.3% of symptoms recorded were severe. Mild symptoms were recorded at 36.2%, with 41.5% displaying no symptoms.

All groups immunised with PP108 spores displayed protection from the severe symptoms of CDI.

As the strain used in this study (R20291) does not cause fatal infection in the study model, body mass of the animals during the period of infection was used to provide a clear representation of the animal's health status. Weight data (**Figure 3.5**) replicates the results from symptom profiles (**Figure 3.4**), with the group receiving inactivated PP108 spores sublingually displaying the least weight loss over the course of infection (**Figure 3.5A**). Groups immunised with live spores either via the oral or sublingual route show a similar profile, mirroring the results from the symptom profiles. The most loss of body mass over the course of infection was recorded in groups given oral doses of inactivated spores (**Figure 3.5B**), which also showed the lowest rate of protection. All immunised groups displayed higher percentage body mass over infection than the PY79 control groups, confirming symptom profile data that all animals receiving PP108 in any form showed greater protection against CDI symptoms than non-immunised animals.

Analysis of faecal samples show that though mice appear protected against symptoms of CDI by immunisation (**Figure 3.4**) this only extends to the symptoms of the disease. Immunised groups did not display resistance to colonisation by *C. difficile*. When enumerating levels of *C. difficile* spores in faecal samples (**Figure 3.6**), there was no difference between spore content in samples from immunised mice when compared to

PY79 control groups (p>0.1). The presence of *C. difficile* in such high numbers in the faecal samples shows that although animals are not as susceptible to the disease, colonisation is not prevented and shedding of high numbers of spores still occurs.

	% Symptoms		
	1	2	3
SL PP108	58.3	38.9	2.8
SL PP108 F	86.0	14.0	0.0
Oral PP108	55.7	41.5	2.8
Oral PP108 F	47.2	50.0	2.8
PY79	41.5	36.2	22.3

Table 3.1. Severity of CDI symptoms in immunised mice: C57BL/6 mice were infected with CD R20291 spores following immunisation with PP108 spores. Symptoms of CDI were scored according to a numerical scale where 1 = healthy, 2 = mild disease symptoms and 3 = severe symptoms. % of symptoms displayed in each category is calculated over the monitoring period of 7 days demonstrating the frequency of each class of symptom within this time period. Symptoms were recorded on 6 days with 6 mice per group, so an occurrence of 2.8% represents one event. Groups (n=6) are denoted by the route and spores used for immunisation, where F represents formaldehyde inactivated spores. No animals reached clinical end point in this study so group numbers remain constant. Refer also to Fig. 3.4.

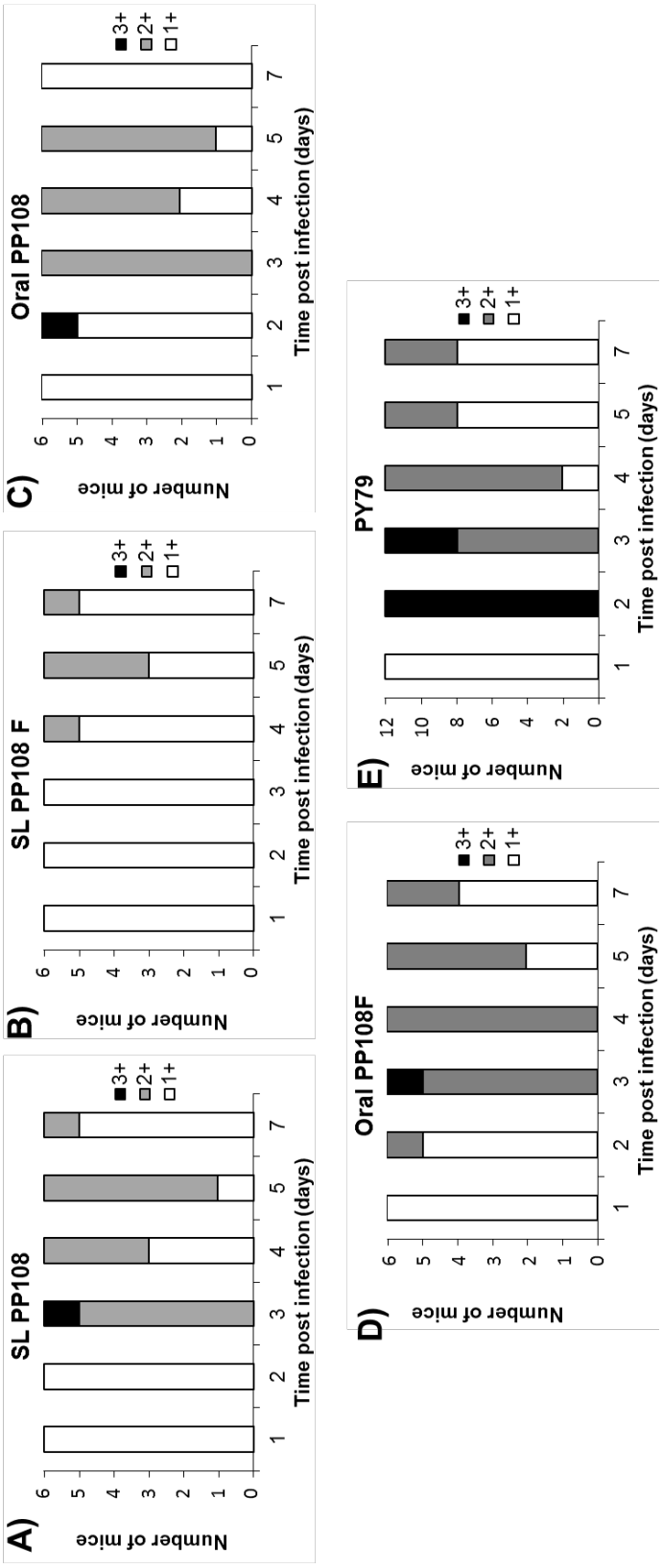


Figure 3.4. Severity of CDI symptoms in immunised mice: C57BL/6 mice were infected with CD R20291 spores following immunisation with PP108 spores. Symptoms of CDI were scored according to a numerical scale where 1= healthy, 2 = mild disease symptoms and 3 = severe symptoms. Graded colour on bars shows the number of mice in each group with particular level of symptoms on each day. No mice reached clinical end point so group size was constant through study. Groups of mice (n=6) were immunised with A) live PP108 via SL route, B) inactivated PP108 via SL route, C) live PP108 via o.g. route, D) inactivated PP108 via o.g. route and E) PY79 spores (combined data for sublingual and oral dose routes n=12). Also refer to Table 3.1.

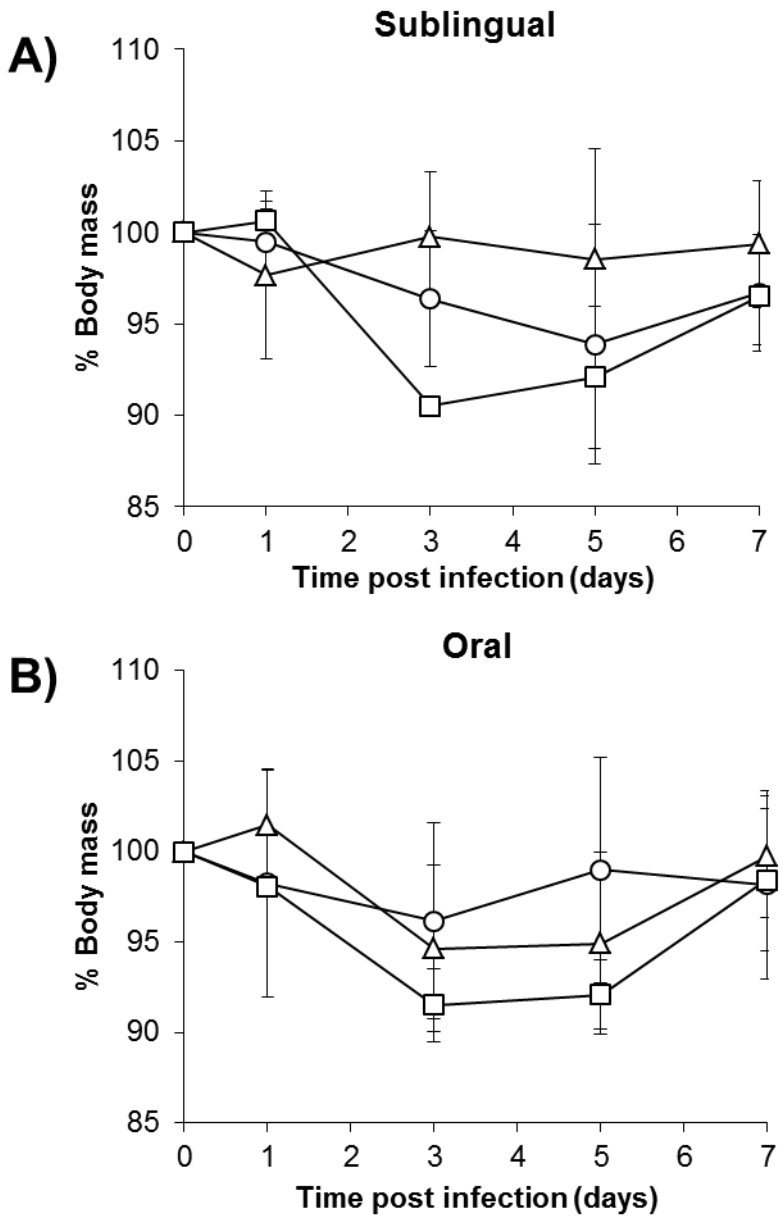


Figure 3.5. % Body mass over period of infection post A) sublingual or B) oral immunisation. Groups received live PP108 spores (○), inactivated PP108 spores (Δ) or PY79 spores (□). Animals treated with an antibiotic cocktail to induce susceptibility to *C. difficile* infection and then infected via o.g. gavage with 10^4 CD R20291 spores. Body mass following infection was calculated as % of body mass at point of infection.

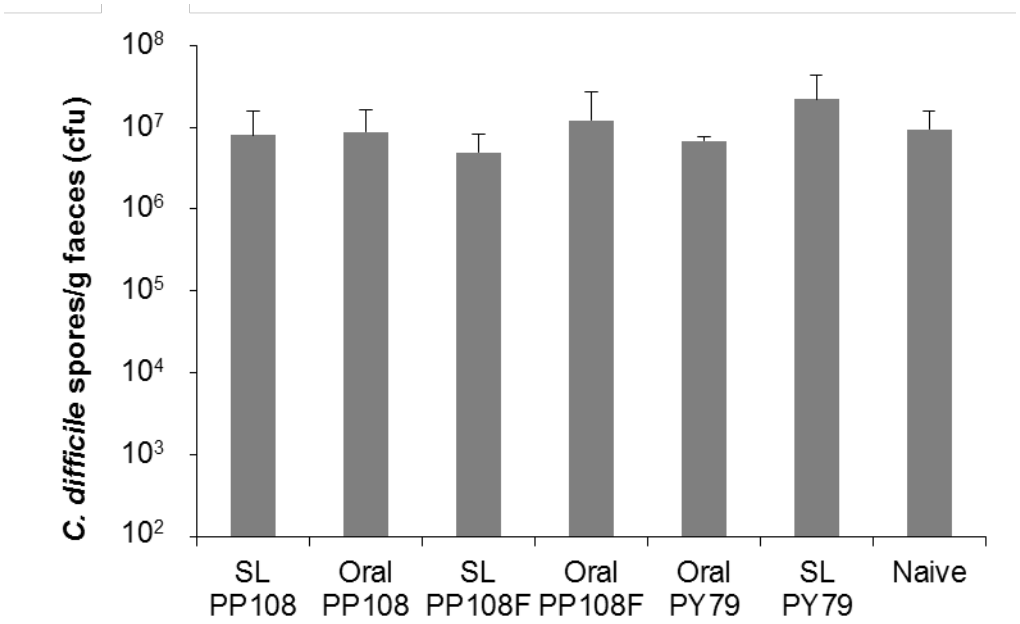


Figure 3.6. Average maximum *C. difficile* spore content in faecal samples from immunised mice. C57BL/6 mice were infected with CD R20291 spores following immunisation with PP108 spores. Daily faecal samples were taken and *C. difficile* spore content assessed by serial dilution on BHISS agar. *Average maximum* – faecal samples were taken daily, and the maximum faecal spore count from individuals within a group was recorded over the infection period (1 week). The mean maximum count was then calculated for each group.

3.2.3 Immune response in immunised mice

Mice received a total of five immunisation doses, with blood and faeces samples taken throughout the dose regimen to analyse immune response generated over time.

αA26-39 specific secreted IgA was measured from faecal sample extracts. Both groups of sublingually immunised mice demonstrate increasing and comparable levels of IgA over the period of immunisation until the final dose where levels of specific IgA are higher in the group receiving inactivated spores (**Figure 3.7A**). The difference between viable and non-viable spores is not statistically significant at any time point however ($p>0.1$). Levels of IgA from faecal samples in sublingually immunised groups are not significantly different from

PY79 control groups until after the third dose of spores. After fourth and fifth doses in sublingual groups, the specific IgA response is significantly higher than respective control groups receiving PY79 spores ($p<0.05$). Mice receiving oral doses of live and inactivated PP108 spores also demonstrated presence of an antigen specific mucosal response (**Figure 7B**). Faecal IgA levels are comparable between the oral PP108 immunisation groups and significantly higher ($p<0.05$) than PY79 control groups after the third immunisation dose. Levels of IgA are significantly higher ($p<0.05$) in immunised groups compared with respective PY79 control groups after fourth and fifth dose of PP108 spores. Both routes of spore delivery require the same number of doses to induce a significant mucosal IgA response.

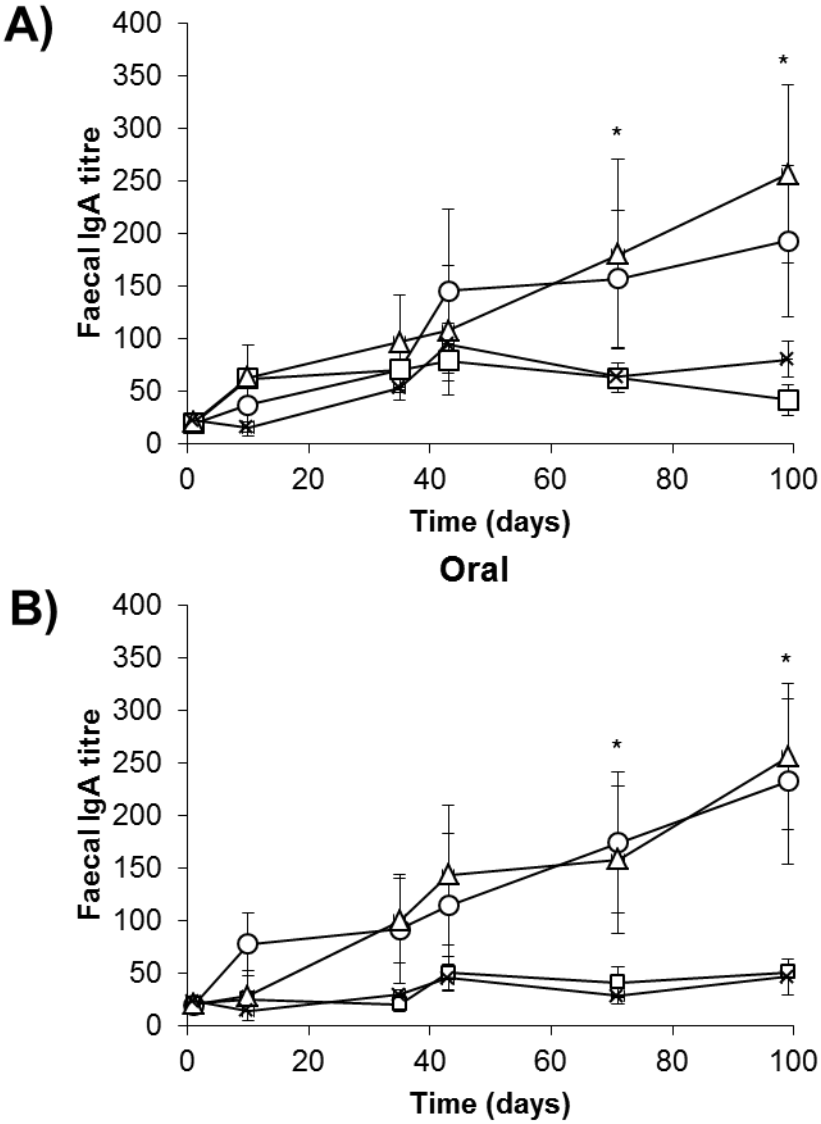


Figure 3.7. A26-39 specific IgA measured from faecal sample extracts using an ELISA. Seroconversion is shown over the immunisation schedule time period in groups receiving A) sublingual or B) oral doses. Doses contained live PP108 (Δ), live PY79 (□), inactivated PP108 (○) or inactivated PY79 (X). * indicates where immunised groups are significantly different from PY79 control groups ($p<0.05$)

Immunisation via mucosal routes gives the potential for systemic responses to be generated concurrently with localised mucosal response. Therefore, antigen specific IgG in blood serum samples was measured throughout the immunisation period to ascertain if a systemic response was also generated. Results show that immunisation via both routes stimulated an antigen specific IgG response (**Figure 3.8**), although this is slightly higher in the sublingual groups (**Figure 3.8**). Mice immunised sublingually with live PP108 spores demonstrated the highest levels of specific IgG after the fourth dose of spores (**Figure 3.8A**). Sublingual immunisation with inactivated spores generated a limited response and specific IgG level decreased after the fourth dose of spores. Despite this decrease, immunised groups displayed a significantly higher response than PY79 control groups from the third dose ($p<0.05$). Orally dosed groups also demonstrated an antigen specific IgG response. Similar to sublingual immunisation groups, groups immunised orally with inactivated spores also show a decrease in response at the final doses (**Figure 3.8B**). Following the pattern set by sublingual groups the lives spore doses generate the highest IgG responses. In both sublingual and oral groups an effect greater than in the PY79 control groups is not achieved until after the third immunisation dose. After the fourth and fifth dose of spores responses from immunised groups were higher than PY79 control groups ($p<0.05$), although this is an aspect of the vaccine that could be improved on as levels remain quite low. At the same time points (fourth and fifth dose) IgG titre from animals dosed with live PP108 spores was higher than those dosed with inactivated spores ($p<0.05$).

The timing of immune response generation is similar between mucosal and systemic responses, both requiring at least three spore doses to generate a significant response.

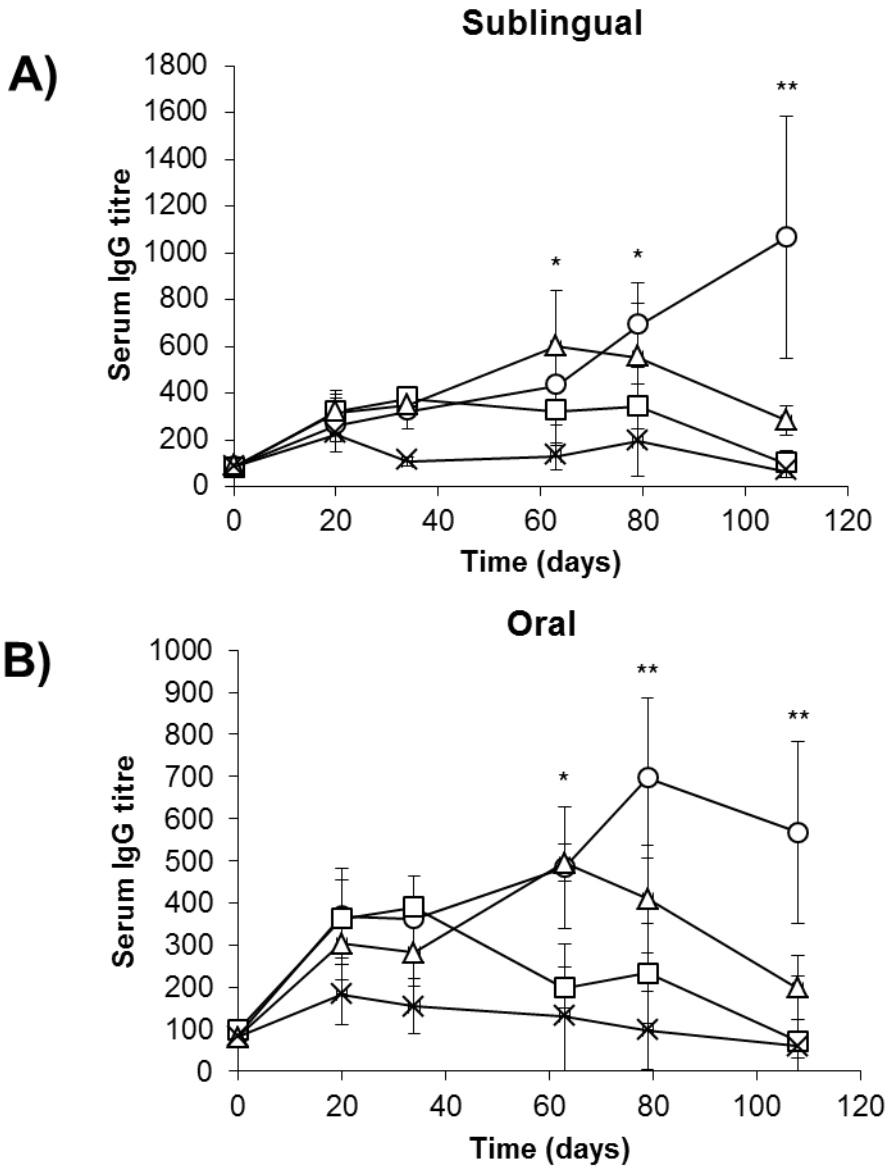


Figure 3.8. A26-39 specific IgG from serum samples was measured using ELISA. Seroconversion is shown over the immunisation schedule in groups receiving A) sublingual or B) oral doses. Doses contained live PP108 (○), live PY79 (□), inactivated PP108 (Δ) or inactivated PY79 (X). Significance when immunised groups are significantly higher than PY79 control groups * = $p<0.05$, ** $p<0.01$.

To ascertain immune responses at the point of challenge, responses at the end point of the immunisation schedule were analysed. In all immunised groups both sublingual and oral delivery of PP108 initiated a significant mucosal immune response (**Figure 3.9**) in comparison to PY79 control groups ($p<0.001$). In sublingually immunised groups inactivated PP108 generated a higher specific IgA response than live PP108 (**Figure 3.9A**), however this is not statistically significant at this sample size ($p=0.09$). In oral groups (**Figure 3.9B**) inactivated PP108 spores also generated only a marginally higher response than live spores ($p=0.5$). Immunisation with inactivated spores delivered sublingually resulted in the highest specific IgA response (**Figure 3.9A**). This result supports the theory that a mucosal response is required for protection against CDI. Levels were not significantly different between PP108 immunised groups, however, so it is likely that there are several mechanisms that contribute to generating protection against CDI.

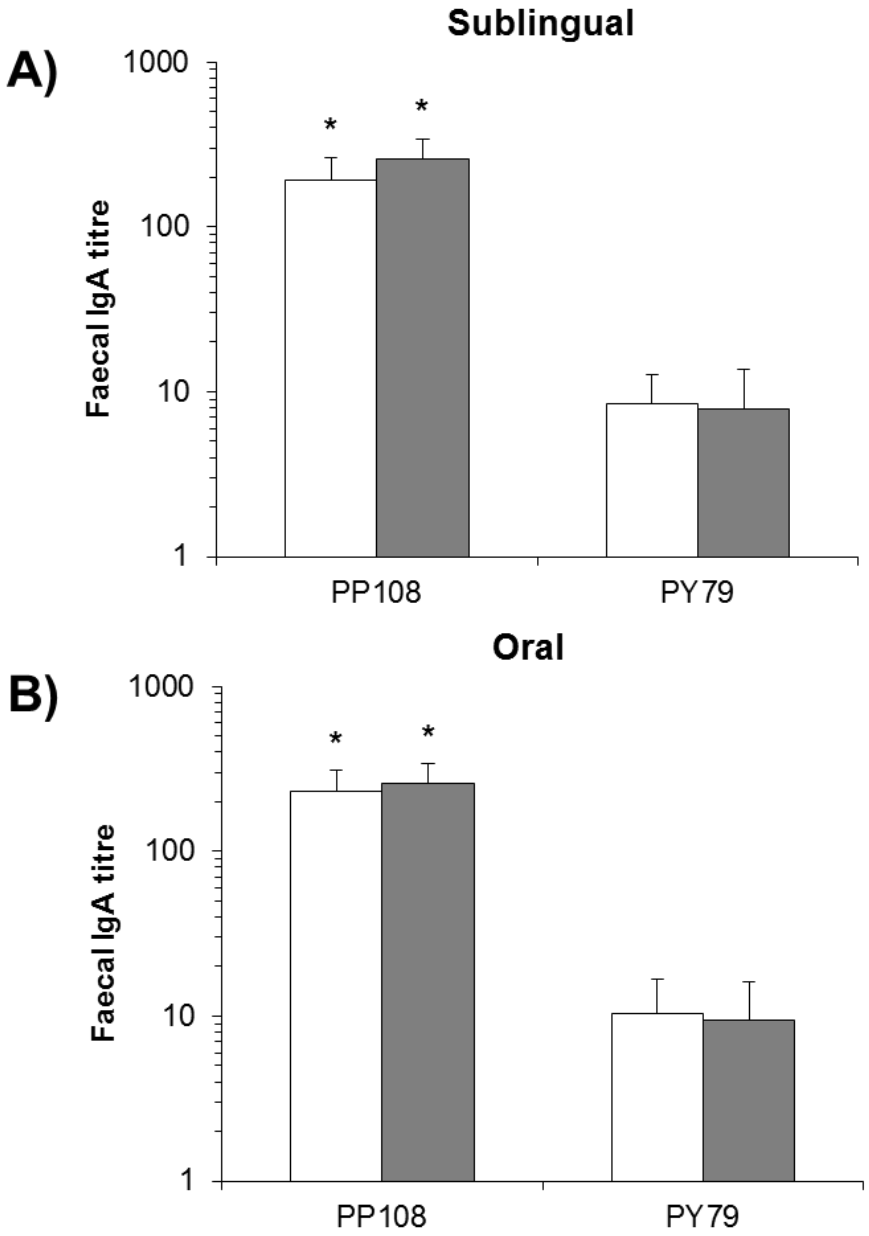


Figure 3.9. **A26-39 specific IgA measured from faecal sample** extracts of A) sublingual and B) orally immunised mice at the end point of immunisation schedule (after 5th dose) using an ELISA. Groups were immunised with live spores (white bars) or formaldehyde inactivated spores (grey bars). Significance when immunised group response is significantly higher than respective PY79 control group, * = $p<0.05$.

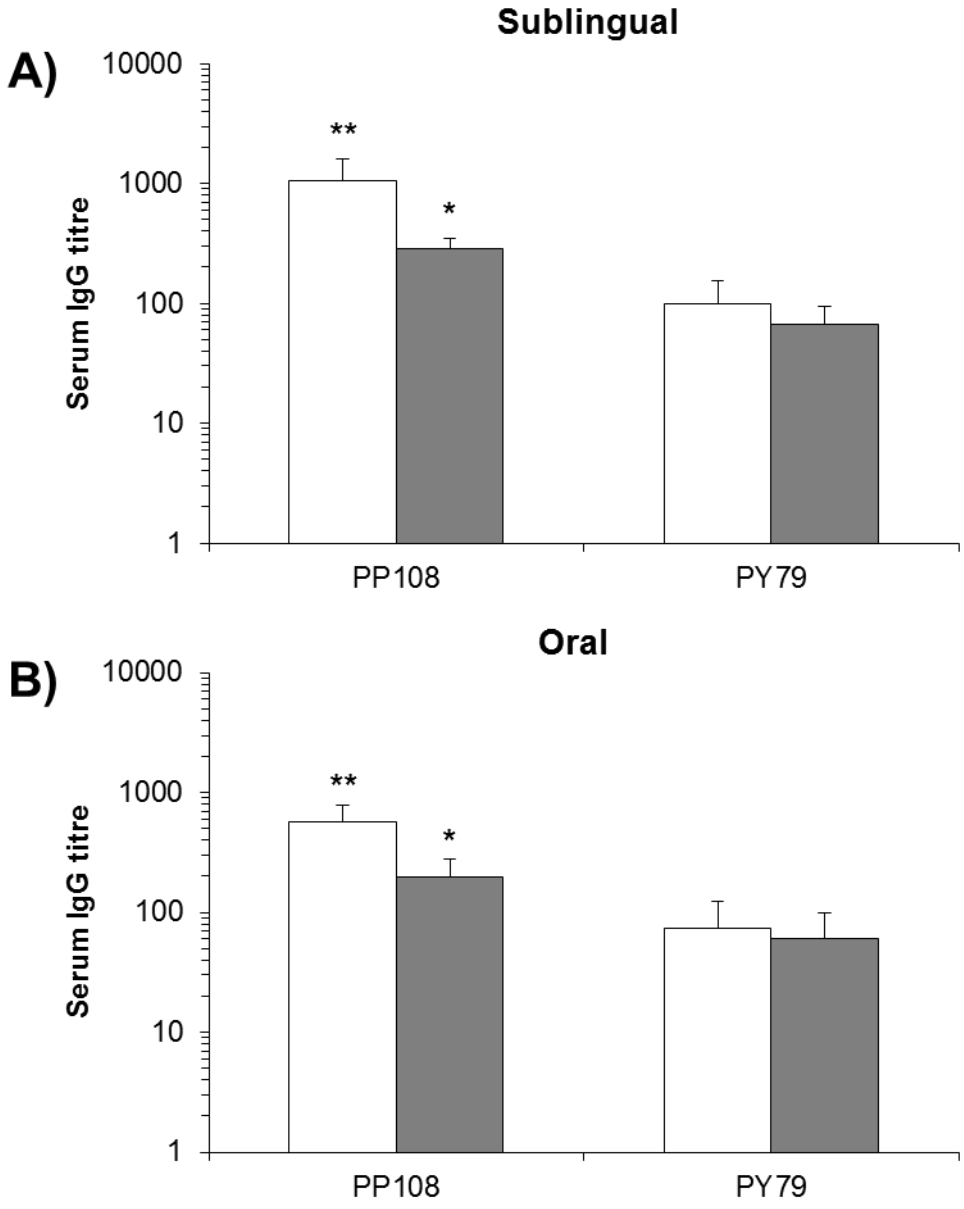


Figure 3.10. A26-39 specific IgG levels in serum samples from A) sublingually and B) orally immunised mice at end point of the immunisation schedule (after 5th dose), measured with an ELISA. Groups of mice received either live spore doses (white bars) or formaldehyde inactivated spores (grey bars). Significance when immunised group response is significantly higher than respective PY79 control group, * = $p<0.05$, ** = $p<0.01$

Final IgG titres were assessed in immunised groups (**Figure 3.10**). In all immunised groups the specific IgG response was significantly higher than in PY79 controls ($p<0.01$ for all groups except inactivated oral doses, where $p<0.05$). While these results are significant, the levels of IgG reported could be an area of improvement for this vaccine candidate. In comparison of live and inactivated PP108 spore immunisations, live spores were responsible for higher levels of specific IgG with both delivery methods (sublingual $p=0.004$, oral $p=0.03$). This deviates from mucosal response data where the highest response was generated by sublingual dosing of inactivated spores (**Figure 3.8A**). The highest specific IgG titre was found in the group immunised sublingually with live spores (**Figure 3.10A**).

To control for the difference in dose regimen between oral and sublingual groups, included in the study were groups that received the oral dose split over three doses (**Figure 3.11**). This enables detection of any changes in response due to dose regimen rather than the immunisation route. No significant difference was evident in mucosal IgA responses from these oral control groups ($p>0.1$). With the oral use of inactivated spores a slight overall decrease in IgA levels was observed in control groups (**Figure 3.11B**). This observation was not replicated in the live spore control group (**Figure 3.11A**).

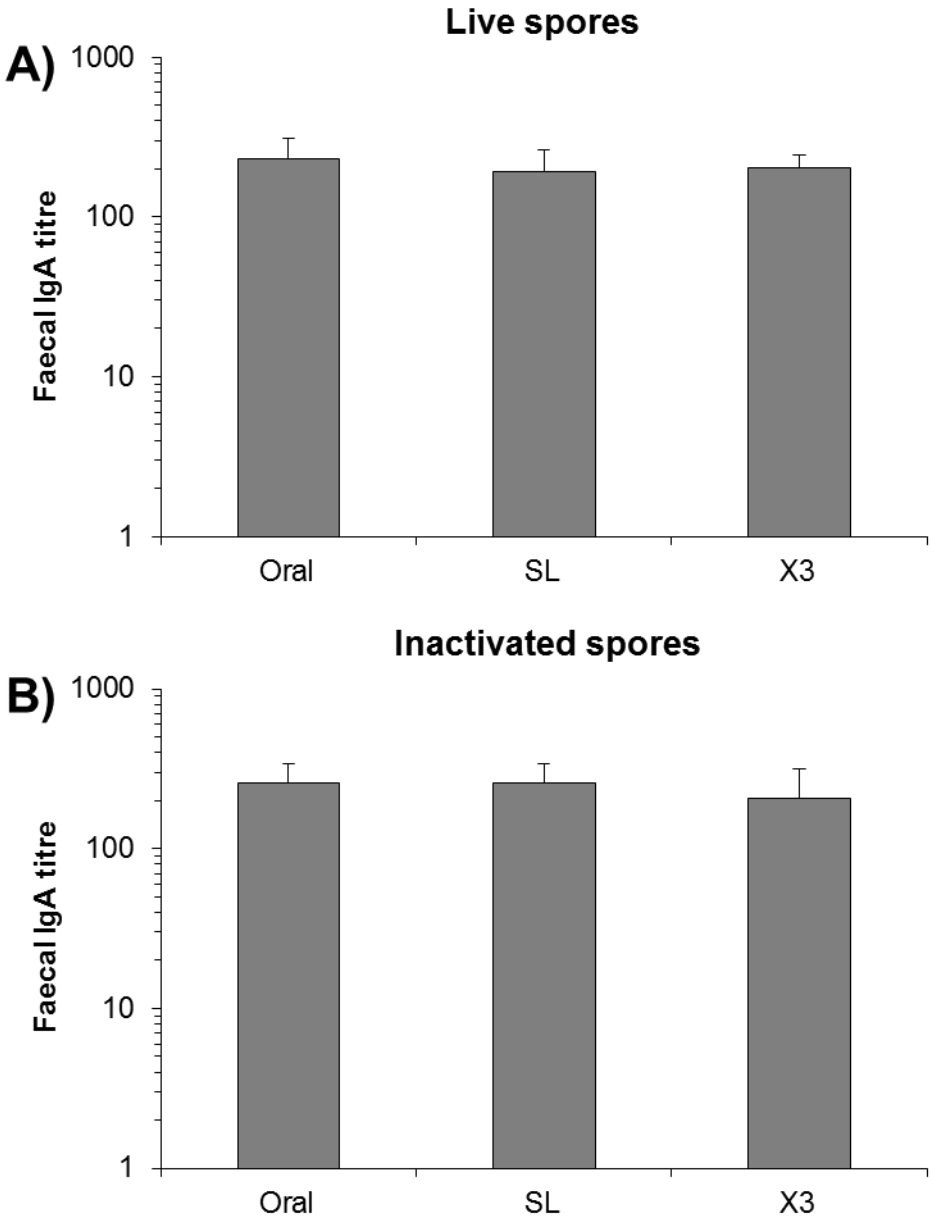


Figure 3.11. Sublingual control groups; A26-39 specific IgA measured from faecal sample extracts of mice immunised with A) live and B) inactivated PP108 spores, at the end point of immunisation schedule using an ELISA. Sublingual doses were administered in three consecutive daily doses due to volume restrictions in dose method, so oral control groups received PP108 spores over three consecutive days (X3) in separate doses to replicate the sublingual delivery. No significant differences exist between groups.

Similarly to mucosal responses, IgG responses in sublingual dosing control groups, where the oral dose was split over three days, were assessed (**Figure 3.12**). In groups receiving live spores, specific IgG levels between groups were not significantly different ($p>0.1$). With the use of inactivated spores in immunisations, the sublingual control group receiving the oral dose as three consecutive daily doses showed higher specific IgG levels ($p<0.05$) than both sublingual and standard oral dosing (**Figure 3.12B**). Levels of antigen specific IgG were lower in groups immunised with inactivated spores than in groups immunised with live spores.

IgG subclass ratio was calculated from IgG1 and IgG2a content of serum samples from immunised mice. This ratio is classically used to give an indication of whether an immune response demonstrates a T_H1 or T_H2 bias.

According to the ratios calculated, neither delivery route displayed consistency towards a particular bias in response (**Figure 3.13**). Sublingual doses with live spores produced an IgG subclass ratio indicative of a T_H2 response, while a decreasing ratio in the sublingual inactivated spore group represents a T_H1 response (**Figure 3.13A**). Ratios from oral groups showed contrasting results, with live spores producing a lower subclass ratio indicative of a T_H1 response, but inactivated spores producing a higher ratio (**Figure 3.13B**).

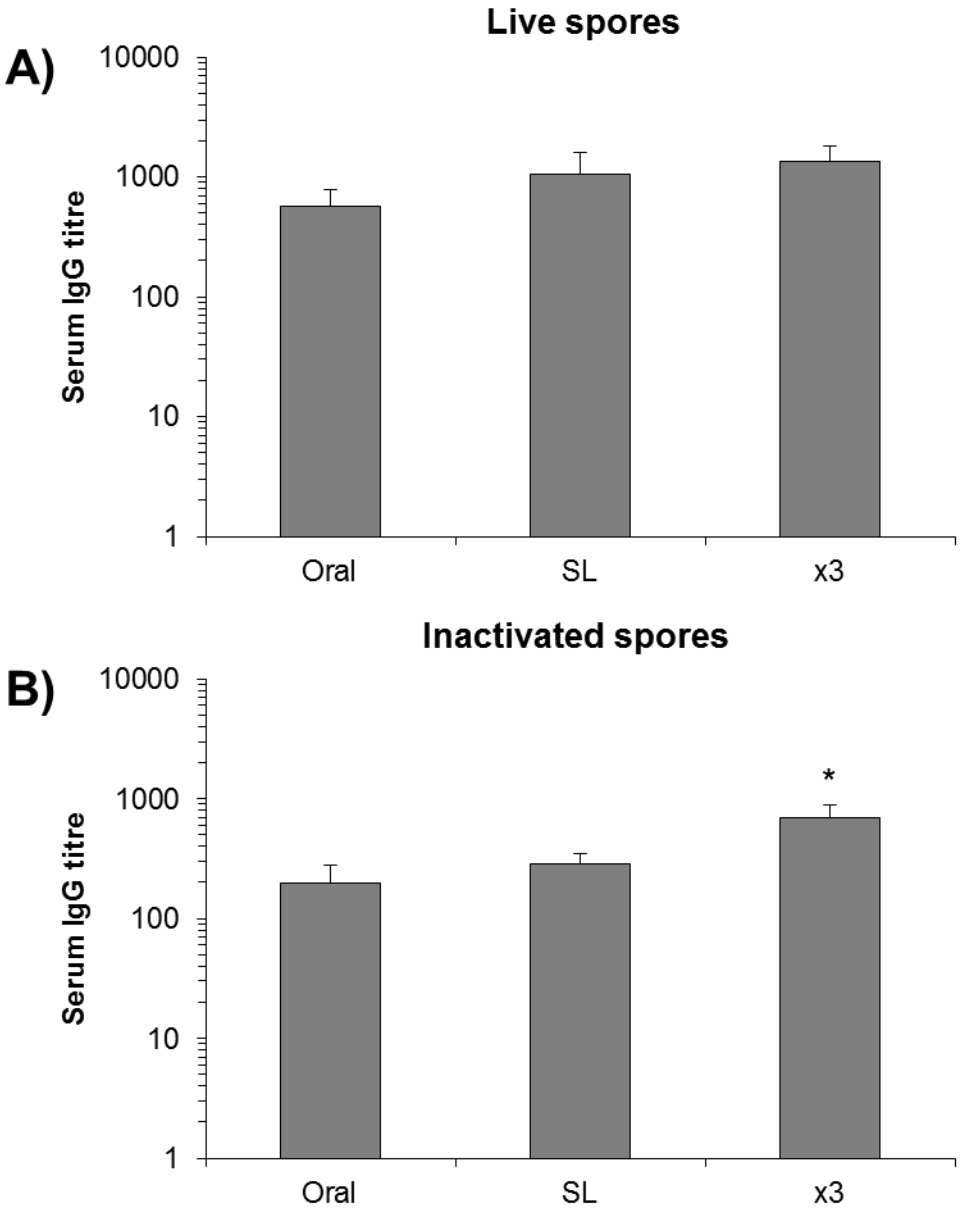


Figure 3.12. Sublingual control groups; A26-39 specific IgG levels in serum samples from mice immunised with A) live and B) inactivated PP108 spores at end point of immunisation schedule, detected via ELISA. Sublingual doses were administered in three consecutive daily doses due to volume restrictions in dose method, so oral control groups were used, with dose delivered over three consecutive days (X3) in separate doses to replicate the sublingual delivery. * shows a significantly higher IgG response, $p<0.05$.

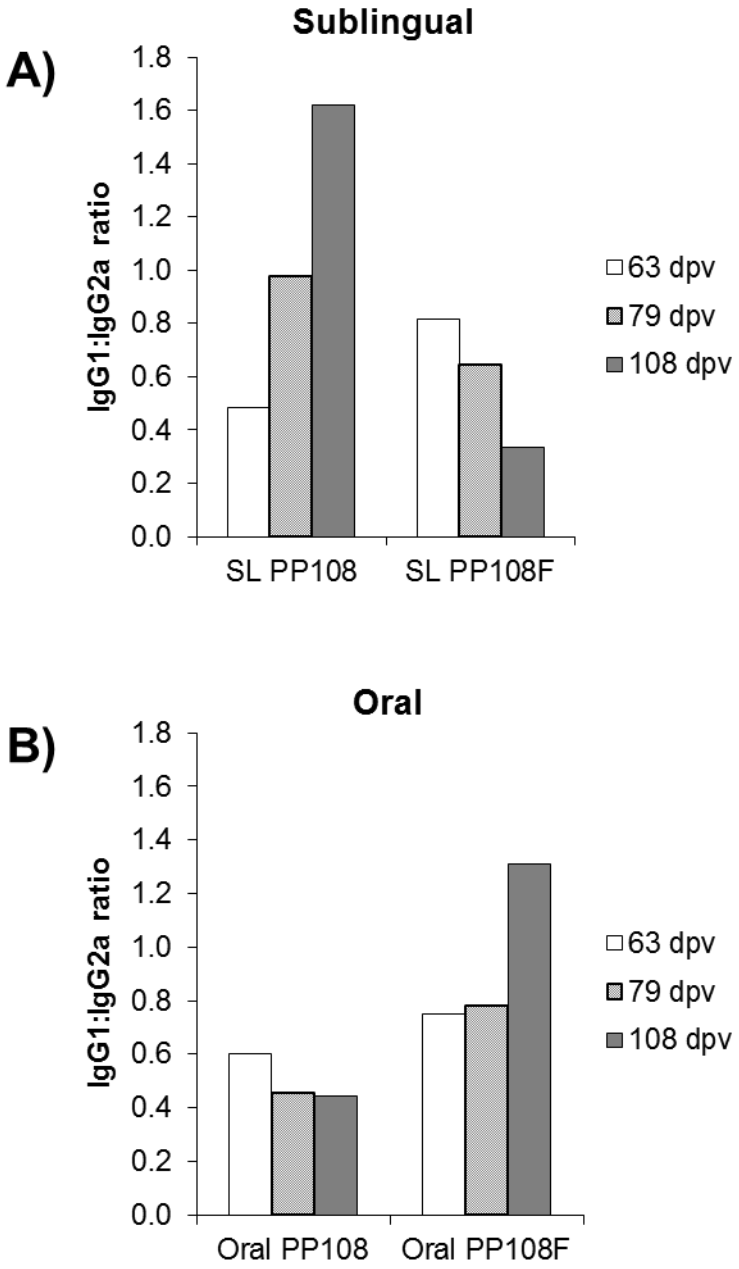


Figure 3.13. IgG1:IgG2a ratios in serum samples from A) sublingual and B) orally immunised mice. Ratios are shown over time course of immunisations, at 63 (white bars) 79 (hatched bars) and 108 (grey bars) days post first vaccine dose (dpv).

To further analyse the response to this vaccination, spleen cells were isolated from immunised animals and stimulated *in vitro* with rA26-39 protein over 96hrs. The supernatants from stimulated cells were then analysed for cytokine content (**Figure 3.14**) using a flow cytometry kit optimised for this application (CBA kit, BD Biosciences). The dominant cytokine in all samples was TNFα, primarily a T_H1 associated cytokine. Levels of IL-6 were lower in two groups: animals dosed sublingually with inactivated spores and the group that were immunised orally with live PP108 spores. Both these groups also demonstrated low IgG subclass ratios.

Interestingly, IL-10 was only present in the supernatant of cells from mice immunised with live PP108 spores (**Figure 3.14A and 3.14C**). IL-10 plays a role in regulation of inflammatory responses and control of the T_H1 response. Of note, IL-6 is a cytokine involved in stimulation of IgA responses. Groups immunised sublingually showed the highest levels of this cytokine, in particular animals receiving inactivated PP108 spores (**Figure 3.14B**). The sublingual inactivated PP108 group also showed the highest level of protection (**Table 3.1**) lending support to the hypothesis that development of mucosal immunity is important in protecting against CDI. The lowest protection rate was seen in animals that received oral doses of inactivated PP108 spores. This group also produced few cytokines, with TNFα being the only one of note (**Figure 3.14D**).

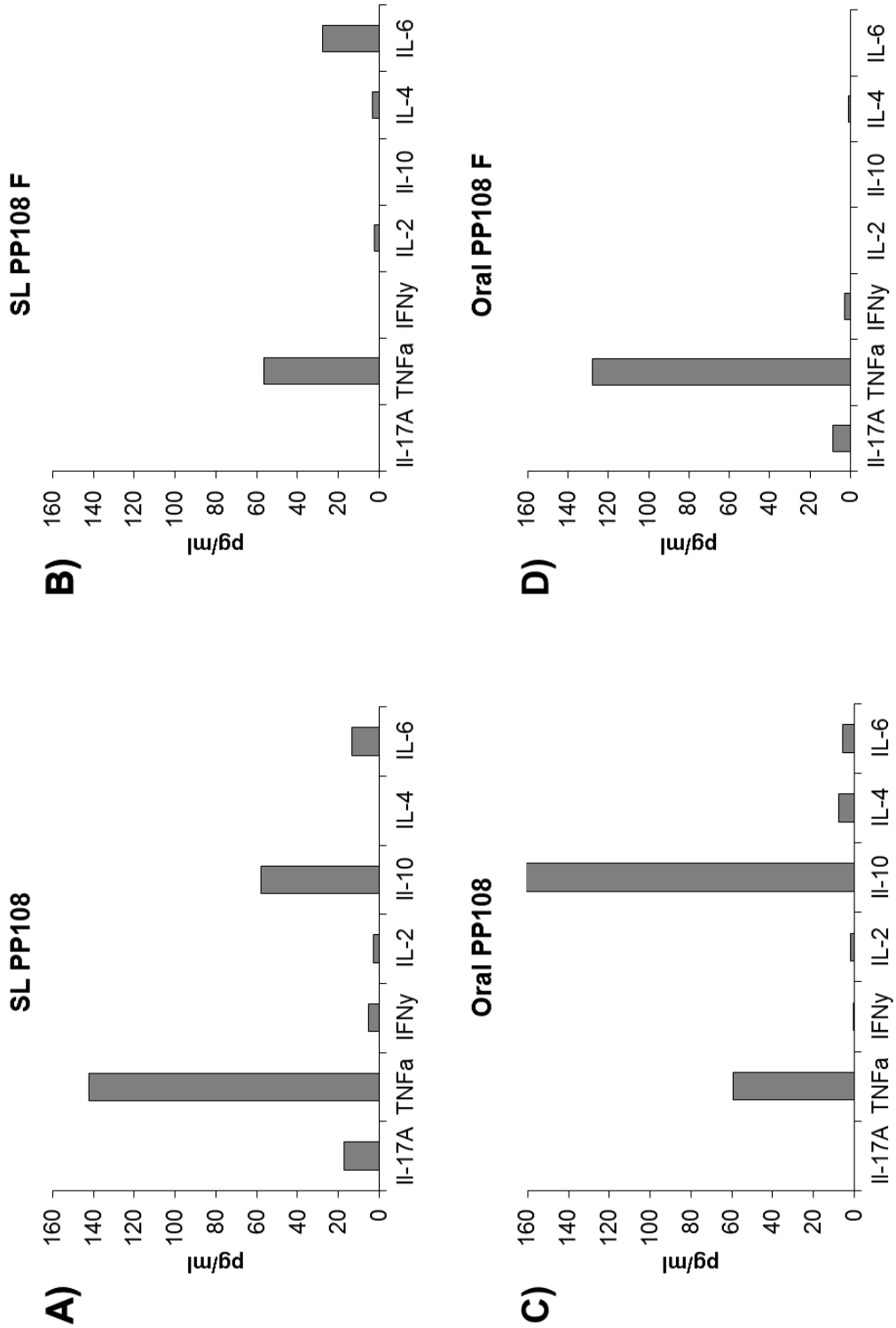


Figure 3.14. Cytokine levels (pg/ml) in supernatants of stimulated spleen cells from immunised mice, incubated with 1µg/ml rA26-39 for 96hrs. Commercial FACs kit (CBA kit, BD) was used to enumerate specific cytokine content of supernatants, calculated against standards in kit.

3.3. Discussion

This work has shown the successful use of a spore based vaccine in generating both mucosal and systemic immune responses, resulting in protection against severe symptoms of CDI in a murine model. Of particular interest was how the route of immunisation and viability of PP108 spores affected the generation of an immune response. This will be discussed alongside the implications for development of mucosal vaccines.

3.3.1. Induction of mucosal responses

Development of a successful vaccine presents many challenges, from finding an effective antigen to the use of the correct adjuvant and employing the best dose regimen. This study has shown that with both sublingual and oral dosing, robust mucosal responses can be seen through production of antigen specific IgA.

Immunisation with the PP108 spore vaccine induces a response against *C. difficile* toxin A, which is sufficient to provide protection against a strain producing both toxin A and B. Previous studies with this vaccine have shown that antibodies against the toxin A fragment additionally recognised toxin B (Permpoonpattana *et al.* 2011a). In the same studies, oral spore doses were shown to be a successful route and method for antigen delivery, resulting in generation of protection. Oral immunisation with the spore vaccine was demonstrated to be superior to the protection generated by injection of the antigen without a supporting adjuvant. Oral delivery of a vaccine is a well-documented route for production of a mucosal response, the oral poliovirus (OVP) vaccine an excellent example of this (Sabin 1985; Holmgren & Czerkinsky 2005). However, the use of the oral route for delivery of a vaccine

has many potential pitfalls, including exposure of the antigen to extremes of pH and degradative enzymes as well as inaccurate delivery to mucosal surface. Development of tolerance to the antigen delivered via this route is also a potential issue. Tolerance to specific antigens that are present as part of our bodies or everyday diets for example, is required to keep the normal state of homeostasis within the GI tract. However, this mechanism could also allow specific antigens delivered as a vaccine to be ignored by the immune system. Therefore this needs to be taken into account especially when designing a mucosal delivery system for a vaccine. Investigation of alternative routes of immunisation will contribute to development of the vaccine in order to provide the best possible response. The work in this study has shown that sublingual dosing of the spore vaccine is a viable and in some respects a superior method of vaccine delivery. Sublingual delivery provides a more direct method of antigen delivery and reduces the exposure of the antigen to the potential degradation that transit through the G.I. tract is associated with. Comparison of protection rates in the challenge study showed that sublingually immunised groups had fewer symptoms than their orally immunised counterparts. More detailed comparison of the dose routes show that sublingual immunisation produces a comparable level of IgA response but also a higher titre of systemic antigen specific IgG. Sublingual doses use fewer spores: the total sublingual dose uses $6x10^9$ spores compared to the $4x10^{10}$ spores in an oral dose. Therefore higher responses are produced from significantly less vaccine presenting sublingual delivery as a more economical route for use of this vaccine. In the comparison of the two delivery routes, the data of this study present sublingual as the superior delivery route. The focus of this study was mucosal immunisation routes, so there is no comparison with a systemic based vaccination. This comparison,

however, would be of interest when considering the broader scope of the immunisation process.

The importance of developing a vaccine to incorporate a mucosal immune response is highlighted by the current situation in CDI management. Antibiotics are becoming less effective and a vaccine would aid the control of this disease. However, despite the focus on parenteral immunisation there is currently no commercial vaccine product available for use. Perhaps a change of perspective for the development of vaccines is required. In a review on mucosal immunity and vaccines, studies show that other pathogens such as HIV, Human Papillomavirus, *Vibrio cholerae* and *Mycobacterium* species require a mucosal response for effective protection (Holmgren & Czerkinsky 2005). A study using mice with specific defects in their immune system suggests that generation of a mucosal response is important to protection from CDI, although not essential (Johnston *et al.* 2013). The authors noted that immunocompetence of the host will play an important role in the mechanism of protection during infection. This is an important factor in the development of a vaccine to prevent CDI, where the target population is elderly and likely to have reduced immunocompetence (Grubeck-Loebenstein *et al*. 2009). Understanding how *C. difficile* interacts with the host, especially immune interactions during infection will contribute towards defining the required response from vaccination. Longevity of immune responses from specific vaccines will also be of interest.

Protection generated by immunisation with PP108 spores was highest in animals that received inactivated, sublingual doses. This group also demonstrated the highest level of IgA production. However, in this study, the highest IgA producers are not the highest IgG

producers. Sublingual delivery of inactivated spores gave better protection and higher IgA levels in immunised mice, but sublingual, live spores induced a higher IgG response when the groups are compared. As the highest level of protection is provided by the inactive spores in the sublingually immunised groups, it could be argued in this case that the production of IgA is the primary protective factor.

Comparison of the orally immunised groups shows that live spores produce higher levels of IgG than inactive spores, despite similar levels of IgA being produced. Live spores in this case produce a higher protection rate, so while IgA production is important for protection, the more robust systemic response from live spores gave this group a boost in protection. Increased protection from live, orally dosed spores could also be attributed to a probiotic effect, as *B. subtilis* spores can colonise the mammalian gut.

This result implies that the balance of the response generated by immunisation is important in providing protection. The results of this study do not allow either the mucosal or systemic response to be solely attributed to generation of protection. However, this does highlight the necessity to consider multiple aspects of the immune response when considering the success or mechanisms of a vaccine.

3.3.2. Improving the vaccine

Mucosal immunity is important in generating a local response at the site of infection and providing a barrier to invading pathogens. Production of IgA and innate mucosal receptors contribute to protection from infection at the mucosal surface (Fagarasan & Honjo 2003). Both routes of immunisation used in this study were shown to induce production of specific IgA in the GI tract. Results from the challenge study using *C. difficile* R20291 show that the

response generated in PP108 immunisations gave protection from the symptoms of infection. However, analysis of stool samples from immunised mice revealed that high levels of spores were present. The PP108 vaccine does not prevent the colonisation of *C. difficile* in the murine model, so dissemination of infectious spores from infected animals can still occur. Risk of spreading the infection is reduced when symptoms are prevented, but colonised asymptomatic individuals form a carrier population that acts as a reservoir for *C. difficile* infection. A more comprehensive solution is required to fully control this disease, preventing colonisation so that reservoirs of *C. difficile* can be eradicated. The PP108 candidate vaccine uses a toxin antigen as a basis for protection, but the spore based delivery system could be extended. The spore vaccine model is a flexible platform and could be engineered to express more than one antigen allowing for a multivalent approach. A multivalent vaccine that could produce toxin neutralising antibodies and be active against colonisation would fully address the problem presented by *C. difficile* infection (Pechine *et al.* 2007). To reduce colonisation, a cell or spore based antigen is required for generation of a targeted immune response. The benefit of creating an immune response against the spore, which is the infecting agent in this disease (Deakin *et al*. 2012), is that the infection can be curtailed before progression to fulminant disease. A vaccine focusing solely on neutralisation of toxins does not address the complex nature of this infection. For example, in a comparison of different *C. difficile* strains in a hamster model it was shown that infection with a non-toxigenic strain resulted in some gut pathology (Buckley *et al*. 2013). Gut injury in the absence of toxin production highlights the need to think of *C. difficile* infection in broader terms than just the ability to produce toxins.

The mechanism of this vaccine platform uses fusion with the spore coat proteins CotB and CotC for display of antigens. However, the recent discovery of the crust layer, covering the

spore coat of *B. subtilis* may hinder the display of the antigens (McKenney *et al*. 2010). Therefore to improve the vaccine by increasing exposure of the antigen, a ΔcotXZY strain *of B. subtilis* could be used, as this mutation would prevent formation of the spore crust.

3.3.3. Mechanisms of immune stimulation

The highest rate of protection against CDI symptoms in this study was reported in animals that received sublingual doses of inactivated PP108 spores. This method of immunisation resulted in production of significant amounts of antigen specific mucosal IgA and systemic IgG as well as induction of TNFα and IL-6 cytokine responses. Activation of the mucosal immune system in the gut is key to the premise of this study. However, care needs to be taken with balance of immune responses as over stimulation of inflammatory responses across mucosal surface could occur (Cerutti *et al.* 2011). An inflammatory environment could aid CDI rather than preventing this infection.

Using data analysing IgG subclasses and *in vitro* cytokine production a T cell response bias towards either a cellular (T_H1) or humoral (T_H2) response can be assigned. *B. subtilis* spores have previously been shown to produce a broad range of responses when dosed either intranasally or oro-gastrically (Barnes *et al.* 2007; Huang et al., 2010). No consensus in type of response is apparent between either routes of dosing or viability of the PP108 spores. This implies that mechanisms behind oral and sublingual routes for immunisation could use different pathways despite both having a mucosal basis. Alternatively, inactivation of the spores could also result in a variation in the mechanism employed in generating an immune response. A consistent pattern of IgG subclass ratio and cytokine expression would make identifying important mechanisms for protection more straight forward, but it is evident

from these data that generalisations between different mucosal routes cannot be made. It should also be pointed out that IgG levels, while significantly higher than the control groups, were quite low. It could be that low levels of IgG potentially confound results.

Cytokine expression profiles of stimulated splenocytes from immunised mice were also included in the analysis of immune responses. *In vitro* experiments showed that only groups that had received live PP108 spore immunisations produced detectable levels of IL-10. The group with the highest level of protection received inactivated spores, and demonstrated no production of IL-10. These observations suggest that this cytokine is unlikely to be required for protection but more likely to be associated with a probiotic effect (Hart *et al.* 2004; Di Giacinto *et al.* 2005). However, links between probiotics and immune modulation are likely to be strain specific so conclusions from other studies should be considered carefully. Links to a probiotic effect raises the question of whether the PP108 strain can also colonise the murine gut during immunisation doses and exert effects through this means. Colonisation of the PP108 strain was not a focus of this study, but *B. subtilis* spores have previously been shown as able to germinate and reproduce in the murine G.I. tract (Hoa *et al*. 2001). The presence of IL-10 in the groups immunised with live spores is of interest, with particular reference to regulation of homeostasis. IL-10 is an anti-inflammatory cytokine that is thought to be essential for immunoregulation in the GI tract. Indeed, IL-10 has been proposed as an option for treatment of dysregulatory bowel conditions such as Crohn's Disease and IBD (Steidler *et al*. 2000; Braat *et al*. 2006). It should be noted that delivery of IL-10 to the required region is one of the issues in developing this as a treatment. Induction of IL-10 in treatment of colitis is also linked with use of some strains of probiotic or commensal bacteria (Di Giacinto *et al.* 2005; Pils *et al*. 2011). IL-10 is also noted for its ability to inhibit synthesis of pro inflammatory cytokines from regulatory

T cells, which in the context of *C. difficile* infection may be a property of the vaccine worth exploiting. The live spore groups did not display the highest levels of protection in the challenge study and only groups immunised with the live spore vaccine displayed production of IL-10 from stimulated splenocytes. However, a mechanism that has potential to reduce or control an inflammatory response should be a consideration in the delivery of this vaccine candidate.

Detection of IL-6 was interesting as it is linked to IgA production (Ramsay *et al*. 1994). Mice immunised sublingually with inactivated PP108 spores showed both the highest level of protection and highest IL-6 production. This indicates that inducing production of IL-6 could be an important factor for this vaccine strategy, strengthening the argument for importance of a mucosal response (Johnson *et al*. 1995; Stubbe *et al*. 2000; Permpoonpattana *et al*. 2011*a*). TNFα was generated by all groups but was lower in sublingual inactivated spore group suggesting that increased levels of this pro inflammatory cytokine may hinder generation of protection.

3.3.4. Immobilisation of antigen

Interestingly, significant protection was demonstrated with the use of inactivated spores. Use of inactivated spores allows the vaccine strain to be used without issues associated with the use of genetically modified (GM) organisms (HSE 2007). In assessment of spore inactivation protocol, it was shown that formaldehyde is an effective agent for inactivation. Gluteraldehyde was investigated as an alternative sporicidal agent to formaldehyde (Power & Russell 1990; Tennen *et al.* 2000), but gluteraldehyde inactivation appeared to reduce immunogenicity of PP108 spores. The incubation time of spores in the formaldehyde

solution is linked to the success of inactivation. Treatment over 24 hours with 4% formaldehyde was less effective than shorter incubations, potentially due to the stability of formaldehyde in solution at 37°C. When inactivated spores were stored at 4°C and then tested for viability, it was shown that complete inactivation was not permanent, with an increase in spore recovery after a week. The increase in recovery was minimal but in a commercial product, this observation would need addressing to avoid a reduction in the amount of antigen present. As the spore vaccine is a GM organism, use in a commercial setting would require complete and permanent inactivation in order to avoid regulatory issues associated with GM organisms. It is also possible that the *in vivo* environment would encourage reversal of the inactivation and germination of spores could occur, although this was not studied in this work.

Spores inactivated with formaldehyde are unlikely to germinate so it is unlikely that protection from the immunisation with inactivated PP108 spores relies on a probiotic mechanism. Orally delivered inactivated spores showed the lowest rate of protection in this study however, implying a benefit of live spores when utilising the oral route of delivery. Germinating spores may contribute to interactions with the microflora in the gut, increasing protection through a probiotic action. It is also possible that live spores germinate (Hoa *et al*. 2001) and could remain in the gut for a prolonged period going through vegetative growth cycles. This would allow for increased exposure to the antigen when spores are produced from vegetative growth. Persistence in the gut of the PP108 strain was not investigated in this study, so prolonged exposure to the antigen is purely speculative. Sublingual delivery of the vaccine avoids swallowing so does not rely on probiotic mechanisms to aid protection and the increased protection rate suggests that inactivation with formaldehyde increases the immunogenicity of the PP108 spores.

Formaldehyde inactivation results in the stabilisation of antigens (Rappuoli 1994), potentially aiding interaction with immune cells and therefore the induction of an immune response. Use of non-viable spores has the added benefit of not interfering with existing microflora as spores will be unable to germinate and initiate vegetative growth.

3.3.5. Vaccine delivery regimen

The method of sublingual dosing used in this study lead to an interesting observation with regard to importance of dose regimen. In mice, small body size means that sublingual doses are limited by volume. This meant that sublingual immunisation doses were delivered in separate doses over three days (3 x $2x10^9$ spores). A control group for oral dosing was added to the study, dividing delivery of the oral dose over three days (3 x $1.3x10^{10}$ spores). This alteration of the oral dose regimen resulted in an increased specific IgG response. This effect was significant with inactivated spores demonstrating that spores do not need to be viable, but that optimal dose regimens will be different for live and inactivated spores. The increase in IgG response with smaller, more frequent doses emphasises the importance of validating the dose regimen. More frequent exposure to smaller amounts of the antigen could be used as a method of boosting immune response in the case of this vaccine candidate. Timing of immunisation doses has been linked to the development of immunological memory (Bakke *et al*. 2004) and should be considered an important optimisation factor in vaccine development.

3.3.6. Use of a CDI vaccine

This study has shown that the PP108 spore based vaccine can provide protection against symptoms of CDI. Use of mucosal delivery routes is a key aspect of the study and provides several benefits. Use of needles is avoided in the delivery of this vaccine and both systemic and mucosal aspects of the immune system are activated. The spore based delivery system serves as a stable and safe adjuvant for the antigen that avoids the use of traditional toxin based adjuvants can have associated safety issues (van Ginkel *et al*. 2000).

Sublingual delivery of this vaccine has several benefits over the oral route. Firstly, fewer spores are used to create a similar or improved immune response. This reduces the cost of a potential product as the same amount of spores would be able to produce more doses of the vaccine. Secondly, the ease of dosing is increased. Oral dosing is a simple procedure, not requiring medical professionals for administration, but if patients have difficulty swallowing this could present a complication. Sublingual doses are placed under the tongue to be absorbed by the associated mucosa, eliminating the need for swallowing a tablet or liquid dose. The global scheme for the eradication of polio seems a grand comparison to make with the need for an effective *C. difficile* vaccine but it does highlight the importance of easy delivery of a vaccine. The eradication scheme has been so successful in part because of the minimal expertise required for delivery of a dose, to the point where vaccinations can be carried out by volunteers with little or no medical training (Koinange *et al.* 1973; Hull *et al.* 1997). Having more people able to give the vaccine means more people can be reached with the vaccine. The issues now faced in completing the eradication program are mostly political, although vaccine derived poliovirus outbreaks are a concern (Kew 2012). *C. difficile* will be difficult to eradicate as a human pathogen, as reservoirs of infection are currently poorly defined (Walker *et al*. 2012). However, definite risk factors

associated with the disease and vulnerable populations have been identified (Johnson 2009; Loo *et al.* 2011), so those most in need of the vaccine can be focused on. The population most at risk of CDI are elderly, immune-compromised and often have other underlying health issues. CDI is most commonly associated with occurrence in hospitals. This presents a clear sub sect of individuals who would benefit from receiving a *C. difficile* vaccination. While a recent study suggests that transmission within hospitals is not as common as previously thought (Walker *et al*. 2012), *C. difficile* remains a key nosocomial pathogen even if the source of infection is not the hospital environment. Ideally, immunisation of individuals would be carried out before admission to hospital or a healthcare facility. This would aid prevention of CDI, whether the infection was contracted from the hospital or due to opportunistic infection by *C. difficile* carried asymptomatically prior to hospitalisation. Immunisation of the population on a larger scale would be expensive but could contribute to reducing reservoirs of carriers. To facilitate immunisations prior to hospital stays, therefore enabling protection to be in place before entering a health care environment, patient out care facilities or even pharmacists could prescribe and deliver the vaccine.

In summary, the results of this study show that both oral and sublingual routes of immunisation are promising options for use with the PP108 spore vaccine. Sublingual dosing presents an encouraging method for delivery of antigens. This study raises questions about the importance of different aspects of the generated immune response in protection against *C. difficile*.

3.4. Conclusion

3.4.1. Generation of protection from symptoms of CDI

The work presented in this chapter builds on a previous study, which focused on developing a vaccine candidate that produced mucosal responses against CDI (Permpoonpattana *et al*. 2011*a*). Results from immunisation of mice in this work have been used to demonstrate that both systemic and mucosal responses are generated when the PP108 spore based vaccine is employed. It should be noted however that while significant levels of IgG were produced in immunised animals, levels could be improved. Protection was also generated against symptoms of CDI in a murine model. Comparison of the two mucosal delivery routes showed that the two methods are similar in their ability to produce an immune response. Sublingual immunisation however, resulted in a greater level of protection in the murine model. The effect of inactivating the spore vaccine was especially interesting. When inactivated spores were delivered via the sublingual route a higher level of protection was generated than with use of live spores delivered by the same route. The effect, however, appears to be route specific as inactivated spores via the oral route gave a poor level of protection in comparison. This indicates that with live oral delivery, a certain aspect of the protection could be attributed to a probiotic mechanism.

Analysis of immune responses generated by the PP108 spore vaccine show that the resulting protection is likely due to multifaceted interaction with the immune system. Interactions and results vary depending on the method of immunisation. Unravelling the complexities of the immune response required for protection against CDI is still an ongoing

process. This study however, highlighted the potential for development in mucosal vaccine research.

3.4.2. Developing immunisation strategy

The immune response produced by the PP108 vaccine is based on the toxins of *C. difficile,* but it should be noted that variations in toxin gene have been reported between different strains. Indeed, toxinotyping is one method of differentiating between strains (Rupnik *et al.* 1998). Multiple strains need to be used in challenge studies to ensure no restrictions in protection exist based on variation in toxin sequence.

This vaccine produces immune responses against the toxins of *C. difficile* and therefore protects against the clinical manifestation of infection. However, it does not address the issue of colonisation by this pathogen. Induction of localised responses at the mucosal surface where *C. difficile* will be present could be used to protect not only from disease symptoms but also against colonisation. This would aid in reducing infections further by eradicating carriers from vulnerable populations so preventing the opportunistic nature of the pathogen. The PP108 vaccine could, for example, facilitate this strategy by being further engineered to display an antigen from the *C. difficile* spore coat. The ability to produce an immune response in the GI tract has been demonstrated in this study. If the response generated by a vaccine enabled eradication of *C. difficile* spores from the gut a huge step could be taken in control of this pathogen.

Chapter 4

Suppression of *Clostridium difficile* infection using *Bacillus subtilis* spores

This work has been published in part:

Use of *Bacillus subtilis* PXN21 spores for suppression of *Clostridium difficile* infection symptoms in a murine model.

Colenutt and Cutting, 2014. FEMS Microbiology Letters ePub 29th May.

4.1. Introduction

4.1.1. *Clostridium difficile* infection and current treatments

Cases of *Clostridium difficile* infection (CDI) have reduced since the introduction of mandatory reporting in 2007 (Public Health England 2013), mostly due to increased awareness of the disease and improved control measures. However, this reduction in cases has now plateaued and new methods of tackling the infection are needed. Current treatment regimens depend on use of the antibiotics vancomycin and metronidazole. Up to 30% of patients treated with these antibiotics experience relapses in infection (Barbut *et al*. 2000; McFarland 2002) and reduced antibiotic susceptibility has been reported in hypervirulent strains (Pépin *et al.* 2004; Valiente *et al.* 2012). New treatments are therefore

required that are more effective at controlling this pathogen. A newly developed antibiotic, fidaxomycin, has been shown to demonstrate a reduction in cases of relapse when compared to treatment with vancomycin (Louie *et al.* 2011). This represents an important step in improving treatment of CDI, but that antibiotic usage is a principal risk factor for contracting this disease should be kept at the forefront of research for novel treatments. Rising levels of antibiotic resistance are increasing pressure on medical resources so alternative options should be considered where possible in order to reduce both use and reliance on antibiotics.

C. difficile is listed as an urgent threat by the CDC based on the risk posed to human health by antibiotic resistance (CDC 2013a). This highlights the importance of alternative treatments for CDI and reducing reliance on antibiotic usage. Measures such as good antimicrobial stewardship would aid in control of cases (Vonberg *et al*. 2008). The issue of antibiotic resistance has received wide coverage in both scientific papers and the popular press. It is not a recent issue; scientists and doctors have expressed concerns about overuse of antibiotics for many years (Neu 1992). Despite this long term awareness, antibiotic resistance is an increasing problem and has the ability to cripple our medical system if managed wrongly. Firstly, inappropriate use of antibiotics can cause a patient more harm than good. Broad spectrum antibiotics will affect a significant amount of an individual's normal microbial flora, not just the pathogen causing disease. This dysbiosis of the microbial flora leaves the patient vulnerable to other infections, *C. difficile* being an excellent example here. Secondly, exposure to high levels of antibiotics has potential to drive evolution of resistance in bacteria. Most of the hospital 'superbugs' are considered as significant threats due to wide scale antibiotic resistance making effective treatment difficult (CDC 2013*a*). *C. difficile* is a textbook example of this, with only two antibiotics in

mainstream use for treatment of CDI and the appearance of 'hypervirulent' strains linked with increased antibiotic resistances (Drudy *et al*. 2007*a*). Methicillin resistant *Staphylococcus aureus* (MRSA) is another example superbug, still listed as a 'serious' threat to public health by the CDC despite falling numbers of infection, due to the limited treatment options for this infection once antibiotic resistances are taken into account (CDC 2013*a*). Certain antibiotics can also act as a predisposing factor to other infections; treatment with oral metronidazole and vancomycin (both used in treatment of CDI) increase the risk of colonisation and infection with vancomycin resistant enterococci (VRE), another nosocomial pathogen (Bhorade *et al.* 1999; Fridkin *et al*. 2001).

One of the more successful alternative treatments for CDI is stool transplantation, or bacterial therapy (Rohlke & Surawicz 2010; Gough *et al.* 2011). This involves reconstitution and implantation of faeces from a healthy donor into the gut of an infected patient. This treatment has been reported to have high long term success rates at resolving CDI (Brandt *et al*. 2012; van Nood *et al*. 2013), but comes with associated safety issues. Recently this process has been refined, with researchers creating 'synthetic stools' where a specific mix of bacterial strains is used in place of a donor stool (Lawley *et al*. 2012; Petrof *et al*. 2013). Bacteriotherapy based treatments could provide a therapy that avoids the use of antibiotics, or at least serves to restore the protective balance of the normal gut flora post antibiotic treatment. Identifying specific strains of bacteria that show efficacy in alleviation of particular symptoms is especially relevant to production of synthetic stool treatments. Alongside the commensal strains typically used in these treatments, it is plausible that probiotic strains found to have specific effects against disease symptoms could be incorporated in these treatments.

4.1.2. Use of probiotics

Probiotics are widely used in treatments and dietary supplements for purported health benefits. *Lactobacillus* and *Bifidobacteria* species are common in probiotic products, especially yoghurt or dairy based drinks that are supplemented with bacteria. The probiotics market is large, estimated to be worth upwards of €1.4 billion in Western Europe alone (Saxelin 2008). This includes probiotics used in animal feed, aqua culture and functional food for human consumption. Probiotics are incorporated into a variety of foods, including dairy products, meat products and cereals (Sanders & Marco 2010). Research into health benefits of probiotics has mainly focused on gastrointestinal disorders, but also extends to potential probiotic effects on skin conditions such as eczema and dermatitis (Boyle *et al.* 2008; Lee *et al.* 2008).

The World Health Organisation (WHO) and the Food and Agricultural Organisation of the United States (FAO) jointly published guidelines for the evaluation of probiotics (Araya *et al.*2002) which state that probiotics are 'live microorganisms which when administered in adequate amounts confer a health benefit on the host.' This report also addressed the need for clearer information regarding probiotic products, setting a list of minimum recommendations for information regarding a potential probiotic strain. Safety concerns such as risk of systemic infection, deleterious metabolic activities, excessive immune stimulation in susceptible individuals and gene transfer from the probiotic strain are all factors for consideration. Recommendations for products included:

1) Determination of antibiotic resistance patterns.
2) Assessment of certain metabolic activities.

3) Assessment of side effects during human studies.
4) Epidemiological surveillance of adverse incidents in consumers.
5) Toxin production tests, if the strain is from a species that produces known mammalian toxins.
6) Determination of haemolytic potential, if any.
7) Risk of infectivity in immunocompromised individuals.

The report provides a framework for generating information about probiotic strains, highlighting the importance of correct identification of strains and avoiding generalisations in attributing specific characteristics to specific strains. Certain groups of bacteria can share a key characteristic, such as *S. thermophilus* and *L. delbrueckii*, where production of β-galactosidase for treatment of lactose intolerance is evident as a characteristic of the group rather than the specific strains (de Vrese *et al.* 2001). However, the guidelines are firm on describing specific strains rather than making generalisations in order to avoid misleading claims. There have been cases in the past where probiotic products have been either mislabelled or found to contain different strains than the product claims (Cutting 2011). The presence of such guidelines should benefit the probiotics industry as well as providing transparency for the consumer. Health claims that are corroborated with scientific evidence and standardised information regarding products will ensure standards are introduced and maintained as the probiotics industry continues to develop. The FAO/WHO report however, was only published as a guideline document and carries no legal weight.

With many probiotic products, or 'functional foods' making health claims that were subjected to little regulation, the European Food Standards Authority (EFSA) introduced new legislation in 2007 that enforced stricter controls on these products. These regulations stipulate that all health claims need to be substantiated with scientific evidence, provided

in dossiers for approval by EFSA (Asp & Bryngelsson 2008). Key to the approval of a health claim would be evidence from human trials; a level of testing many products would not have been subjected to previously. It is worth noting that these regulations apply only to Europe, and that in other countries, regulations, if they exist at all are less stringent. This led to some consternation within the industry, with a feeling of unfair competition from global markets outside of the EU that are not subject to such regulations. An important distinction to make at this point is that the EFSA regulations apply to products being sold as foods, not as medicines. Medical science could benefit from these regulations if significant research is pursued by companies marketing probiotics. Intestinal dysbiosis caused by antibiotic treatment is a risk factor for development of CDI (Chang *et al.* 2008; Johnson 2009), with disruption of the gut flora also linked with other conditions such as chronic inflammatory disorders including IBD and Crohn's disease (Round & Mazmanian 2009). Probiotic research represents an avenue to establish how managing the gut flora could provide a novel approach to managing GI tract conditions both at acute infection and chronic condition level.

Specific use of probiotics as a medical intervention is still in experimental stages, despite the concept of probiotics being well established. There is a shortage of large scale clinical evidence regarding whether probiotic treatment can be successful and in which situations use of specific probiotics could be could be applied. Results from existing studies suffer as poor study design presents a challenge in obtaining significant results. There are however, numerous published studies covering the use of a variety of probiotics (**Table 4.1**).

Study	Participants (n)	Strain/product	Results	Comments
Gao *et al*	255	*Lactobacillus acidophilus* CL1285® + *Lactobacillus casei* LBC80R® (Bio-K)	Reduction in cases of CDAD compared to placebo group (1.2% vs 23.8%), over time period of study. Dose response effect was shown with higher dose (100 billion cfu vs 50 billion cfu) yielding superior outcomes (1.2% vs 9.4%).	Study was single centred and follow up time was 21 days - possible some late developing CDAD cases could have been missed. Age range of subjects (50-70yrs) is in line with at risk population.
Sampalis *et al*	437	*Lactobacillus acidophilus* CL1285® + *Lactobacillus casei* LBC80R® (Bio-K)	Reduction in cases of AAD (21.8% vs 29.4%) compared with placebo group and in duration of AAD events (0.67 days vs 1.19 days) in patients within study.	Multicentre study, with adults of all ages (18+). Only 46 cases of AAD were tested for presence of C. difficile- efficacy applies to prevention of AAD, not CDAD. Follow up time was 21 days.
Hickson *et al*	135	Milk drink (Danone) containing *Lactobacillus casei, Lactobacillus bulgaris* and *Streptococcus thermophilus* mix	Reduction in *C. difficile* positive cases of AAD compared to placebo (0% vs 17%).	Testing for *C. difficile* in diarrhoea cases was a secondary consideration. Primary aspect of the study was AAD occurrence. Age group of study participants was 50 years plus, and exclusion criteria from the study were very stringent.
Plummer *et al*	138	*Bifidobacterium bifidum* and *Lactobacillus acidophilus* mix	Reduction in *C. difficile* positive diarrhoea samples compared to placebo (2.9% vs 7.25%).	Low recruitment levels means statistical power of results is reduced. Study participants are defined as elderly rather than defining age bracket. Single centre study.
Surawicz *et al*	180	*Saccharomyces boulardii*	Reduction in *C. difficile* positive cases compared with placebo (9.4% vs 31%)	Mean age of study particpants was 47 years, and lower incidence of *C. difficile* was not significant (p=0.07)

Table 4.1. Examples of studies investigating effects of using probiotics for the prevention of *C. difficile* infection or antibiotic associated diarrhoea.

In order for probiotics to be considered seriously as treatment options, more research is required. This means defining what is expected from probiotic strains in specific situations; are they to be used as supplementary treatments to antibiotics, used in prophylactic treatments, or applied as a primary treatment option? Clear objectives are required in order to make results easily applicable to a specific medical problem. The most beneficial studies will, if possible, demonstrate the mechanisms behind positive results. Elucidating probiotic mechanisms will aid successful application to existing medical problems. In all studies, emphasis needs to be placed on specific findings and avoiding generalisations to all use of probiotics. Where meta-analysis of data is used in the absence of larger scale studies, differences between studies need to be highlighted to avoid misleading conclusions.

Suggested mechanisms of action for probiotics in the GI tract include stabilisation of epithelial tight junctions, action of bacteriocins, immunomodulation and effects on immune cell signalling, displacement of pathogens from epithelial surfaces and alteration of the gut pH, among others (D'Souza 2002; McFarland 2009; Islam *et al.* 2012). Guidelines from the WHO (Araya *et al.* 2002) encourage strain specific information with regard to probiotic treatments in order to clarify findings as mechanisms will differ between probiotic strains. Ideally, studies should include identification of the product used to strain level so that available information is as accurate as possible. Generalisations between all types of probiotics do not present a valid argument for or against use of probiotics. Distinct strains and genera of microorganisms will behave differently and have unique properties. It therefore cannot be assumed that mechanisms of action or the successes of one strain or product can be applied to others without evidence from relevant studies. The makeup of the microflora of the human gut will vary with factors such as age, diet and ethnicity

(Woodmansey *et al.* 2004; Wu *et al.* 2011; Bäckhed *et al.* 2012), so the structure of study populations should also be addressed before applying data from studies to the general population.

While efforts are being made to regulate and substantiate claims made around use of probiotics in food products, scientific literature presents conflicting opinions as to whether probiotics are a viable option for treatment of gastrointestinal disorders (Miller 2009; Rowland *et al.* 2010). Available data sets are often limited and a number of general improvements to studies could be made to improve available data.

1. Study design needs to be consistent. Comparison of different probiotics and how successful they are is difficult when large differences exist between studies testing them. Outcomes and parameters of studies need to be clearly defined; using CDI as an example, does a study address the issue of antibiotic associated diarrhoea (AAD) or CDI in particular? How is CDI confirmed? How long does the study last and is long term assessment included? Specifics of the study should be clear; the strain, the dose regimen and quantity of bacteria used are all important factors in developing treatments.
2. Study populations. Clinical studies relating to treatment of a certain condition should include at risk populations. Stringent exclusion criteria to control the study can mean that those most likely to develop CDI are not included in the studies. Ideally, studies need to be conducted including individuals from populations at risk of CDI for proper analysis of clinical efficacy. Size of the study also needs to be considered in order to ensure robust and significant results.
3. Controls. Where specific treatments are being tested, placebo groups have to be included for the data to be significant.

Fulfilling all of these requirements is challenging, especially when human subjects are being used in studies. The health of individuals needs to be the foremost consideration. Use of alternative models for *in vivo* studies is not a perfect solution, but may enable more sophisticated preliminary work. Without well considered studies, debate about probiotic use will continue due to lack of conclusive evidence on either side.

A recent study investigating use of a microbial preparation in prevention of AAD and CDI (Allen *et al.* 2013) demonstrates consideration of the scale involved in such research. A total of 2941 participants from five separate centres took part in this randomised, placebo controlled study. All participants were over 65 and had recently been exposed to antibiotic treatment, representing a specific population vulnerable to CDI. The microbial preparation used in treatments was shown to not reduce frequency of AAD or CDI between treatment and control groups. The scale of this study allows robust conclusions to be stated. However, a mix of strains was used in the microbial preparation, which prevents clarity. For example, a strain in the mix may have beneficial effects, but not at the levels used in the composition of the probiotic. No beneficial effect attributable to the probiotic treatment was reported in this study, but if there were, use of a mix of microbes could have led to difficulty in elucidating a mechanism. If microbial therapies are to be considered as viable treatment options, more studies of this scale need to be carried out. Refinement of study protocols will produce superior data for consideration.

Research into *Saccharomyces boulardii* is a good example of the debate surrounding probiotic use. *S. boulardii* has been shown to have efficacy in reducing episodes of *C. difficile* associated diarrhoea (Surawicz *et al.* 1989). Further work demonstrated that this yeast produces a protease that can cleave the toxins of *C. difficile*, rendering toxins ineffective at binding to receptors (Castagliuolo *et al.* 1996). These results led to work

combining the probiotic strain with antibiotic treatments to focus on reducing relapses of infection (Surawicz *et al.* 2000). However, more recent studies by a different investigator (Pozzoni *et al.* 2012) have shown that this yeast is not effective at reducing cases of AAD. A key difference here is the condition being assessed. AAD is a general condition whereas *C. difficile* is a specific cause of diarrhoea. It is distinctions such as this that are important in clarifying the role that probiotics can play in treatments. There is also concern over the use of *S. boulardii* in patients that are immunocompromised, with potential for opportunistic infections (Muñoz *et al.* 2005). When conflicting cases like this occur, it is not surprising that debate exists around the use of or potential of probiotics in medical therapies.

This thesis will use a *Bacillus subtilis* strain to investigate its potential as a probiotic in treatment of *C. difficile*. *Bacillus* probiotics are of particular interest in this work as they have the ability to form spores. Spores are an incredibly resistant, stable form of the bacteria allowing for simple storage and dosing of the probiotics (Cutting 2011). Refrigeration of spores is unnecessary due to their heat stable nature. These spores can be administered in an oral dose, eliminating the need for trained medical professionals or needles for delivery of treatment. A key point of interest with *B. subtilis* spores is their ability to absorb proteins to their spore coat. In previous studies (Duc *et al.* 2007; Huang *et al.* 2010), this ability has been used to absorb antigens for use in vaccines. In the context of *C. difficile* treatment, this ability draws a parallel to use of toxin binding resins (Hinkson *et al.* 2008; Weiss 2009). In a 2011 study investigating the use of *B. subtilis* as a vaccine delivery system (Permpoonpattana *et al*. 2011*a*), it was shown that wild type *B. subtilis* PY79 spores, administered orally, could prolong survival of hamsters infected with *C. difficile*. These results raised the question of whether spores of *B. subtilis* could be used as

a probiotic treatment against *C. difficile* infection. The work of this thesis aims to address this question.

4.1.3. Use of animal models

One of the key issues surrounding use of probiotics is the lack of standardised studies and trials. Use of human trials often results in complex results that stem from the complex medical histories of the patients involved. It is difficult to arrange a standardised study for an opportunistic disease that presents with different stages of clinical manifestation in individual patients. Use of animal models would provide a controlled method for preliminary exploration of probiotic strains. This type of study could then be beneficial in identifying how probiotics may be used before use in humans. If animal studies can provide elucidation of mechanisms behind probiotic action, application to human use should be an easier, more successful step.

In the study of *C. difficile*, two species of animal model are available: the hamster and the mouse (Sambol *et al.* 2001; Chen *et al.* 2008; Goulding *et al.* 2009). These can be used to represent different aspects of the disease, with benefits and limitations present in each model.

Mice are less sensitive to *C. difficile* infection than hamsters, with the severity of induced infection dependant on the antibiotic regimen and the strain of *C. difficile* used. Hamsters however, will succumb to fatal disease within 48 hours of infection with a toxin producing strain. Clear signs of disease are evident in hamsters, including diarrhoea, pilo erection, hunched posture and lack of responsiveness. Presence of toxins and bacteria can be confirmed using faecal samples. Hamsters are held as the gold standard for studies in *C.*

difficile infection, as the symptoms are similar to those displayed in humans. The short time period that hamsters can survive with this infection imposes a limit on studies using this model. The short window of survival prevents study of manifestations of infection such as the carrier state (Riggs *et al.* 2007; Lawley *et al.* 2009*a*), where individuals can be infected with *C. difficile* but not display symptoms. Furthermore, human disease is only fatal in severe cases, which calls into question the comparability of the hamster model with the human CDI condition.

In mice, doses of multiple antibiotics are required to induce susceptibility to fatal *C. difficile* infection. Manifestation of disease is seen in a strain and dose dependent manner, which adds a level of complexity to studies using this model and perhaps makes this model more comparable to the human clinical situation. Infections can be monitored over several days rather than hours, meaning that studies have more scope to collect data. Multiple aspects of disease can be studied in the murine model, from asymptomatic colonisation to fatal disease, providing a much wider range of possibilities for study. The murine model is also more accessible, with associated reagents commercially available for use. This probiotics study will use primarily the murine model of CDI described by Chen and colleagues (Chen *et al.* 2008), but also includes data from a hamster study to broaden the base of the study's findings.

4.1.4. Experimental Objectives

This study will address the hypothesis that *B. subtilis* spores can be used as a probiotic treatment for disease caused by *C. difficile* infection. The suitability of this strain will be assessed prior to use as treatment of disease, in terms of safety and ability to survive in the GI tract. If successful in alleviating symptoms, this work aims to then investigate the mechanism behind the probiotic action.

Use of a murine model of infection will provide a controlled platform to carry out preliminary probiotic trials. Many of the criticisms surrounding probiotic use are due to the lack of clarity and control in research meant to provide evidence for or against probiotic usage. While it was not possible to address all the points raised in the WHO report (Araya et al. 2002) or indeed fulfil the requirements set out by ESFA this study aims to provide clear preliminary evidence for the suitability of this strain as a treatment of CDI.

4.2. Results

4.2.1. Suitability of probiotic strain

From a single oral dose of 10^{10} spores, spores of the probiotic strain *B. subtilis* PXN21 can still be detected in the faeces up to two weeks later (**Figure 4.1**). Elevated levels of spores in faecal samples over a two week period demonstrate evidence of transient colonisation. The plateau in numbers between days three and six represents the ability of the strain to maintain levels by establishing a growth cycle before faecal spore levels decrease. Numbers of *B. subtilis* spores recovered from faecal samples were generally lower in antibiotic treated individuals, but not significant at this sample size.

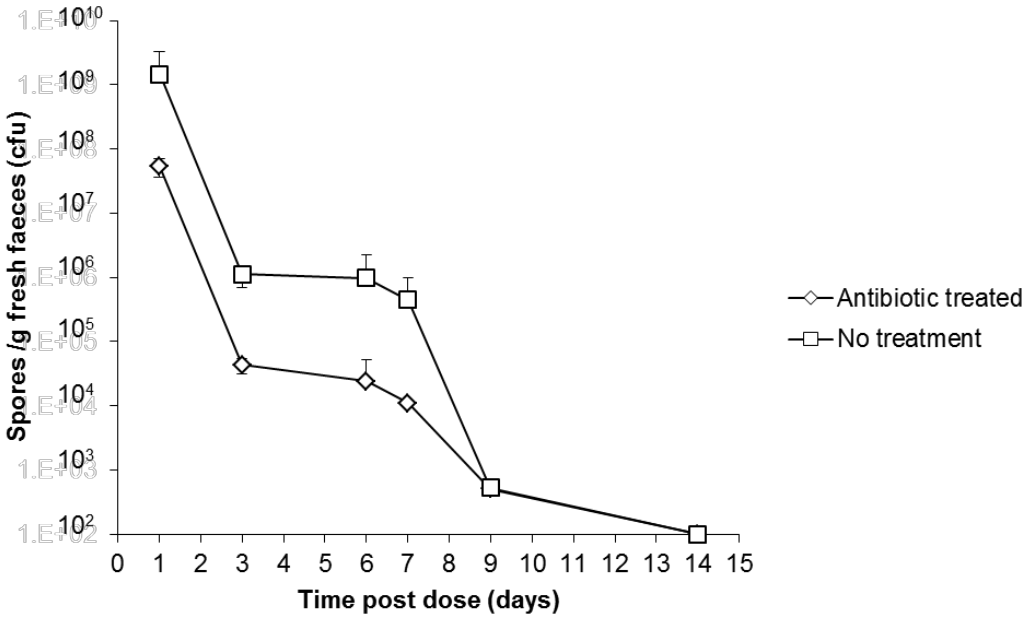


Figure 4.1. Detection of *B. subtilis* PXN21 spores in faecal samples from C57Bl/6 mice. Groups (n=4) received either antibiotic cocktail treatment or no treatment prior to receiving a single oral dose (10^{10}) of *B. subtilis* PXN21 spores. Faecal samples were collected and treated with ethanol to leave only spores viable in samples. Spore content was measured by serial dilutions of samples plated on DSM agar and the PXN21 strain was identified by colony morphology.

The *B. subtilis* PXN21 strain was also tested against a panel of antibiotics to ensure conformity with EFSA guidelines for the species. Minimum inhibitory concentration (MIC) breakpoints were compared to guideline points from both EFSA and CLSI. Control *B. subtilis* strains PY79 (lab stock) and NCIMB strain 3610 were tested alongside the study strain for comparison. Ampicillin is the only antibiotic tested where the MIC was higher than the recommended breakpoints. This result was replicated in the control *B. subtilis* strains used, implying an intrinsic resistance rather than a transmissible property. Experimental MIC breakpoints show that the *B. subtilis* PXN21 strain has no resistances of concern, all being lower than guideline points, so conforms to current legislature for use as an oral probiotic.

Strain	Ampicillin µg/ml	Vancomycin µg/ml	Erythromycin µg/ml	Tetracyline µg/ml	Clindamycin µg/ml	Chloramphenicol µg/ml	Streptomycin µg/ml	Kanamycin µg/ml	Gentamycin µg/ml
EFSA Breakpoint	**n/a**	**4**	**4**	**8**	**4**	**8**	**8**	**8**	**4**
CLSI Breakpoint	**0.25**	**4**	**0.5**	**4**	**0.5**	**8**	**n/a**	**n/a**	**4**
PXN21	>64	0.25	0.125	4	2	4	8	0.5	0.25
PY79	>64	0.5	2	8	1	8	8	0.5	<0.625
NCIMB 3610	32	0.25	0.5	4	1	4	32	0.5	0.125

Table 4.2. Antibiotic MIC values for *B. subtilis* strains, with comparison to standard levels as set out by EFSA (EFSA, 2012) and the CLSI (CLSI, 2010).

4.2.2. Colonisation resistance

Non-fatal colonisation of *C. difficile* was induced in a murine model of infection by antibiotic treatment followed by infection with *C. difficile* R20291 spores (**Figure 4.2**). Levels of *C. difficile* spores present in faecal samples demonstrated that oral delivery of *B. subtilis* PXN21 spores both prior to and post infection had no significant effect on reducing *C. difficile* colonisation. In all groups, peak levels of spores were detected on day two post-infection, and although *C. difficile* levels were highest in mice receiving no probiotic treatment, this was not significant ($p = 0.1$).

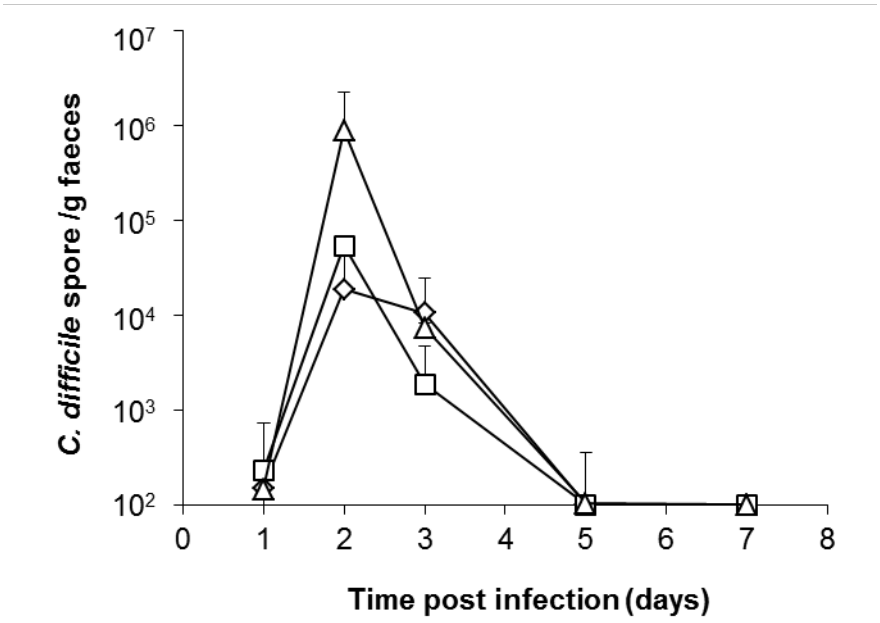


Figure 4.2. Colonisation resistance. Susceptible C57Bl/6 mice were infected with 10^4 *C. difficile* R20291 spores. Groups ($n = 6$) were given daily doses of *B. subtilis* PXN21 spores pre infection (◊), during infection (□) or no treatment (Δ). Faecal samples were collected daily and ethanol treated samples were serially diluted then plated on BHISS agar for enumeration of *C. difficile* spores.

4.2.3. Attenuation of symptoms in a model of fatal disease

A study model utilising *C. difficile* strain VPI 10463 to induce a fatal infection in mice was used to assess the ability of *B. subtilis* PXN21 to influence the clinical outcome of infection. This model of infection utilised pre-dosing with a cocktail of antibiotics including vancomycin that was designed to mimic the clinical situation of antibiotic therapy in humans (Chen et al. 2008). PXN21 spores were administered to mice before and after infection with VP1 10463 and both pre and post infection treatment increased survival compared to non-treated animals (**Figure 4.4A**). Pilot studies showed that administration of PXN21 spores did not reduce the efficacy of vancomycin in treatment of CDI in mice (**Figure 4.3**). Delivery of PXN21 spores prior to infection resulted in a survival rate of 41.6% while 66.6% survival was achieved in animals treated post *C. difficile* infection (**Figure 4.4A**), this compared to a survival rate of 16.6% in non-treated groups. Interestingly, weight profiles of infected animals added further complexity to these results (**Figure 4.4B**). Animals that survived infection having received probiotic treatment prior to infection displayed less weight loss than those treated with PXN21 spores post infection. Post infection treated animals displayed a similar weight profile to the non-treated group, despite having the highest rate of survival.

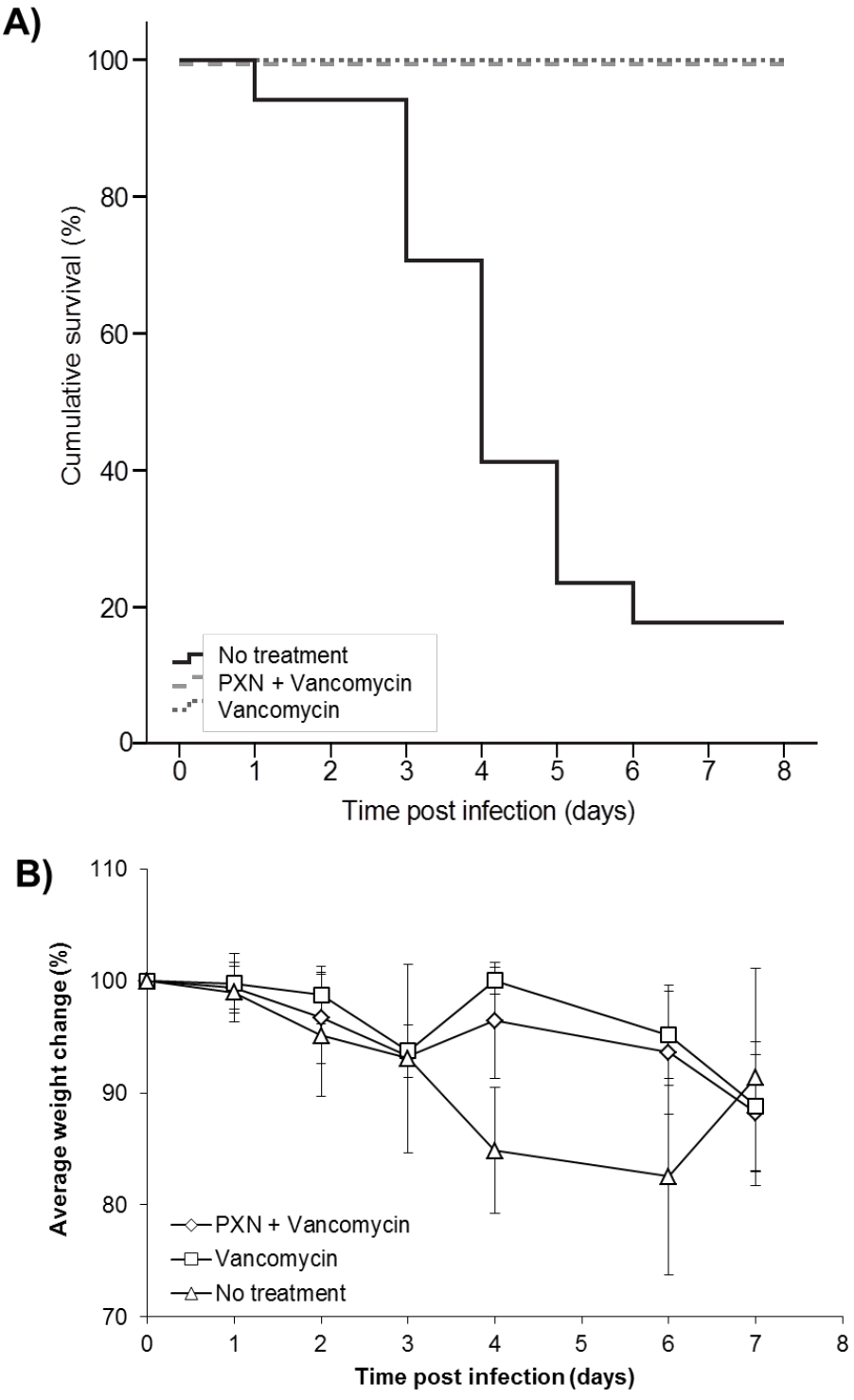


Figure 4.3. Effect of PXN spore treatment on antibiotic treatment. Susceptible C57Bl/6 mice (n≥ 6) were infected with 10^4 *C. difficile* VPI 10463 spores. Groups received daily doses of oral vancomycin (100µg/dose), oral vancomycin (100µg/dose) with *B. subtilis* PXN21 spores (10^9/dose) or no treatment. A) Survival was monitored over 7 days post infection. Both vancomycin treatments at 100% survival over 7 days. B) % weight change was calculated based on recorded weight throughout infection monitoring period compared to weight at point of infection. Weights were recorded daily over 7 day infection period.

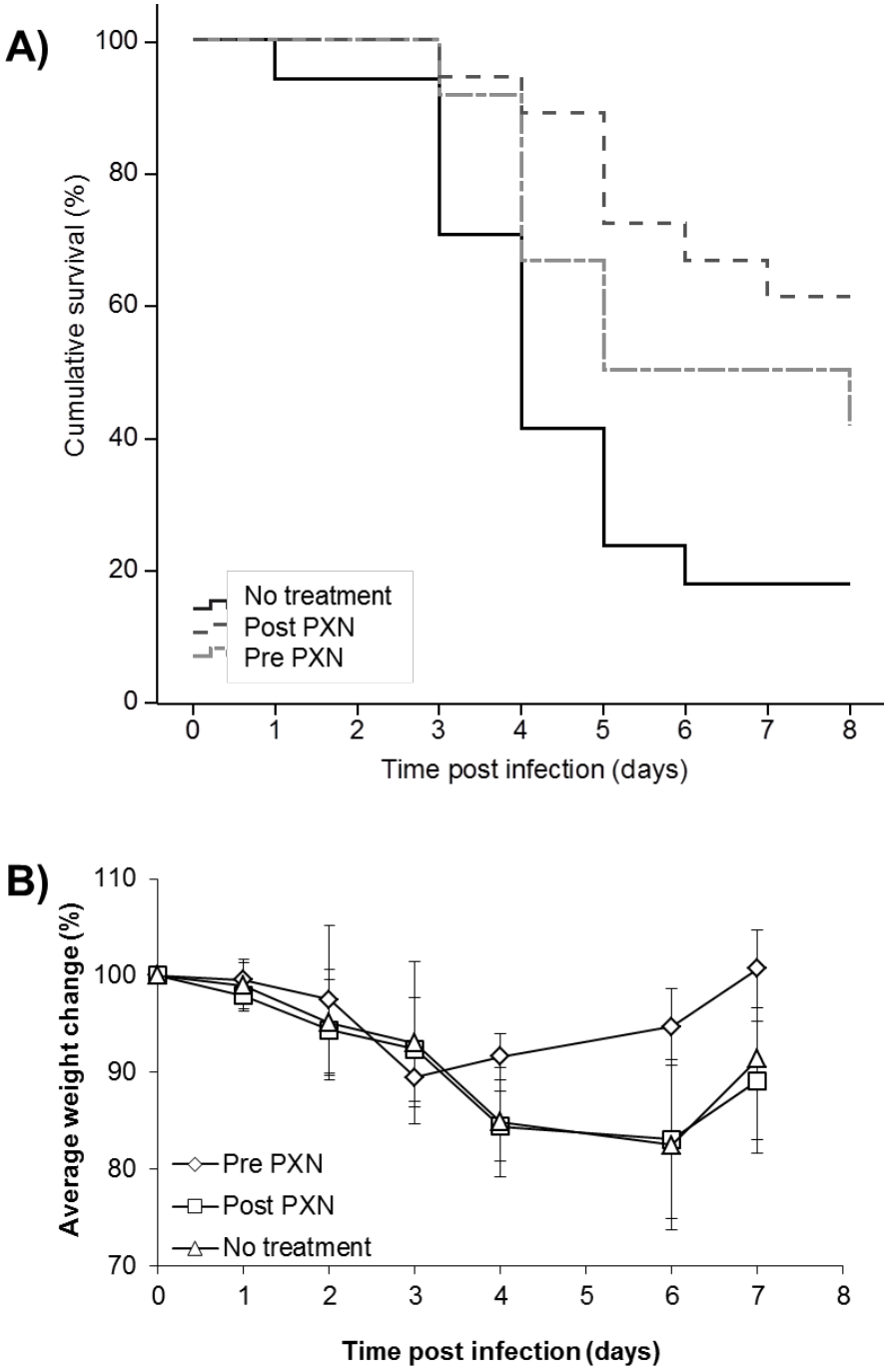


Figure 4.4. Pre and post infection probiotic treatment. Susceptible C57Bl/6 mice (n≥ 6) were infected with 10^4 *C. difficile* VPI 10463 spores. Groups received either daily doses of PXN21 spores pre infection, post infection, or no treatment. A) Kaplan-Meier survival plot over seven days post infection. B) % weight change was calculated based on recorded weight throughout infection monitoring period compared to weight at point of infection. Weights were recorded daily over the 7 day infection period. All probiotic doses contained 10^9 spores and were delivered orally.

Treatment with *B. subtilis* PXN21 spores was also used in a hamster model (**Figure 4.5**), although the methodology was adapted to fit with the differences with the model. CDI is fatal in hamsters, and the probiotic treatment does not prevent infection, so results were based on survival time. Average length of survival was increased slightly in animals pre dosed with *B. subtilis* PXN21 spores. Increased survival times were not statistically significant (p=0.7) but a trend of increased survival in hamsters pre dosed with *B. subtilis* PXN21 spores supported data from the murine model that pre dosing probiotics (**Figure 4.4B**) has links to a successful probiotic mechanism.

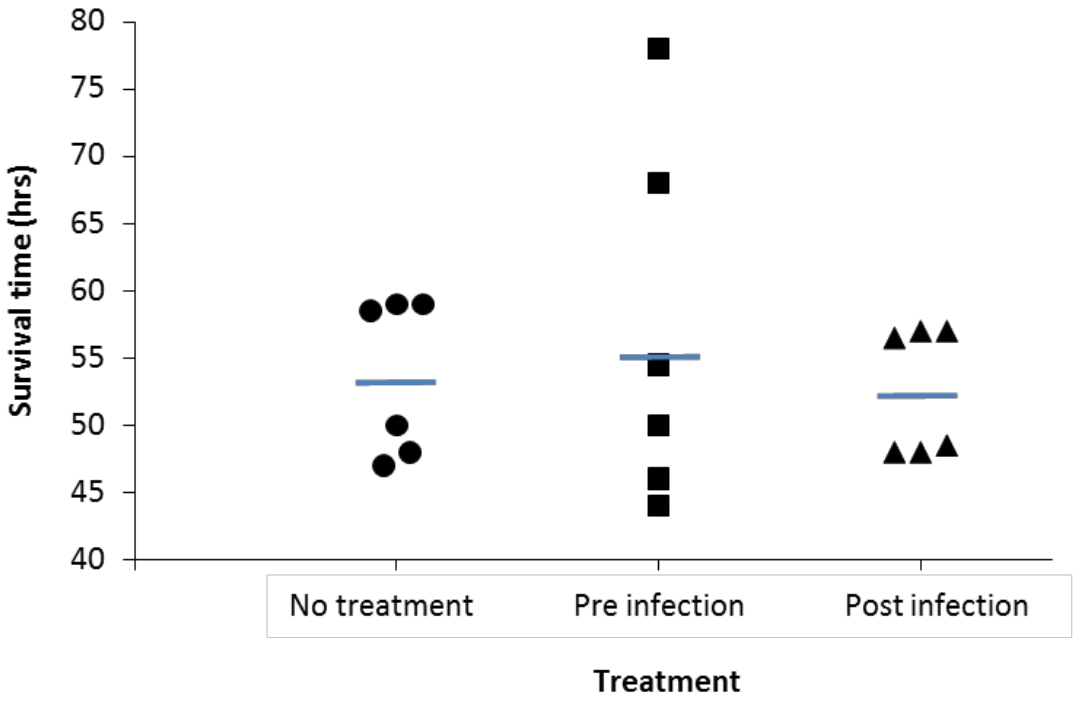


Figure 4.5. Survival time of hamsters infected with 100 *C. difficile* 630 spores. Pre-infection group (□) received daily oral doses of 10^9 *B. subtilis* PXN21 spores in the 5 days prior to infection. Post infection group (Δ) received oral doses of *B. subtilis* PXN21 spores at 12 hour intervals post infection. N=6, each point on scatter plot represents 1 animal. Blue lines show mean average survival time.

To provide further information for optimisation of dose regimen, further aspects of dose were considered, including dose size. Two doses were tested; the original 10^9 dose, and a 100 fold reduction in this, using 10^7 spores per dose (**Figure 4.6**). Survival rate in both the probiotic treated groups was 66.6% seven days after infection (**Figure 4.6A**). Reduction of the number of spores per dose gave no change in the rate of survival but the progression of disease appeared slower. Disease progression reached the fatal stage at day three of infection in the 10^9 dose treatment group; however the first fatality did not occur until day five in the 10^7 dose group. Weight profiles appear to reflect this observation from survival data (**Figure 4.6B**). Loss of body mass occurs in animals given doses of 10^7 spores, but at a reduced level and at a later stage of the experiment. The data indicates less severe symptoms in the 10^7 dose group.

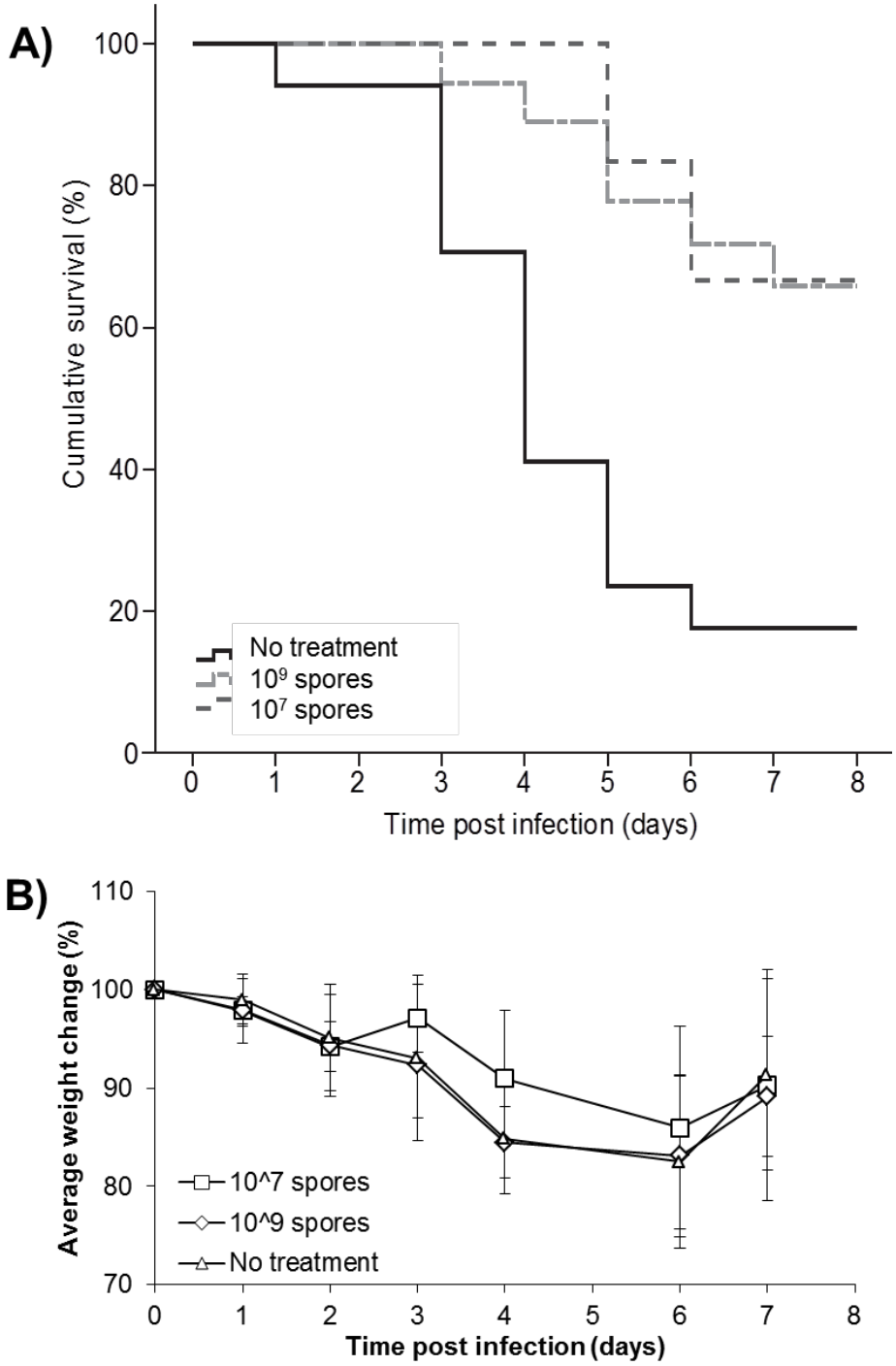


Figure 4.6. Dose response probiotic treatment. Susceptible C57Bl/6 mice (n≥ 6) were infected with 10^4 *C. difficile* VPI 10463 spores. Groups received either daily PXN21 spore doses of 10^9, 10^7, or no treatment. A) Kaplan-Meier survival plot over seven days post infection. B) % weight change was calculated based on recorded weight throughout infection monitoring period compared to weight at point of infection. Weights were recorded daily over 7 day infection period.

4.2.4. Live *B. subtilis* spores are required for suppression of infection

Administering PXN21 spores post infection was more effective in preventing CDI than pre-dosing. Since killed spores have been shown to have adjuvant properties and can enhance the immunity of prototype vaccines (Barnes *et al.* 2007; Huang *et al.* 2010), the effect of spore viability on survival rate in mice administered PXN21 spores post infection was assessed. As shown in **Figure 4.7A** dosing of mice post infection with killed spores showed almost no improvement in survival rate. By contrast, dosing with live spores markedly improved the survival rate. As shown earlier (**Figure 4.4A**) treatment post infection with live PXN21 spores increases survival rate but did not induce an improvement in the weight profile of infected animals. To further support the need for viable bacteria in this treatment, when vegetative cells of *B. subtilis* PXN21 were used (**Figure 4.7A**) survival rate increased to 83.3%. Animals receiving vegetative probiotic doses displayed less weight loss over the course of infection (**Figure 4.7B**), demonstrating reduced severity of symptoms.

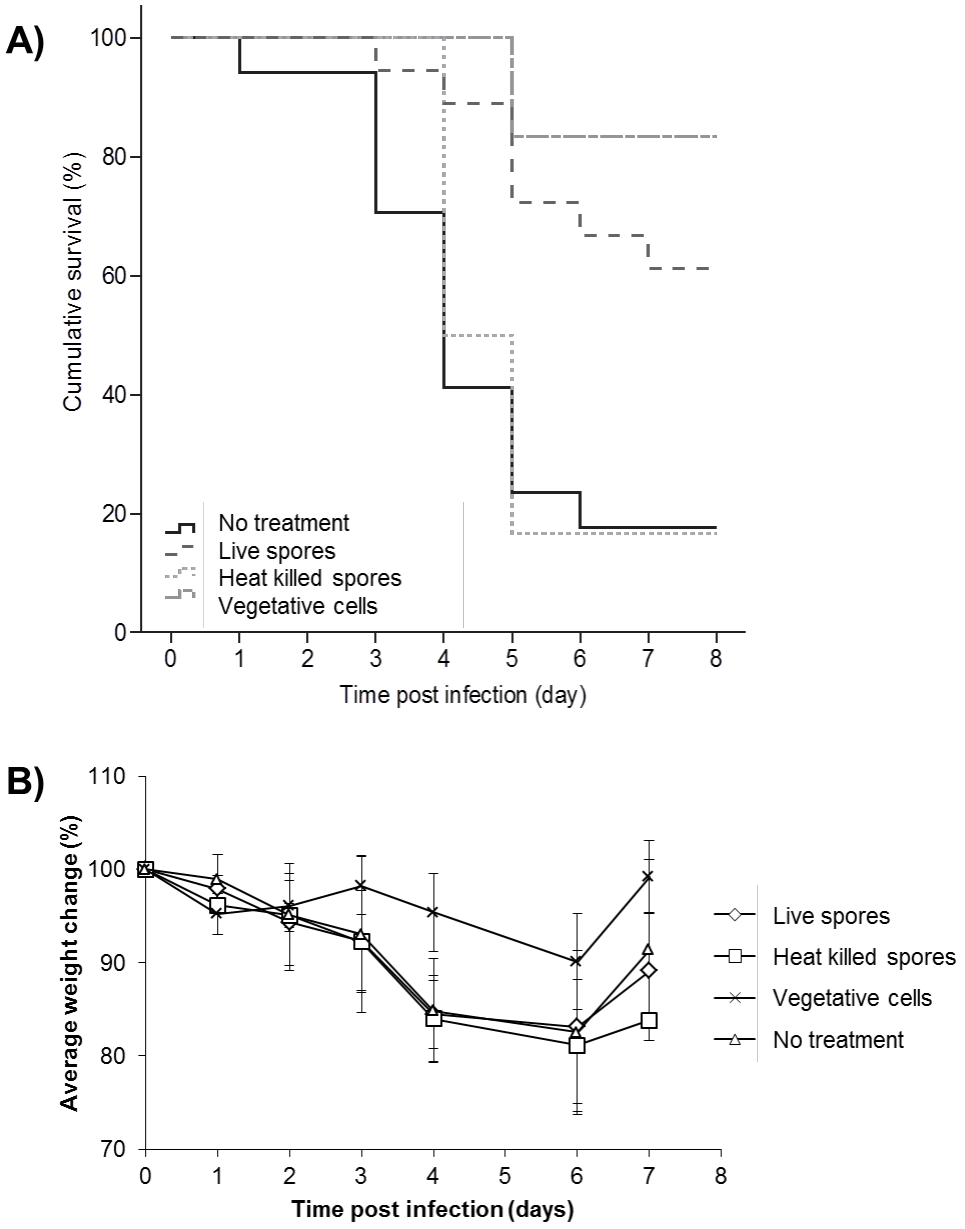


Figure 4.7. Viability of probiotics. Susceptible C57Bl/6 mice (n≥ 6) were infected with 10^4 *C. difficile* VPI 10463 spores. Groups received either daily *B. subtilis* PXN21 doses of live spores, heat killed spores, vegetative cells or no treatment. A) Kaplan-Meier survival plot over seven days post infection. B) % weight change was calculated based on recorded weight throughout infection monitoring period compared to weight at point of infection. Weights were recorded daily over 7 day infection period. All probiotics doses contained 10^9 spores or cells, and were delivered orally.

4.2.5. Histological sections

H&E stained tissue sections were produced to assess pathology affecting gut tissues in groups receiving *B. subtilis* PXN21 spores. Images from tissue sections suggest that in animals given PXN21 spores the gut tissues display reduced pathology, with less damage to cell structure than in untreated animals (**Figure 4.8**). Tissues from healthy, non-infected mice exhibited well defined tissue structure and cell integrity (**Figure 4.8A**). Toxin mediated damage is evident in the breakdown of tissue structure and disruption of epithelial cell membranes (**Figure 4.8D**). The level of damage in terms of cell structure and tissue integrity was reduced in groups receiving PXN21 spores both before (**Figure 4.8B**) and after infection (**Figure 4.8C**). However, PXN21 treatment did not completely protect tissues and some cellular damage was present in PXN21 treated animals, with minor disruption of cell membranes apparent.

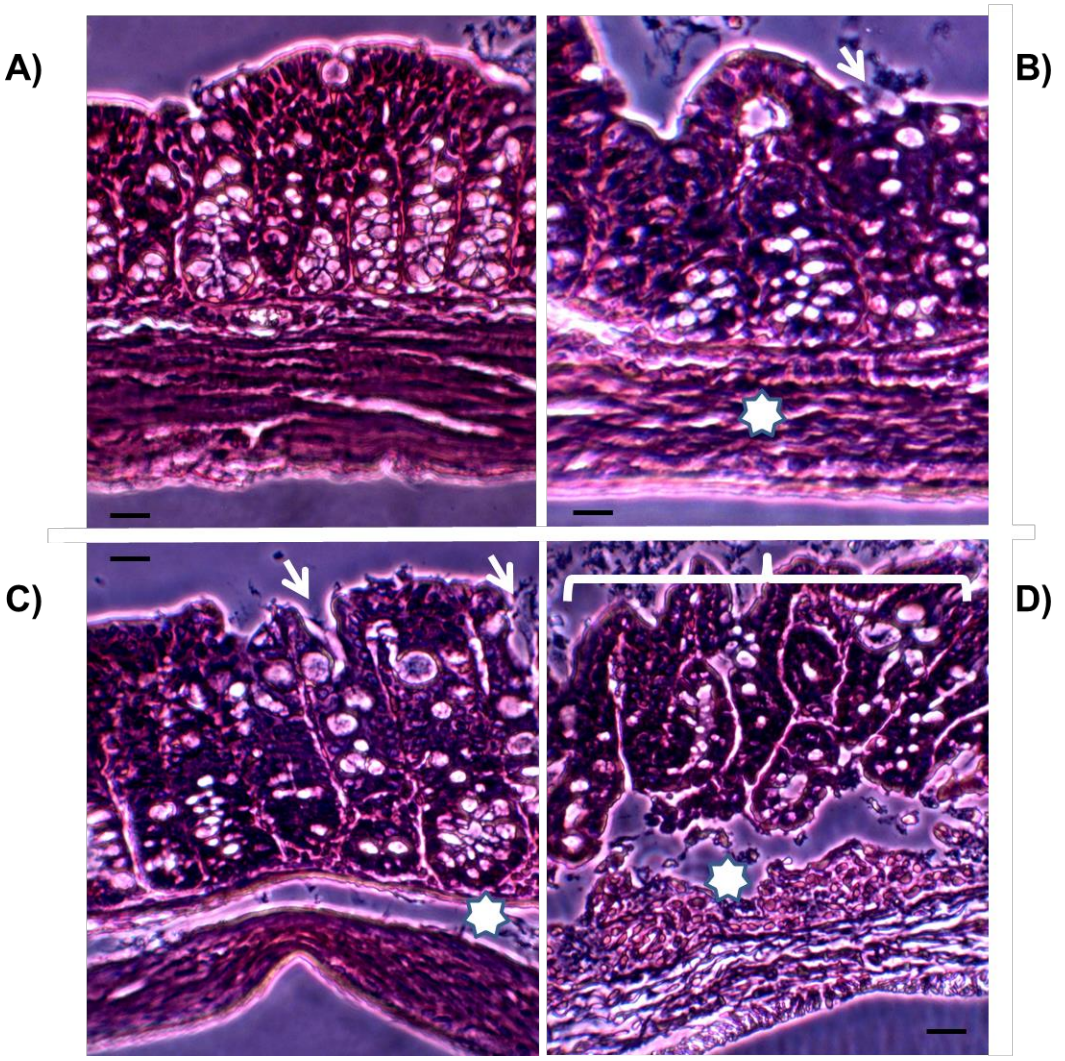


Figure 4.8. Hematoxylin and eosin stained sections of colon from *C. difficile* VPI 10463 infected mice. (A) uninfected mice, displaying healthy tissue and intact epithelial lining to colon, (B) mice treated with PXN21 spores pre-infection with some damage to epithelial structure but with lining still intact although evidence of mild edema in submucosa, (C) mice treated with PXN21 spores post infection show slight damage to integrity of epithelial lining and some submucosal disruption and (D) untreated mice displaying extensive damage to colonic epithelial lining and erosion of the sub mucosa (shown by white arrows and stars). Scale bars = 25µm

4.2.6. Mechanisms of probiotic action

The ability of spores to bind proteins has been demonstrated previously (Huang et al. 2010), and was examined as a possible mechanism for protective action in CDI. *In vitro* experiments showed that the whole toxins, rather than protein fragments or antigens could be absorbed on to the spore coat of *B. subtilis* PXN21 spores (**Figure 4.9**). Incubation of spores with commercially purified toxin A and B was used to test absorption to spore coats. Western blots of extracted spore coats after incubation with the toxins showed the presence of both toxins A and B on the spore coat. In this experiment, toxin B was detected on the spore coat with greater efficacy than toxin A (**Figure 4.9B**). Due to the significant differences between an *in vivo* and *in vitro* situation, these *in vitro* results should be applied with caution to the *in vivo* situation.

It was proposed that *B. subtilis* spores could replicate the *in vitro* scenario of absorbing toxins to the spore coat, binding free toxin in the gut to prevent causation of damage to cellular targets. However, on investigation, developing an assay to assess this was extremely difficult. A small amount of toxin is detectable via immunoblotting on spores isolated from caecal extracts (**Figure 4.9C**). However, the amount of free toxin also detected in the caecum of infected animals makes levels of absorbed toxin appear insignificant. Further study of toxin absorption may provide definitive evidence as to whether this mechanism actually contributes *in vivo* to the increase in survival seen in groups that have received *B. subtilis* PXN21 spore doses.

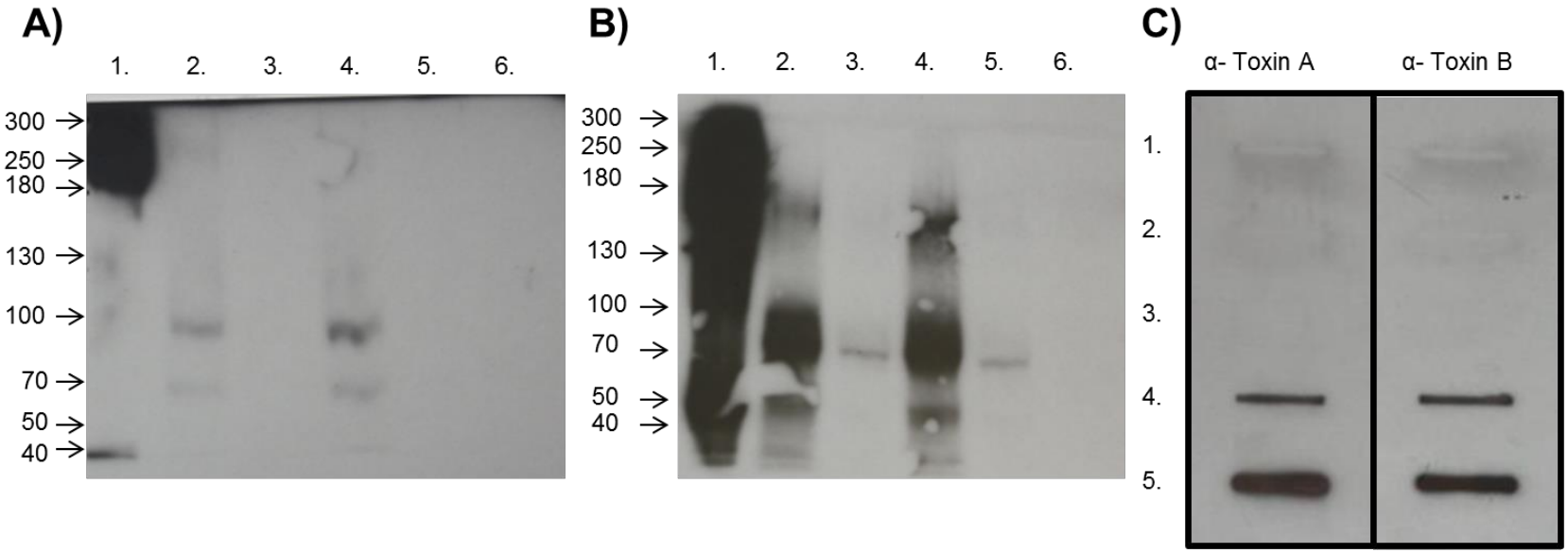


Figure 4.9. Toxin binding to spore coats visualised by extracting spore coats and probing with toxin specific antibodies.
A) Toxin A incubated with purified *B. subtilis* PXN21 spores, samples as follows, lane 1: 0.2 µg toxin A, lane 2: PXN21 + 2 µg toxin A, lane 3: PXN21 + 0.2 µg toxin A, lane 4: PXN21 + 2 µg toxin A and B, lane 5: PXN21 + toxin A and B and lane 6: PXN21 without toxin.
B) Toxin B incubated with purified *B. subtilis* PXN21 spores, samples as follows, lane 1: 0.2 µg toxin B, lane 2: PXN21 + 2 µg toxin B, lane 3: PXN21 + 0.2 µg toxin B, lane 4: PXN21 + 2 µg toxin A and B, lane 5: PXN21 + toxin A and B and lane 6: PXN21 without toxin.
C) DotBlot of caecal sample extractions. Membrane has been probed with α toxin A (left panel) and α toxin B (right panel) antibodies to detect toxins within the samples. Samples are taken from the caecum of mice 1) infected with *C. difficile* and treated with PXN21 spores, 2) infected with *C. difficile* but receiving no treatment and 3) that have not been infected. Controls are 4) purified toxin A/B (300ng) and 5) supernatant from caecum samples.

An alternative mechanism for how PXN21 spores might supress symptoms of CDI includes stimulation of the innate immune responses. *In vitro* assays were carried out to assess the ability of the *B. subtilis* PXN21 spores to interact with cellular receptors and produce measurable immune responses. Firstly, interaction with TLR2 was analysed. In this assay a response was detected when both vegetative cells and spores were incubated with macrophages. Interestingly, this reaction was reduced when heat killed spores were used (**Figure 4.10**). Live spores germinate over the time course of this experiment. The reaction stimulated by heat killed spores is solely attributable to spores rather than vegetative cells or germinating spores, showing that these spores are recognised by TLR2.

Induction of pro inflammatory cytokines IL-6 and TNFα by macrophages incubated with *B. subtilis* PXN21 spores was also studied. Both cytokines were shown to be produced when incubated with live spores (**Figure 4.11**). Levels of IL-10 were also assessed, but no detectable levels were found in this assay. To control for germination of the spores during the assay, SC2376 *gerD-cwlD*, a strain congenic to *B. subtilis* PY79 that carries a severe defect in germination (Mauriello *et al.* 2007) was included. Heat killed spores were tested for induction of responses. Live cells were able to induce production of IL-6, but this effect was reduced with the use of germination deficient spores. Interestingly, heat killed spores failed to stimulate any detectable levels of IL-6, highlighting a difference between germination deficient and heat killed spores. TNFα production was limited to macrophages that were incubated with viable *B. subtilis* PXN21 spores (**Figure 4.11B**), linking production of this cytokine to germination and vegetative growth of the strain. Heat killed and SC2376 *gerD-cwlD B. subtilis* spores failed to induce TNFα production in this assay. Production of cytokines demonstrates that the *B. subtilis* PXN21 strain is capable of interacting with receptors of the innate immune system. Differences between viable and inactive spores

imply that these interactions will strongly depend on the state of growth displayed by the strain. It is worth noting that these are simplistic *in vitro* assays and direct comparison to the extremely complex *in vivo* situation should be done with care.

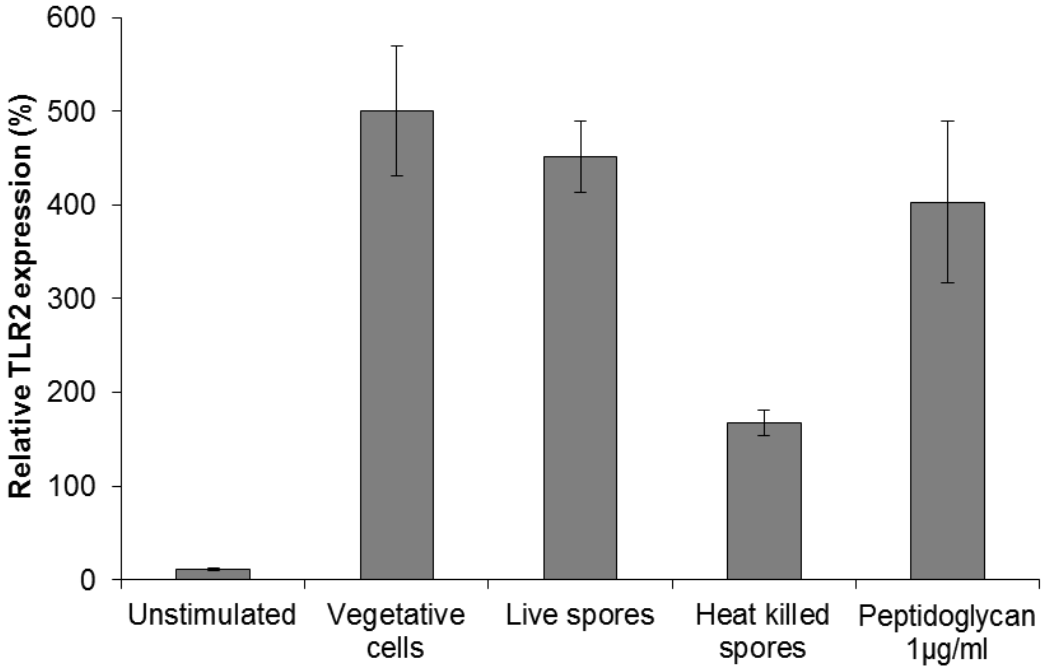


Figure 4.10. TLR2 expression in murine macrophages (RAW264.7) after incubation with *B. subtilis* PXN21. Vegetative cells, spores and heat killed spores were added to cells at a concentration of 10^7/ml and incubated for 4hours. RNA was extracted from cells and TLR2 gene expression was calculated relative to β-actin expression, using qPCR. Results are from three individual experiments.

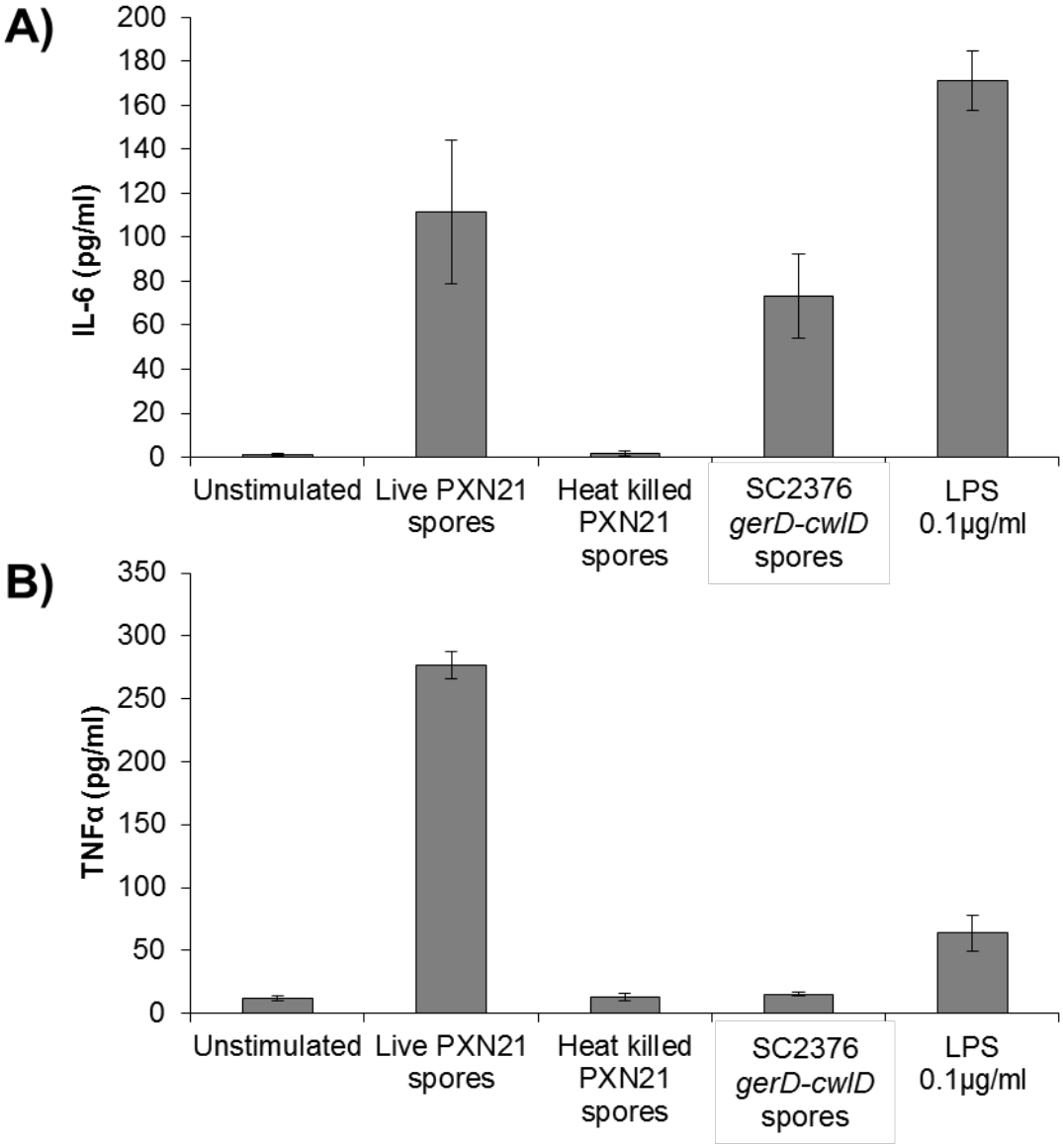


Figure 4.11. Cytokine production in murine macrophages (RAW264.7) after incubation with *B. subtilis* PXN21. Live and heat killed spores were added to cells at a concentration of 10^7/ml and incubated for 24hours. Commercial ELISA kits (eBioscience) were used to quantify levels of A) IL-6 and B) TNFα in supernatants of cultured cells. Results are from three individual experiments.

4.3. Discussion

This work demonstrates that oral administration of probiotic *B. subtilis* PXN21 spores is effective in reducing CDI symptoms in a murine model of disease. Variation displayed in survival rates between different treatment regimens highlights the need for optimisation of this probiotic treatment. Attempts to elucidate the mechanisms by which the probiotic effect is achieved and how the findings of this study can be applied to development of commercial treatments will be discussed.

4.3.1. Probiotic mechanisms

B. subtilis PXN21 is a component of a commercial probiotic product and numerous other products also carry spores of *B. subtilis* as an active ingredient (Permpoonpattana *et al.* 2012). Additionally, some traditional food such as Natto, a fermented soy bean based food stuff, contain live *B. subtilis* spores and have been recognised as having associated health benefits (Hosoi & Kiuchi 2004). There are also studies that show oral consumption of probiotic bacteria can have some effect in reducing symptoms or occurrence of CDI. As an adjunct to antibiotic therapy, species of *Lactobacillus, Bifidobacteria* and *Saccharomyces boulardii* appear to reduce the incidence of CDI in human studies although no study has yet shown full protection (Pochapin 2000; Surawicz *et al.* 2000; Wullt *et al.* 2003). In addition, no consensus has been reached as to how these bacterial supplements exert their effects. Several different mechanisms can be considered when explaining the ability of a strain such as *B. subtilis* PXN21 to reduce symptoms of CDI.

Firstly, probiotic strains could influence the gut microbiota such that growth of *C. difficile* is inhibited, possibly by out-competing for available nutrients or by secretion of antimicrobial

factors. This is the basis of treatments that focus on supplementation of the gut flora in order to prevent *C. difficile* from maintaining a niche within the GI tract. Supplementation of the gut flora with a complex or even minimalist mix of microorganisms mimics the protection against colonisation by *C. difficile* that the normal healthy microflora of the gut provides. Single strains of bacteria are less likely to be able to successfully act as a substitute for the complex microflora of the gut. However, used alongside conventional antibiotics, particular strains could potentially exert enough influence in the gut environment to reduce the symptoms of infection and aid restoration of the gut flora. The PXN21 strain was shown to be able to transiently colonise the murine gut providing evidence for the strain being able to survive and exert an effect on the environment. The temporary nature of the colonisation shows that use of this strain is unlikely to permanently alter composition of the microflora, although further study would be required to confirm this. The state of the microflora is an important factor in susceptibility to CDI, therefore the long term impact on the microflora must be an important consideration of probiotic usage.

It is possible that the probiotic strain served as competition for *C. difficile* during the infection, but there is no direct evidence for this aside from the need for treatment with viable bacteria. It is possible however that competition between probiotic and pathogen was spatially defined with the probiotics filling the role of the commensal microflora by associating with the mucosa (O'Hara & Shanahan 2006). Histological sections from infected animals showed protection of the mucosal surface in probiotic treated animals, and a potential competition based mechanism behind this could be that presence of the probiotic strain prevents access to the tissue by *C. difficile* toxins.

A second potential probiotic mechanism is stimulation of a robust innate immune response in the gut associated lymphoid tissue (GALT). Immune regulation plays a large role in homeostasis in the GI tract with the commensal microflora influencing how the system responds. Probiotic bacteria will have a direct effect on immune regulation in a disrupted environment. In this work, both spores and vegetative cells of *B. subtilis* demonstrate the ability to interact with receptors of the immune system and stimulate responses. This was shown through basic *in vitro* assays, which are difficult to compare with the complex *in vivo* environment, but do provide indications of potential interactions for *B. subtilis* within the GI tract. The number of bacteria used in probiotic treatment also affects immune regulation within the gut. Spores of *B. subtilis* stimulated production of TNFα and IL-6 in a macrophage cell line, both of which are proinflammatory cytokines. IL-6 production in particular has been linked to stimulation of mucosal responses and IgA production (Ramsay *et al.* 1994; Mantis *et al.* 2011). Histological examination of tissue shows the least damage occurs in probiotic treated animals, implying a protective effect at the surface of the tissue. Presence of the *B. subtilis* strain could stimulate a mucosal immune response, contributing to protection of the mucosal surface. The normal gut flora acts to mediate intestinal immune responses, but when dysbiosis occurs this control is lost (Schiffrin & Blum 2002; Pils *et al.* 2011).

The impact of interactions with the immune system is difficult to assess with data available from this study. In order to clarify the role of interaction with the immune system, immune deficient mice could be used in a similar study. Use of Myd88$^{-/-}$ mice in the study of *C. difficile* infection has been described previously (Lawley *et al.* 2009*a*). Mice with this mutation in innate immune signalling were more susceptible to disease. If use of probiotics in an infection model involving these mice resulted in increased survival as demonstrated in

this work, this further study would give an indication that the probiotic response was not due to interaction with the innate immune system in the gut, and so clarifying an aspect of the mechanism. Stimulation of an innate immune receptor, TLR5, has been used previously to prevent symptoms of CDI (Jarchum *et al.* 2011). Jarchum and colleagues used targeted TLR stimulation for successful treatment of CDI, thereby demonstrating how important specific interactions with the innate immune system could be in developing a treatment for CDI.

Finally, it is possible that in toxin mediated disease, probiotics may moderate symptoms by reducing levels toxins present in the gut lumen. This is particularly relevant for probiotic spores since these entities have been shown able to efficiently absorb toxins onto the spore surface. This action draws a comparison to the novel treatment Tolevamer, a polymer that is able to bind toxins of *C. difficile* (Hinkson *et al.* 2008). While demonstrating the ability of PXN21 spores to bind toxin *in vivo* proved difficult, it is plausible that this mechanism could contribute to the probiotic effect. However, in this study, given the large amount of free toxin in the gut lumen it is unlikely that this is the sole mechanism by which the PXN21 strain produces a probiotic effect.

The studies shown in this thesis support the role of innate immunity in the positive probiotic response, since PXN21 spores are shown to up regulate expression of TLR2. Peptidoglycan is one of many ligands associated with TLR2 induction and was used as a control for *in vitro* experiments. Spores carry peptidoglycan in their cortex, a layer lying beneath the spore coat, although when autoclaved spores were used induction of TLR2 was substantially reduced. Vegetative cells of PXN21 showed strong induction of TLR2, most likely due to peptidoglycan exposed on the cell envelope. This suggests that spore germination and release of the growing vegetative cell might be critical to induction of

TLR2. This assumption is based on the fact that when the spore is inactivated by autoclaving it is not broken but remains intact, so it is unlikely that peptidoglycan within the spore cortex is released in significant quantities. In other studies TLR2 was shown to be activated by live *B. subtilis* PY79 spores but not by heat-killed PY79 spores nor spores unable to germinate (Huang *et al.* 2008). Interestingly, in other work live spores of *B. subtilis* PY79 were shown to significantly delay symptoms of CDI in a hamster model of infection (Permpoonpattana *et al*. 2011*a*). Although hamsters did eventually die from CDI PY79 spores were clearly shown to delay fatalities and most likely this is due to germination and release of live *B. subtilis* vegetative cells. A clear result of TLR2 activation would be induction of pro-inflammatory cytokines and this study confirmed that i) both IL-6 and TNF-α were induced and ii) induction did not occur with heat-killed PXN21 spores. Again, proof that this must arise from germination of live spores to the vegetative cell comes from indirect evidence using germinating and non-germinating spores of PY79.

4.3.2. Probiotic treatment regimen

Survival rates of infected animals in this study depend on the dosing strategy employed. This highlights dose regimen as an important issue and also shows that optimisation studies are imperative to identify the most successful method and timing for use of treatments. The mechanism of probiotic action will have a significant impact on design of the treatment regimen. Evaluating how the probiotics increase survival rate in *C. difficile* infected mice is important to the application of the treatment.

Factors to be considered in optimising use of the PXN21 strain in a CDI treatment include timing and size of doses and also viability of the probiotics.

The dose size is an important aspect of this treatment, linking with two potential mechanisms, impact on the microflora and stimulation of an innate immune response. Unlike other work where an increased dose resulted in an increased probiotic effect (Gao *et al.* 2010), use of a smaller dose appears more efficacious with *B. subtilis* PXN21. The key to recovery from CDI is restoration of homeostasis within the GI tract, both in terms of normal microbiota reasserting itself and the return to normal immune regulation. Reducing the size of the probiotic dose suggests that commensal microflora have less competition for nutrients and space as they repopulate the gut. This would aid recovery of the system once the infection has cleared and therefore contribute to a faster improvement in health. Higher numbers of bacteria have the potential to increase immune responses, which may lead to over expression of inflammatory factors within the disrupted gut, as occurs in chronic conditions such as IBD (Duchmann *et al.* 1995; O'Hara & Shanahan 2006). The introduction of probiotics further alters the GI tract from its original state, even if the probiotics do produce the immediate benefit of attenuating effects of CDI. Reducing the dose of spores in probiotic doses limits stimulation of the immune system decreasing the risk of a damaging inflammatory response. This effect can be linked to results from pre infection treated groups, where surviving animals demonstrated an improved weight profile. In pre-infection dosing, the probiotic strain has time to adjust to the environment and find a niche to colonise, so numbers are limited to capacity of the system, avoiding overstimulation. Increased success with a reduced dose may however be a function of the physical size of the model used.

Timing of treatment is also a significant factor in success of the probiotic treatment, with benefits that can be attributed to probiotic colonisation apparent in the comparison of dose regimens. Probiotics delivered prior to infection did not increase survival rate as

effectively as post infection treatment. However, surviving individuals treated prior to infection demonstrated less weight loss than animals receiving probiotics post infection. This observation suggests that the mechanism behind protection depends on the dose regimen employed, hence the difference in survival rates. Probiotic doses delivered prior to infection provide time for the strain to colonise the gut. Colonisation allows the strain to establish itself within a niche and it is then able to play a role in a suitable response, tailored to the situation when infection occurs. With probiotics dosed after infection, there is not sufficient time to adapt to the environment, so responses and interactions in the GI tract are not stabilised. The weight profile of the two groups reflects the difference in response to treatment; pre-treatment allows for a more stabilised environment, resulting in healthier animals. Post infection treatment acts acutely, preventing death, but does not support the recovery of microflora to a balanced, homeostatic state.

The reduced rate of survival in the pre-treated group compared to the group receiving treatment after infection is likely due to some individual variation existing. That survival rate is increased compared with the non-treated group shows that at the very least, the dosing of probiotic spores prior to infection exerts a lasting beneficial effect. These results are supported by data from the hamster model both in this and previous work (Permpoonpattana *et al.* 2011*a*), where individuals given *B. subtilis* before infection showed an increase in survival time.

A fundamental finding in this study is the importance of the viability of *B. subtilis* spores when used as a probiotic treatment, highlighting that it is not just when treatment is delivered but also what is delivered. Heat killed spores showed none of the beneficial effects demonstrated with the use of viable spores or vegetative cells. The probiotic effect can therefore be attributed to the ability of viable spores to germinate and establish

vegetative growth in the GI tract. This links to stimulation of innate immune responses through presence of peptidoglycan, amongst other ligands, from vegetative growth. *In vitro* data shows IL-6 production is reduced when non-germinating spores are used and is eradicated completely when heat killed spores are used to stimulate cells. TNFα is produced only when the cultured macrophages are incubated with live spores that germinate over course of the experiment. A similar effect is seen with study of TLR2 expression where use of heat killed spores reduces the expression of TLR2 in comparison to use of live spores or vegetative cells. Increased responses due to vegetative growth and germinating spores may be due to the increase in bacterial number but can also be attributed to ligands produced by growing cells. Heat killed spores in this study demonstrated only a limited ability to stimulate expression of TLR2. Inactivated spores are unable to germinate and enter a vegetative growth phase. Lack of a vegetative phase prevents further interactions within the gut and limits the time period that spores will remain in the gut. Interaction with the immune system is important information in considering a probiotic treatment. It is plausible that presence of the non-commensal probiotic strain induces immune responses that contribute to a response against *C. difficile*, in effect priming the immune system to act against an infection. Based on results from this work, it is unlikely that *B. subtilis* spore specific interactions are responsible for the increase in survival when *C. difficile* infected mice are treated with probiotics. Vegetative growth of the strain carries more importance than the spores themselves. *In vivo* studies show no improvement in condition when heat killed spores are used, so any interactions specific to spores with innate immune receptors have little impact. The strain itself proves beneficial; however spores as a dormant form do not contribute significantly to the probiotic effect.

The majority of studies on the use of probiotics use viable bacteria, as viability is inherent to the current definition of probiotics (Araya *et al.* 2002). However, there are studies that have reported beneficial effects with use of non-viable bacteria (Xiao *et al*. 2002; Ventola *et al.* 2012; Lahtinen 2012). Benefits are specific to strains and the method of inactivation used. The findings of this work however, show that *B. subtilis* PXN21 spores need to be viable to exert a beneficial effect. This highlights a key point in probiotic research; generalisations cannot be applied to the use of different strains, as properties of individual strains will vary.

Occurrence of disease attributable to *C. difficile* has been assessed on several levels in this study. Despite this, no single defined mechanism can be linked to the increased survival of probiotic treated animals, although options for further study do exist to that could help clarify this. It is likely that several mechanisms contribute to the beneficial action of the probiotics. This is not surprising, as the G.I. tract itself is a complex environment. Gaining understanding of how the probiotic effect is achieved would enable more informed use of this treatment in terms of dosing regimens.

4.3.3. Application to a commercial product

Results from this work show that the *B. subtilis* PXN21 strain can be used to attenuate the symptoms of disease in *C. difficile* infected mice. Following this success, application of this to a treatment for human disease is a possibility to be addressed.

Factors to be considered in development of a product include not just the success of a treatment but also the ease of delivery, storage and overall cost.

Differences in survival rates of groups treated with either viable or non-viable bacteria make it clear that vegetative growth of the strain is essential for the probiotic effect. Survival rate was increased further when vegetative cells were used rather than spores in the probiotic dose. It is worth noting however, that these doses contained freshly grown cultures of cells, which would be difficult to replicate if this type of treatment was to be developed commercially. Logistics of probiotic treatment doses need to be considered. *B. subtilis* spores make a very attractive probiotic treatment; robust, dormant forms of the bacteria can be stored for long periods without loss of viability as would occur in vegetative cells. Also, spores can be delivered orally without risk of sensitivity to the extreme pH and digestive enzymes of the stomach and GI tract. If *B. subtilis* PXN21 as a probiotic treatment was to be used in a human, clinical situation, vegetative cells may well provide better protection against symptoms as demonstrated in this study. However, the ease of use that spores provide may outweigh the potential benefits of doses containing vegetative cells. Parallels can be drawn to an existing product Enterogermina™, which is a suspension of *B. clausii* spores. This product is marketed by Sanofi Aventis for the treatment of gastrointestinal disorders (Hong *et al.* 2005), and serves conveniently as a model for a spore based product. Two billion viable *B. clausii* spores are present either in a vial or capsule, for oral administration. Use of a pure suspension of spores avoids the need for dairy based matrices to support the probiotic strain, a model that is commonly used in probiotic products. This gives several advantages, firstly the creation of a simple product with no refrigeration required. Secondly, avoiding the use of an associated dairy based food product makes the product more accessible especially to those with lactose or dairy intolerances. A similar product could be developed using *B. subtilis* PXN21 spores that demonstrates the same ease of use.

Despite increasing survival rate in infected mice, treatment with *B. subtilis* PXN21 does not prevent infection so cannot be used as a standalone treatment. Antibiotics must still be relied upon to eliminate infections of *C. difficile*. *B. subtilis* PXN21 spores could therefore be considered for use as an adjunct therapy alongside or prior to antibiotic infection. Giving doses of these spores to individuals at risk of developing *C. difficile* infection could help reduce infections or bolster the gut against severe disease, as seen from the murine study results. The murine study represents a very simple controlled infection situation which allows basic research to be carried out before involvement with the clinically complex human situation. Evidence of a beneficial effect from the PXN21 strain in this model could feed into further study and potentially lead to design of a product that could also prove beneficial to human health. The murine model therefore is useful but only trial in human subjects would provide definitive results on the potential of the PXN21 strain for treatment of CDI.

It is clear from the results in this study that the *B. subtilis* PXN21 strain alone will not prevent or cure *C. difficile* infection. However, use of the PXN21 strain to reduce symptoms of CDI is of significant interest and can be applied to further research into *C. difficile* treatments. Development of synthetic stool treatments uses a range of bacterial strains and species that in combination are able to treat infection. Identification of strains such as PXN21 that are able to reduce symptoms of disease caused by CDI could potentially be useful to production of such treatments, allowing further application of this work.

4.4. Conclusion

B. subtilis PXN spores can be used as an oral treatment to reduce the severity of CDI symptoms in a murine model of infection. Success of this treatment is demonstrated through increased survival rates. Use of the murine model provides a good platform for this work, allowing in depth study of the disease progression in this model. A precise mechanism was not defined for the probiotic treatment, however it is likely that multiple mechanisms contribute to increased survival rates in probiotic treated groups. Complex interactions occur in the gut and as such unravelling the mechanics of interactions is challenging.

A key observation was that *B. subtilis* spores must be viable in order to obtain the probiotic effect. This suggests that interaction with the immune system and ability to colonise a niche within the GI tract relies on vegetative growth of the probiotic strain. Results from use of a variety of treatment strategies highlighted the importance of optimising the dosing regimen. The number of bacteria per dose, timing and regularity of treatment are all important factors for the successful use of this probiotic strain.

Use of the *B. subtilis* PXN21 strain does not prevent infection and will not replace use of antibiotics. A single bacterial strain is unlikely to be able to exert a strong enough effect to inhibit *C. difficile* infection. Investigating the use of a single strain is still relevant however, as the complex interactions within the GI tract will only be understood once they have been stripped down to simple steps and then built back up again. This idea, however, represents a very large body of work. The study here reflects critical basic information about how infection can be managed. In order for *C. difficile* to be controlled, a more complex treatment has to be used, mimicking how the microflora could normally supress

invasive pathogens. A successful *C. difficile* treatment should address both restoration of homeostasis in the gut and prevention of toxin mediated disease.

Spores of *B. subtilis* provide a stable, easy to store and easy to deliver probiotic treatment. *Bacillus* strains are already frequently used in probiotic applications and this history of use demonstrates their safety for human consumption. The findings from this work show the potential of the *B. subtilis* PXN21 strain in treating CDI. Application of these findings could result in production of a single strain probiotic supplement, or used in design of a complex probiotic mix.

Chapter 5

The Spore-Associated Protein BclA1 Affects the Susceptibility of Animals to Colonisation and Infection by *Clostridium difficile*

This work has been published in part:

The spore-associated protein BclA1 affects the susceptibility of animals to colonization and infection by *Clostridium difficile*.

Phetcharaburanin, Hong and Colenutt *et al.* Molecular Microbiology, 92(5). 1025-1038

I would like to acknowledge my co-authors for their contributions to planning and carrying out practical work, with specific reference to in vivo experiments.

5.1. Introduction

5.1.1. Clostridium difficile spores and infection

The spore of *C. difficile* is the dormant state of this organism and is the primary agent of transmission (Gerding *et al.* 2008). This has been supported by recent studies where a mutant strain of *C. difficile*, unable to produce the Spo0A protein (a regulatory protein essential for spore formation) fails to persist and transmit the disease (Deakin *et al*. 2012).

The Spo0A protein has also been implicated to have role in biofilm formation, with a similar mutant strain unable to form biofilms as robustly as the wild type (Dawson *et al.* 2012). Interestingly, mice infected with *C. difficile* can exist in two physiological states, a carrier state, where low levels of *C. difficile* spores are shed in the faeces and a 'supershedder' state where large numbers of spores are shed (Lawley *et al*. 2009). This 'supershedder' state is induced following antibiotic treatment and most closely resembles the human clinical situation. Capable of withstanding heat, desiccation and noxious chemicals, spores facilitate transmission of *C. difficile* outside of the host and therefore present a major burden to hospitals in containment and disinfection (Vonberg *et al*. 2008).

Although toxin A and toxin B are considered the two main virulence factors, others cannot be excluded; for example, it has been shown that hamsters challenged with spores of the non-toxigenic strain CD1342 showed mild caecal pathology characterised by local acute epithelial cell loss, haemorrhagic congestion and neutrophil infiltration (Buckley *et al*. 2013). Similarly, hamsters colonised with non-toxigenic strains, M3 and T7, were protected against challenge with toxigenic B1 group strains (Nagaro *et al.* 2013). The mechanism for how these non-toxigenic strains confer protection remains both intriguing and unclear.

Given the importance of spores in the transmission of *C. difficile* an effective treatment should address presence of the spores in the GI tract. Antibiotics will have no effect on spores, only vegetative growth of *C. difficile*. Current vaccination strategies are focused almost exclusively on parenteral delivery of toxoids and the generation of protective humoral (IgG) responses (Gerding 2012) based on a response against toxins, a feature of vegetative growth. Although it is possible that anti-toxin serum IgG could reach the mucosa by transudation (Woodrow *et al*. 2012) systemic delivery of toxoids will otherwise fail to

produce polymeric secretory IgA (sIgA), the predominant mucosal antibody that has been shown to neutralise toxins (Johnson *et al.* 1992; Kelly *et al*. 1992). An alternative strategy in developing a vaccine against CDI would be to investigate use of antigens from the surface of the bacterium rather than factors produced in vegetative growth. This would have the benefit of creating a response against the presence of the bacteria rather than waiting until toxins are produced. Addressing decolonisation with a vaccine is complex although if achieved would be beneficial in the case of *C. difficile* where recurrences of infection are common. Recurrences could be due to spores remaining in the gut post antibiotic treatment, so a vaccine that aims to produce sterile protection would provide an obvious benefit here. CDI is a complex infection with multiple levels of clinical manifestation; for example, it has been shown that strains of *C. difficile* show differences in pathology in the hamster model of infection (Goulding *et al*. 2009) and that some high toxin producing strains (such as NAP1/027) can produce mortality in hamsters but without diarrhoea (Razaq *et al*. 2007). A further complication is that *C. difficile* infection can recur in about 30% of infected patients (Gerding *et al.* 2008b). Interestingly, antibodies to the surface layer proteins of *C. difficile* vegetative cells have been found associated with reduced levels of recurrence implying that useful antigenic targets associated with the vegetative cell may exist (Drudy *et al*. 2004).

5.1.2. Exosporium and BclA proteins

The role of the spore in transmission of the disease suggests that this dormant life form may play a key role in colonisation, a process better divided into three stages; establishment of infection, maintenance of infection (persistence) and spore shedding. Spores of *C. difficile* resemble those of other Gram-positive spore-formers but differ

somewhat in the abundance of enzymes they carry on their surface layers, including three catalases and a bifunctional peroxiredoxin-chitinase (Permpoonpattana *et al.* 2011*b*; Permpoonpattana *et al.* 2013). Spores also carry a poorly defined surface layer known as an exosporium whose function has been linked to germination, adhesion and resistance properties of the spore (Henriques & Moran 2007; Lawley *et al.* 2009; Escobar-Cortés *et al.* 2013). In some spore formers a major component of the exosporium is the BclA (bacillus collagen-like protein of anthracis) glycoprotein that carries collagen-like repeats of the amino-acid triplet GPT used for attachment to oligosaccharides and which forms a hair-like filament (Steichen *et al.* 2003; Sylvestre *et al.* 2002, 2003). A second collagen-like protein, BclB has also been identified in *B. anthracis* and has been linked to exosporium assembly (Waller *et al.* 2005; Thompson & Stewart 2008).

The genome of *C. difficile* 630 carries three *bclA* genes (*bclA1-3*) (Sebaihia *et al*. 2006) although in a proteomic analysis of *C. difficile* 630 spores, only BclA1 was identified as present (Lawley *et al*. 2009*b*). The *C. difficile* exosporium has been visualised as a loose sac like material surrounding the spore, but the appearance of hair like structures formed by BclA in other species have not been reported in *C. difficile* strains. Recent research (Pizarro-Guajardo *et al*. 2013) demonstrated that the BclA glycoproteins are located on the spore surface as part of the exosporium of *C. difficile* spores. The study also reports on the orientation of the glycoproteins, with the BclA N-terminal domain (NTD) buried inside the exosporium layer and the C terminal domain (CTD) angled to the surface of the exosporium. Parallels with work in *B. anthracis* can be drawn here, as similar orientation of the BclA proteins has been demonstrated (Boydston *et al.* 2005). Interest in the exosporium proteins of *C. difficile* has stemmed from studies in other species where

specific properties have been attributed to the presence of an exosporium. As the outer layer of the spore, this proteinaceous layer is important as it represents the primary interactions the spore potentially has in an environment. In studies with *B. cereus*, the exosporium layer has been implicated in adherence and persistence of the spores on abiotic surfaces, although variation is demonstrated between different strains (Faille *et al*. 2010). Adherence properties of spores are of interest in particular to the food manufacturing industry, where residual spores can be problematic in causing food poisoning cases. Decreased adherence reported after deletion of the BclA glycoprotein in *B. cereus* demonstrated the role this glycoprotein could play in the adherence of spores (Lequette *et al*. 2011). *B. anthracis* is another species that carries an exosporium, of which the BclA glycoprotein is a significant component (Sylvestre *et al*. 2002). The *B. anthracis* BclA protein was demonstrated to have immunogenic properties and has been trialled as a candidate antigen for use in a vaccine (Steichen *et al.* 2003; Brahmbhatt *et al*. 2007*a*). BclA is not necessary for virulence in *B. anthracis*, but has been shown to have an effect on adherence and germination characteristics of spores in this species (Brahmbhatt *et al.* 2007*b*).

5.1.3. ClosTron mutagenesis

In the past, work with the Clostridia has been restricted due to difficulties in genetic manipulation. Extensive study and gains in knowledge of the *Bacilli* has been enabled by genetic studies and while parallels can be drawn between the *Bacilli* and the Clostridia, species specific information is far more valuable. The development of the ClosTron system (Heap *et al*. 2007) for targeted inactivation of genes has facilitated accessible functional genomic studies in the Clostridia. This system uses an intron based process to produce

stable mutations by insertional inactivation of genes. Further refinement of the technique (Heap *et al*. 2010) has broadened the scope of the system, with additional plasmids to address such issues as intrinsic antibiotic resistances and production of multiple mutations in a single strain.

The ClosTron system is designed for use across the Clostridia, with research into *C. difficile* vastly benefitting from the availability of such a tool. A diverse range of studies have been undertaken using ClosTron with *C. difficile*, including characterisation of master regulator Spo0A (Underwood *et al*. 2009; Dawson *et al.* 2012; Deakin *et al.* 2012) and importance of toxins in disease (Lyras *et al.* 2009; Kuehne *et al.* 2010l). Functional genetic studies contribute significantly to understanding of this pathogen, enabling better treatments and control measures to be developed.

In the work of this thesis, mutant strains of *C. difficile* created using the ClosTron system will be characterised. ClosTron inactivation of the *bclA* genes should result in the absence of the proteins from the surface of the spore allowing analysis of how removal of the BclA proteins can affect the spore properties. A recent characterisation of the *C. difficile* BclA proteins used enzyme digests to remove BclA from the spores (Pizarro-Guajardo *et al*. 2013). This approach demonstrated that the proteins were present in the exosporial layer of the spore and allowed the orientation of the proteins to be determined. However, when defining the properties of the spores the collagenase treatment represents a deviation from wild type presentation of spores. In this thesis, spores will be purified using a Histodenz (Sigma) gradient to ensure that while a pure suspension of spores is obtained, they remain as similar to wild type as possible. The aim of the genetic approach to removing of the BclA proteins is to keep the rest of the spore structure as undisturbed as possible.

5.1.4. Experimental Objectives

The genome of *C. difficile* 630 carries three *bclA* genes (*bclA1-3*) which in this work will be mutated and characterised. To define gene function insertional mutagenesis of each gene will be carried out. Mutated strains will be analysed for changes in properties that may affect infectivity. Strains will be tested to ensure maintenance of resistance properties and then *in vitro* germination and adhesion will be studied. Infectivity will be assessed through the use of two different animal models – mice and hamsters. These studies will be carried out based on the hypothesis that the BclA proteins have a significant effect on the properties of the *C. difficile* spore.

5.2. Results

5.2.1. The *C. difficile* bclA genes

Three genes encoding BclA-like proteins, annotated as *bclA1, bclA2* and *bclA3* are present on the genome of strain 630 (**Figure 5.1**) and encode proteins with predicted masses of 67.8, 49.0 and 58.2 kDa respectively. Similar to the BclA proteins found in *B. anthracis* and *B. cereus*, all three *C. difficile* BclA proteins consist of a long, central, collagen-like region with multiple GXX repeats flanked by N- and C-terminal domains of varying length (**Figure 5.2A and 5.2B**). Most of the triplet repeats are GPT, with nearly all containing a threonine residue that could provide multiple potential sites for *O*-glycosylation as seen in *B. anthracis* (Daubenspeck *et al*. 2004) (**Figure 5.3**). Bioinformatic comparison of BclA protein sequences from *B. anthracis, B. cereus* and *C. difficile* revealed that most of the similarity between the proteins is limited to the collagen-like central region since both the C- and N-terminal domains of the *C. difficile* BclA proteins seem to be distinct from those found in other species (**Figure 5.4**). In *B. anthracis* BclA these terminal regions are implicated in trimerisation of the BclA monomers and their attachment to the exosporial basal layer (Boydston *et al*. 2005; Thompson & Stewart 2008). Importantly, the *C. difficile* BclA proteins do not appear to carry a sequence at their N-termini that resembles the motif (LIGPTLPPIPP) that targets the BclA and BclB proteins of *B. anthracis* to the exosporium (Thompson *et al*. 2011*a*; Tan *et al.* 2011; Thompson *et al.* 2011*b*).

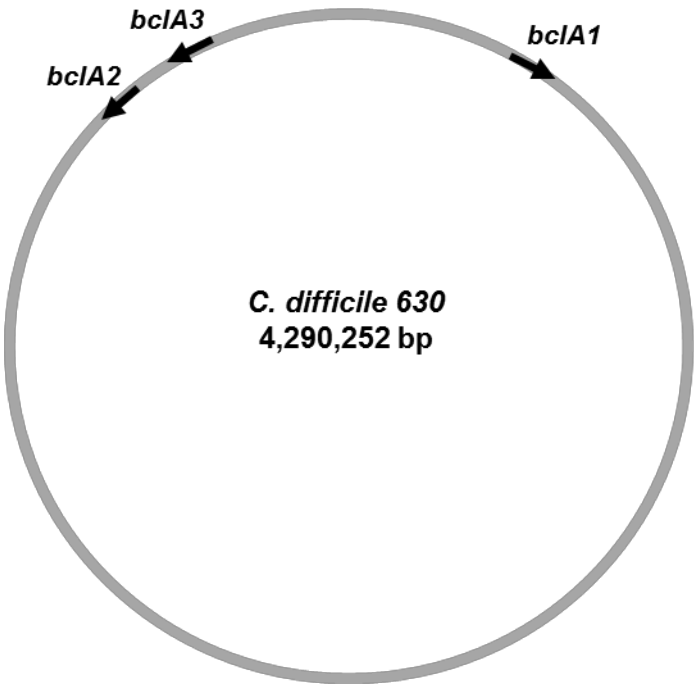


Figure 5.1. *C. difficile bclA* genes

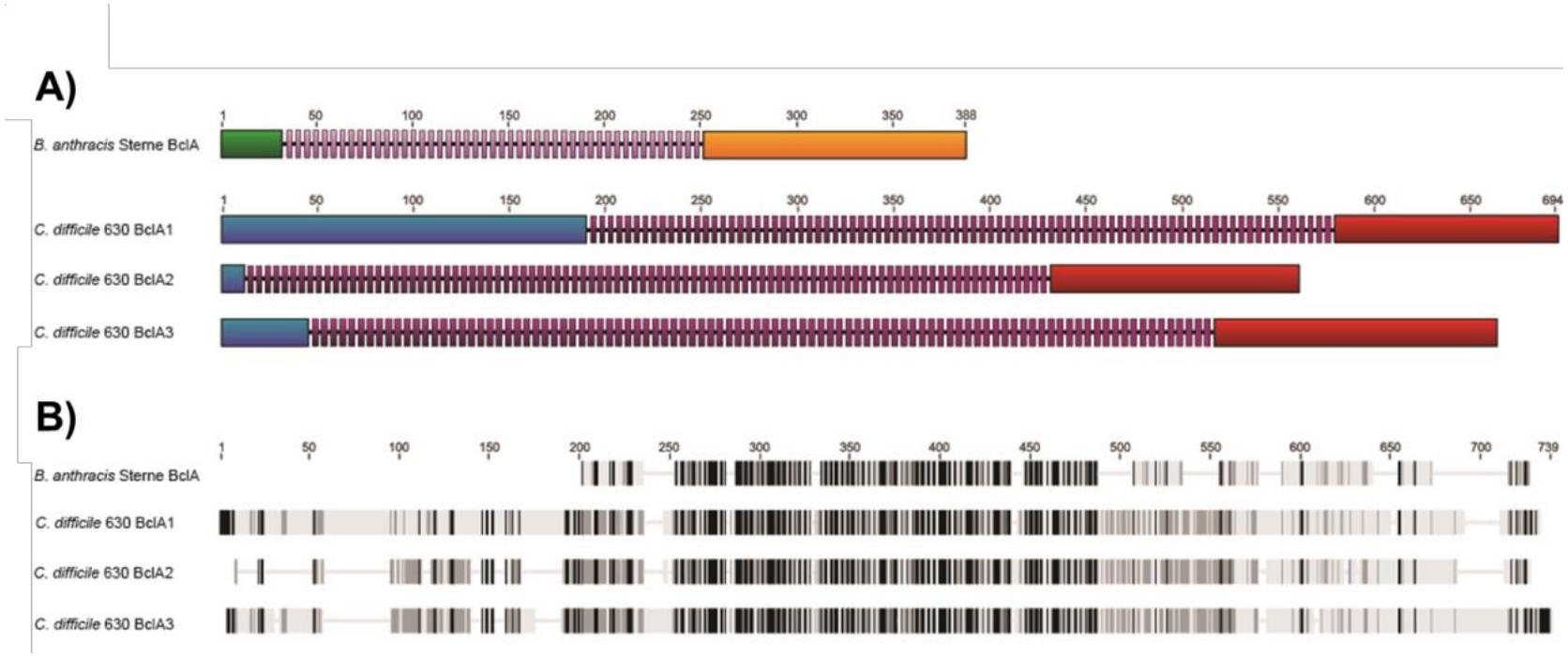


Figure 5.2. BclA proteins A) Schematic representation of the domain structure of BclA proteins from *B. anthracis* Sterne (AY995120.1) and *C. difficile* 630 (BclA1, CAJ67154.1; BclA2, CAJ70128.1; BclA3, CAJ70248.1). The central, collagen-like region (purple) contains multiple GXX repeats and is flanked by the N-terminal (green/blue) and C-terminal (orange/red) domains. In *B. anthracis* the C-terminal domain mediates trimerization of the BclA monomers while the N-terminal domain is implicated in anchoring the proteins to the exosporial basal layer. The function of these domains in *C. difficile* remains to be confirmed.

B) Pairwise alignment of the same BclA protein sequences from *B. anthracis* Sterne and *C. difficile* 630. Most sequence similarity is limited to the central, collagen-like region. Key: Black, 100% similarity; dark grey, 80-100%; light grey, 60-80; white, <60%. Pairwise identity was generated using ClustalW and a Biosum62 scoring matrix and between all four proteins was 39.1%.

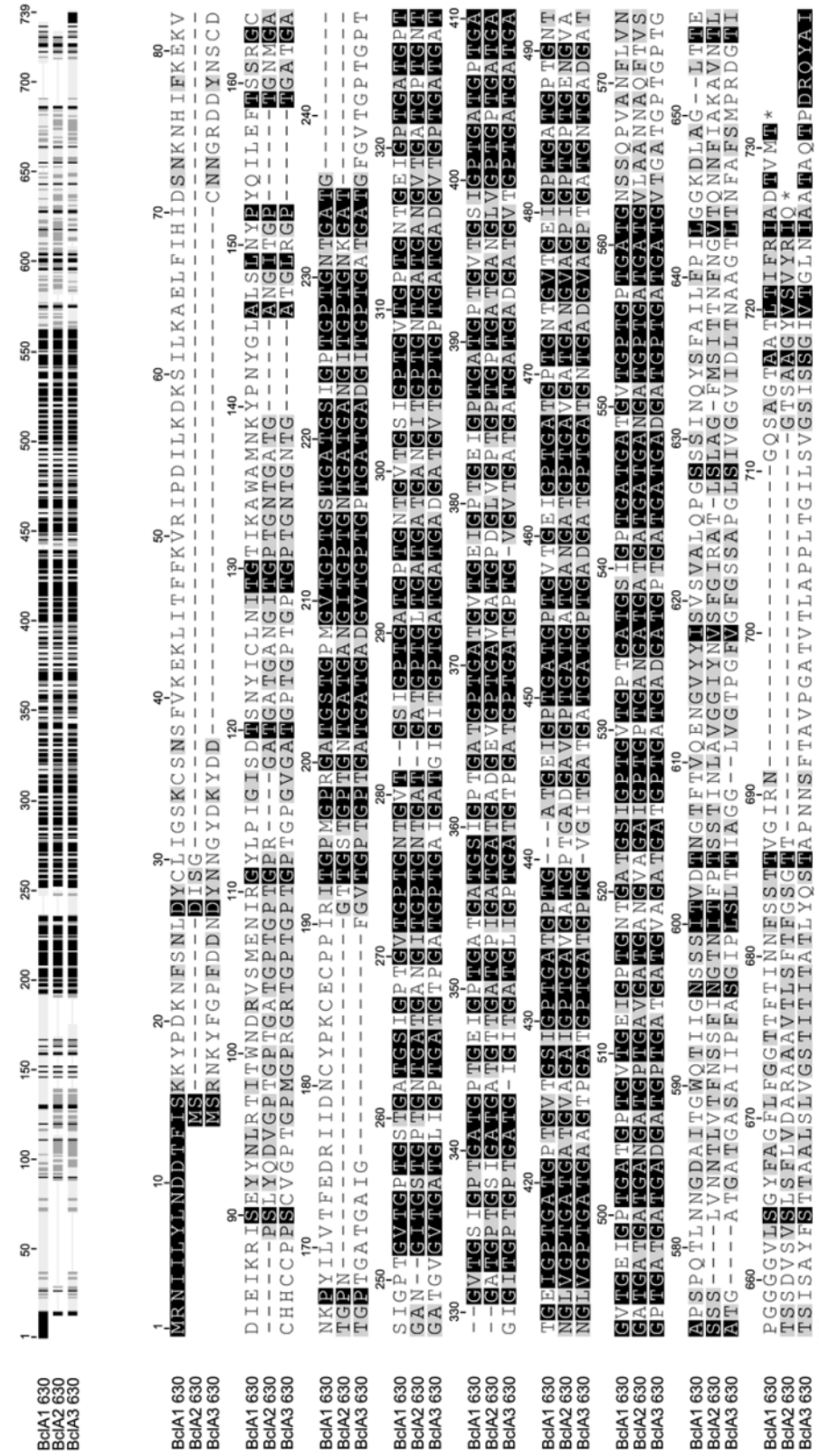


Figure 5.3. *C. difficile* 630 BclA proteins

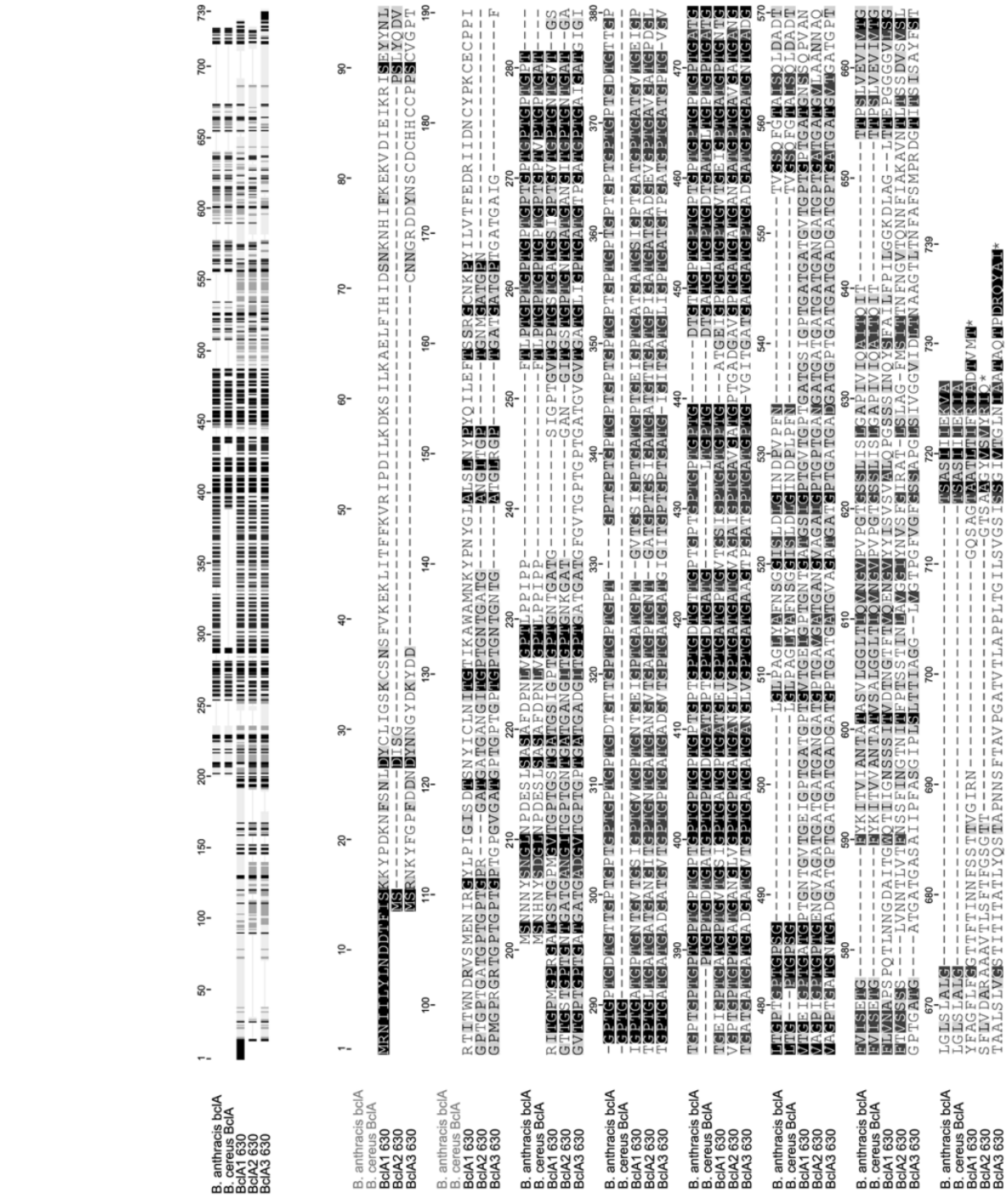


Figure 5.4. *B. anthracis, B. cereus* and *C. difficile* 630 BclA proteins

5.2.2. Phenotypes of *bclA* mutant spores *in vitro*

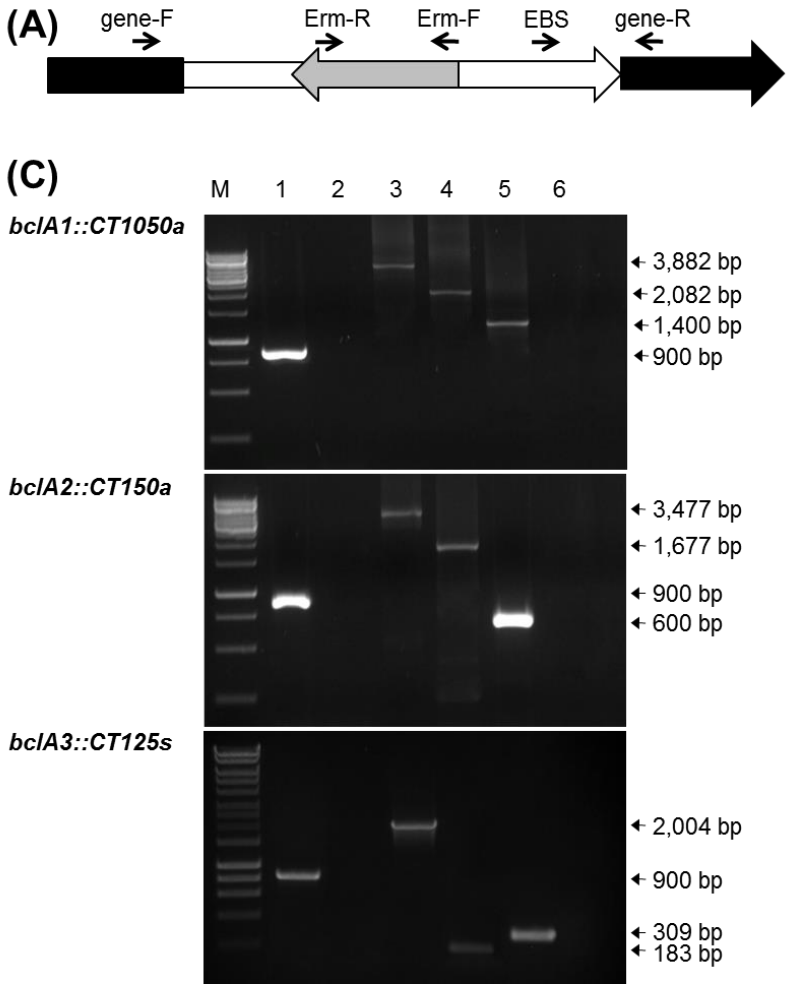
ClosTron mutagenesis was utilised to insertionally inactivate the three *bclA* genes creating the mutants, *bclA1::CT1050a, bclA2::CT150a* and *bclA3::CT125s* referred to hereafter as *bclA1*$^{-}$*, bclA2*$^{-}$ *and bclA3*$^{-}$ (**Table 5.1** and **Figure 5.5**). Mutants were examined for their growth and sporulation phenotypes in parallel with the isogenic Spo^{+} parent strain, 630Δ*erm.* Growth and sporulation of mutants in liquid medium was essentially identical between strains with approximately 10^{4}-10^{5} spores/ml produced after 5 days (**Figure 5.6**). Transmission electron microscopy (TEM) was used to examine the structure of wild type and mutant spores (**Figure 5.7**). Compared to 630Δ*erm* spores (**Figure 5.7A**) it was clear that for the *bclA1*$^{-}$ and *bclA2*$^{-}$ mutants there were substantial aberrations in the spore coats. In both cases sheets of coat-like material were present in the medium (**Figure 5.7C** and **5.7E**) as well as angular projections of material at the spore surface (**Figure 5.7B** and **5.7D**). The *bclA3*$^{-}$ mutant did not present any apparent defect compared to wild type spores nor was any coat-like material shed into the medium (**Figure 5.7F**).

Gene	Locus Tag[1]	Encoded Protein[2]	Mutant allele[3]	Retargeted sequence
bclA1	CD0332	Putative exosporium glycoprotein (83 kDa)	bclA1::CT1050a	ACTCCTGTCGCTCCT GTTGGACCTGTTGC T<intron>CCTGTTGG TCCTATA
bclA2	CD3230	Putative exosporium glycoprotein (67 kDa)	bclA2::CT150a	GCTCCATTTGCTCCT GTTGCTCCTGTCGC C<intron>CCTGTTGC TCCTGTC
bclA3	CD3349	Putative exosporium glycoprotein (79 kDa)	bclA3::CT125s	GTCGTGATGATTATA ATAGCTGTGATTGC< intron>CATCATTGCT GTCCAC

Table 5.1. Clostron insertional inactivation of *bclA* genes [1] as described in Sebaihia *et al* (2006) and schematically in Figure 5.1.

[2] predicted mwt of full-length protein in brackets.

[3] The mutant allele is shown with a) CT designing ClosTron insertion, b) the number showing the bp within the ORF immediately preceding the ClosTron insertion and c) letter *a* indicating insertion in the antisense strand.



(B)

Primers	Sequence 5'-3'
Erm-F	ACGCGTTATATTGATAAAAATAATAATAGTGGG
Erm-R	ACGCGTGCGACTCATAGAATTATTTCCTCCCG
EBS Universal	CGAAATTAGAAACTTGCGTTCAGTAAAC
bclA1-F	CATGCCATGGCCATGAGAAATATTATACTTTATTTA
bclA1-R	GCTGATTGCCCATTTCGTAT
bclA2-F	CATGCCATGGCCATGAGTGATATTTCAGGT
bclA2-R	ACCAAACCATCTGGACCTGT
bclA3-F	CATGCCATGCGCGTGCTTTTAATAATGAGTA
bclA3-R	GAGCCGAACTACCAAATCCA

Figure 5.5. Inactivation of *bclA* genes in *C. difficile* 630 using the ClosTron system

A) gene annotation. B) oligos used for screening mutants by PCR. C) PCR validations of *bclA1::CT1050a* and *bclA2::150a* using mutant (odd number lanes) and 630Δ*erm* (even number lanes) genomic DNA amplified with following pairs of primers; lanes 1,2: ERM-F and ERM-R, lanes 3,4: gene-F and gene-R and lanes 5,6: gene-R and EBS universal. M is a gene marker.

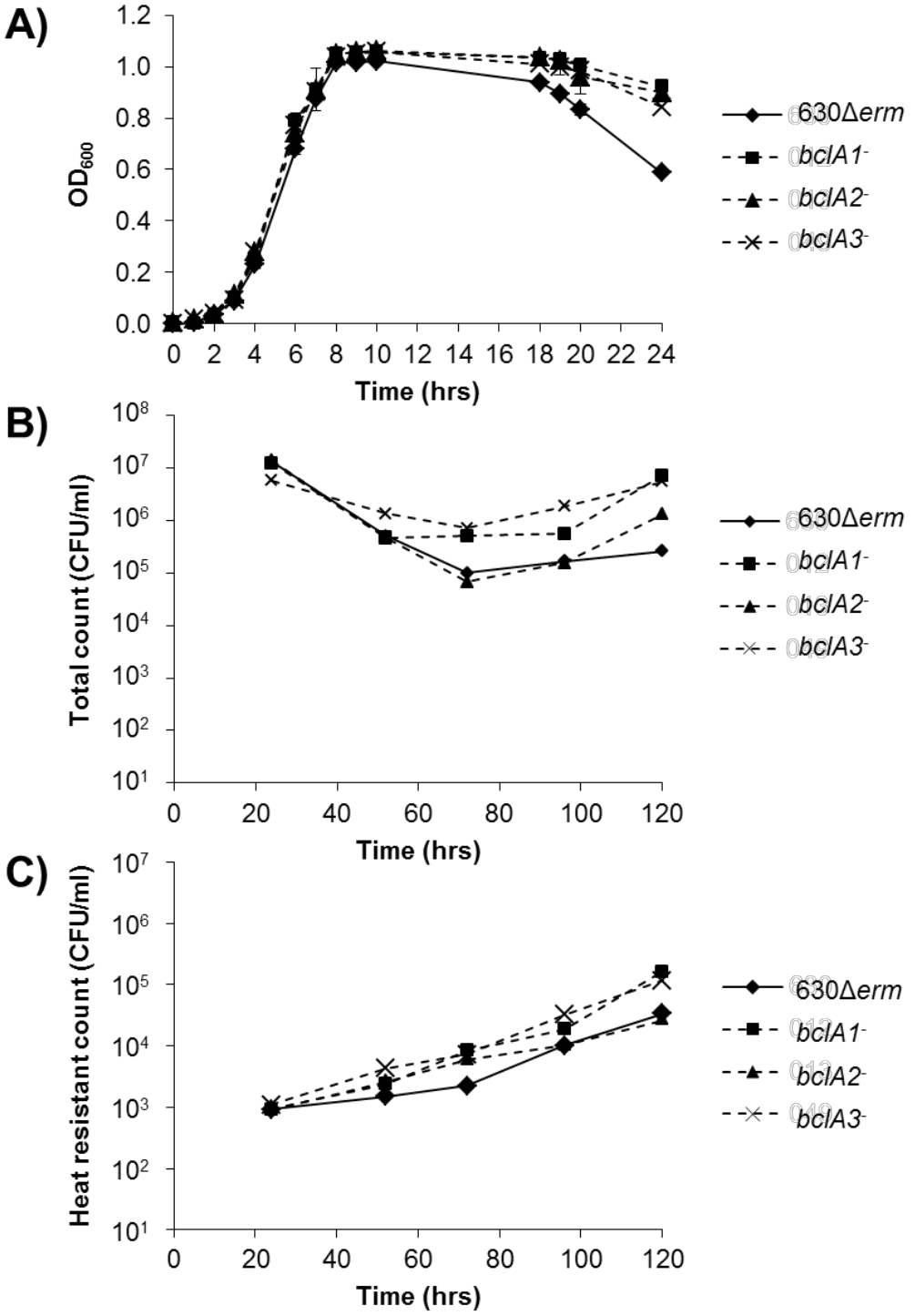


Figure 5.6. Growth of wild type and isogenic mutant *C. difficile* strains grown in BHI medium at 37°C over 24h.

A) OD_{600}

B) Total counts

C) Heat-resistant (spore) counts. Heat resistance measured at 60°C 20 min.

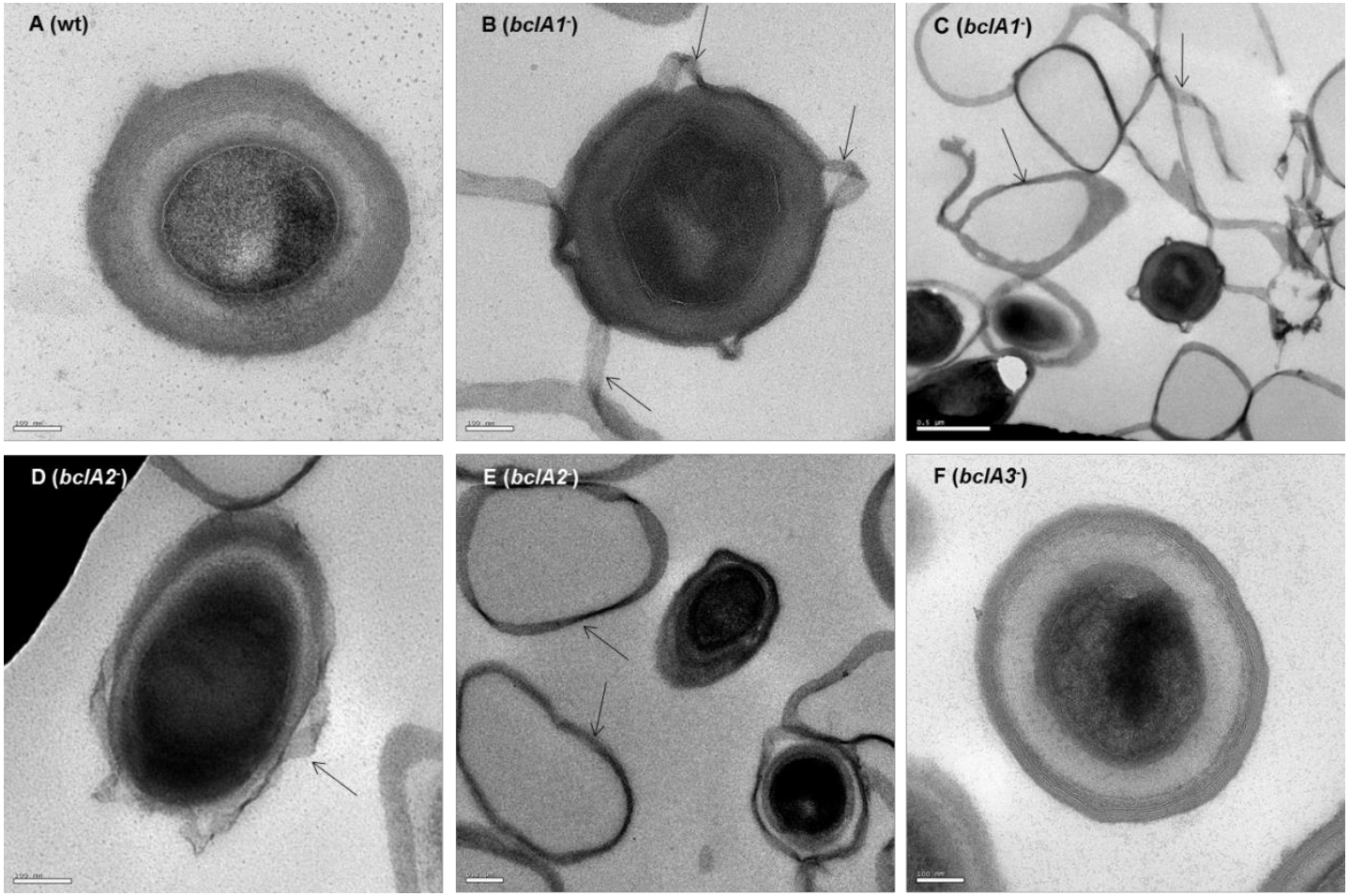


Figure 5.7. Spore ultrastructure analysis by TEM

A) high-magnification image showing purified 630Δ*erm* spores with a normal morphology. Bar: 100 nm.B) and C) *bclA1*$^{-}$ purified mutant spores showing clear defects including a sheet-like material on the outermost layer (arrows indicated). Bars: 100 nm (B) and 0.5 mm (C). D) and E) ill-formed *bclA2*$^{-}$ purified mutant spores with a sheet-like material (arrows indicated). Bars: 200 nm (D) and 0.2 mm (E).

F) a *bclA3*$^{-}$ mutant spore showing normal morphology. Bar: 100 nm.

Heat, ethanol and lysozyme treatments were used to determine the robustness of the mutant spores alongside the wild type strain. Histodenz-purified spores of all mutants showed no significant susceptibility to treatment with heat, ethanol and lysozyme (**Table 5.2**).

	Untreated	± SD	Heat	± SD	Ethanol	± SD	Lysozyme	± SD
630Δerm	1.08 X 10^7	1.41 X 10^6	1.46 X 10^7	2.03 X 10^6	5.20 X 10^6	1.05 X 10^6	2.27 X 10^6	7.23 X 10^5
bclA1$^-$	1.03 X 10^7	1.77 X 10^6	3.90 X 10^6	4.58 X 10^5	2.87 X 10^6	1.01 X 10^6	4.80 X 10^6	6.08 X 10^5
bclA2$^-$	9.20 X 10^6	1.21 X 10^6	4.40 X 10^6	6.24 X 10^5	3.07 X 10^6	4.04 X 10^5	2.23 X 10^6	1.18 X 10^6
bclA3$^-$	1.00 X 10^7	1.57 X 10^6	4.20 X 10^6	5.57 X 10^5	2.13 X 10^6	3.51 X 10^5	4.17 X 10^6	9.61 X 10^5

Table 5.2. Resistances of spores from *bclA*$^-$ strains.

[1]Spores purified using Histodenz (Sigma) were tested for resistance to heat, ethanol and lysozyme.

Heat: 10^7 spores suspended in sterile water were incubated at 60^{o}C for 24h.

Ethanol: 10^7 spores were suspended in 70% ethanol and incubated at RT with agitation for 24h. After incubation period, spores were washed once with sterile water.

Lysozyme: 10^7 spores were suspended in a buffer (20mM Tris HCl (pH8.0) and 300mM NaCl) containing lysozyme (1 mg/ml) and incubated with agitation at 37^{o}C for 20 min.

Serial dilutions for enumeration of surviving spores were plated on BHI agar supplemented with 0.1% sodium taurocholate. Plates were incubated in anaerobic conditions at 37^{o}C for 48h.

Purified spores were used to assess their ability to germinate in BHI medium supplemented with 0.1% sodium taurocholate as a germinant (**Table 5.3** and **Figure 5.8)**. As controls spore germination experiments were conducted in parallel in the presence of the inhibitor sodium chenodeoxycholate. Germination correlates to a loss in OD_{600} as spores rehydrate and become phase dark. 630Δ*erm* spores germinated relatively slowly with a 16% reduction in OD_{600} over a 30-minute period. All three mutants germinated faster than the wild type strain with the *bclA1*$^-$ and *bclA2*$^-$ mutants exhibiting the highest germination rates with 40% and 34% loss in OD respectively. In the presence of inhibitor though spores of wild type and mutant strains remained stable and all exhibited a maximum OD drop of 5-6% over 30 minutes. Germination of sonicated spores of the wild type strain was also evaluated in parallel and had an OD drop of 29% indicating that disruption of the spore surface layers enhanced germination (**Figure 5.8D**).

	% drop in OD	
Spores	+ inhibitor	+ germinant
630Δ*erm*	6	16
bclA1$^-$	6	40
bclA2$^-$	6	34
bclA3$^-$	5	20
630Δ*erm* (sonicated)	6	29

Table 5.3 Germination phenotypes.
% drop in OD was determined as % loss in OD_{600} in the presence of inhibitor (0.1% sodium chenodeoxycholate) or germinant (0.1% sodium taurocholate). All samples had an initial OD of approximately 1.0.

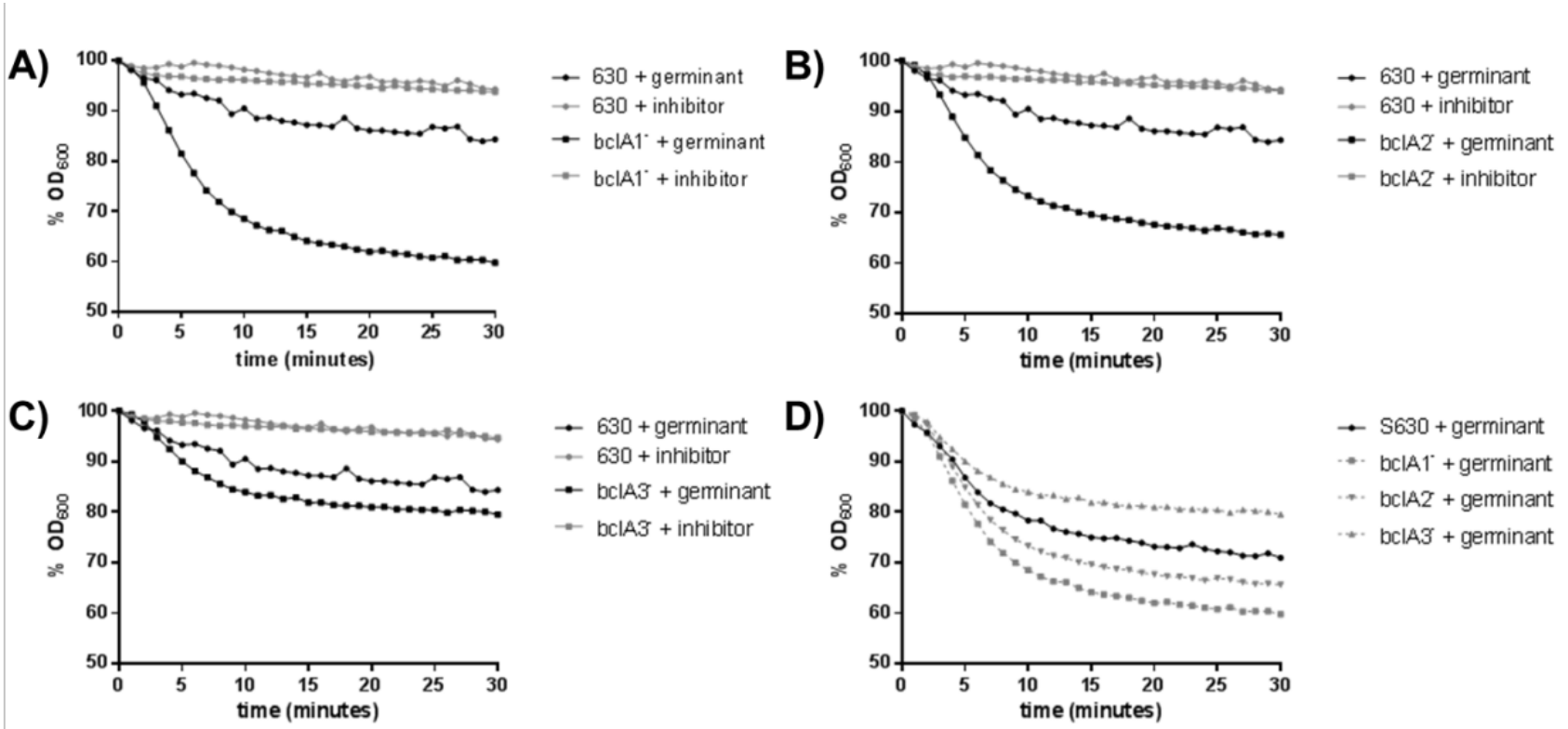


Figure 5.8. Germination of spores in BHI media, with either 0.1% sodium chenodeoxycholate (germination inhibitor) or 0.1% sodium taurocholate (germinant). A-C) shows fall in OD of *bclA*⁻ mutant spore suspension compared with WT 630Δ*erm* spores. D) shows fall in OD of *bclA*⁻ mutant spore suspension compared to sonicated (S) WT spores. Loss of OD_{600} from start OD (100%) represents germination of spores as phase brightness is lost at start of germination process.

% fall in OD was determined as recorded OD_{600} at time interval/initial OD_{600}) X 100. All samples had an initial OD of approximately 1.0.

The hydrophobicity of purified spores was measured and all three *bclA* mutants were significantly (p<0.035) less hydrophobic than spores of the wild type 630Δ*erm* (**Figure 5.9**). It has been shown recently that sonication of *C. difficile* spores is efficient at removing the exosporium (Escobar-Cortés *et al*. 2013). For comparison, sonicated spores were used to demonstrate that sonication significantly (p<0.024) reduced the surface hydrophobicity of 630Δ*erm* and *bclA* mutant spores with the exception of the *bclA3*$^{-}$ mutant (p=0.185).

Polyclonal antibodies raised against recombinant BclA1, BclA2 and BclA3 proteins were used to confirm that each protein was i) located on the surface of 630Δ*erm* spores, ii) absent in vegetative cells and, iii) not present in spores of the corresponding isogenic mutant (**Figure 5.10**).

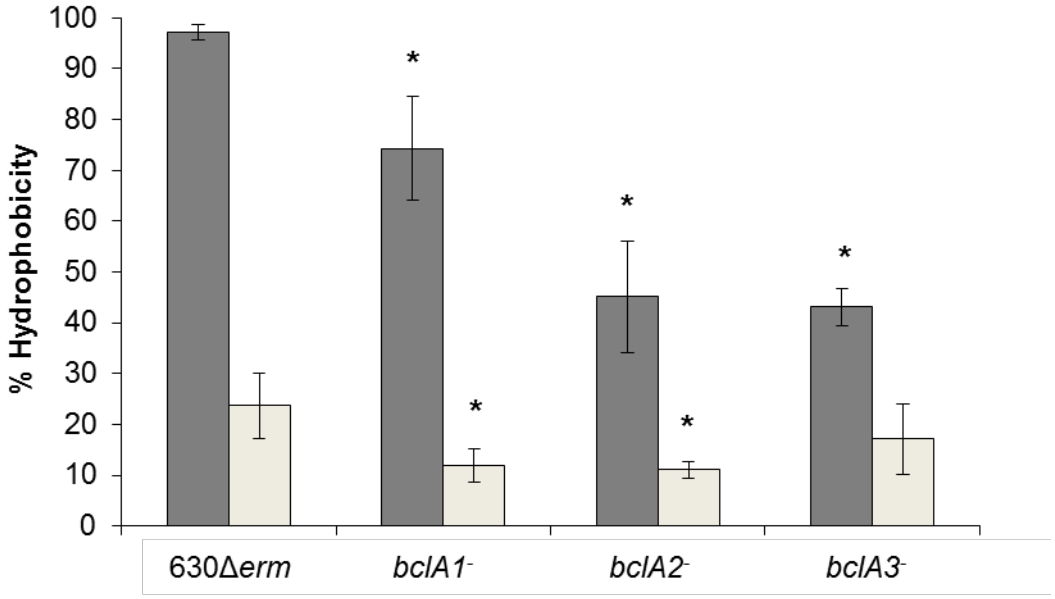


Figure 5.9. Spore hydrophobicity. The SATH assay was used to calculate % hydrophobicity of Histodenz-purified spores of wild type and mutant spores with (white column) or without sonication (grey column). The analysis was performed three times. * indicates values significantly different between *bclA* mutants and 630D*erm* (*bclA1*$^{-}$, 0.036; *bclA2*$^{-}$, 0.0064; *bclA3*$^{-}$, 0.0006) and sonicated mutant and 630D*erm* spores (*bclA1*$^{-}$, 0.02; *bclA2*$^{-}$, 0.0243).

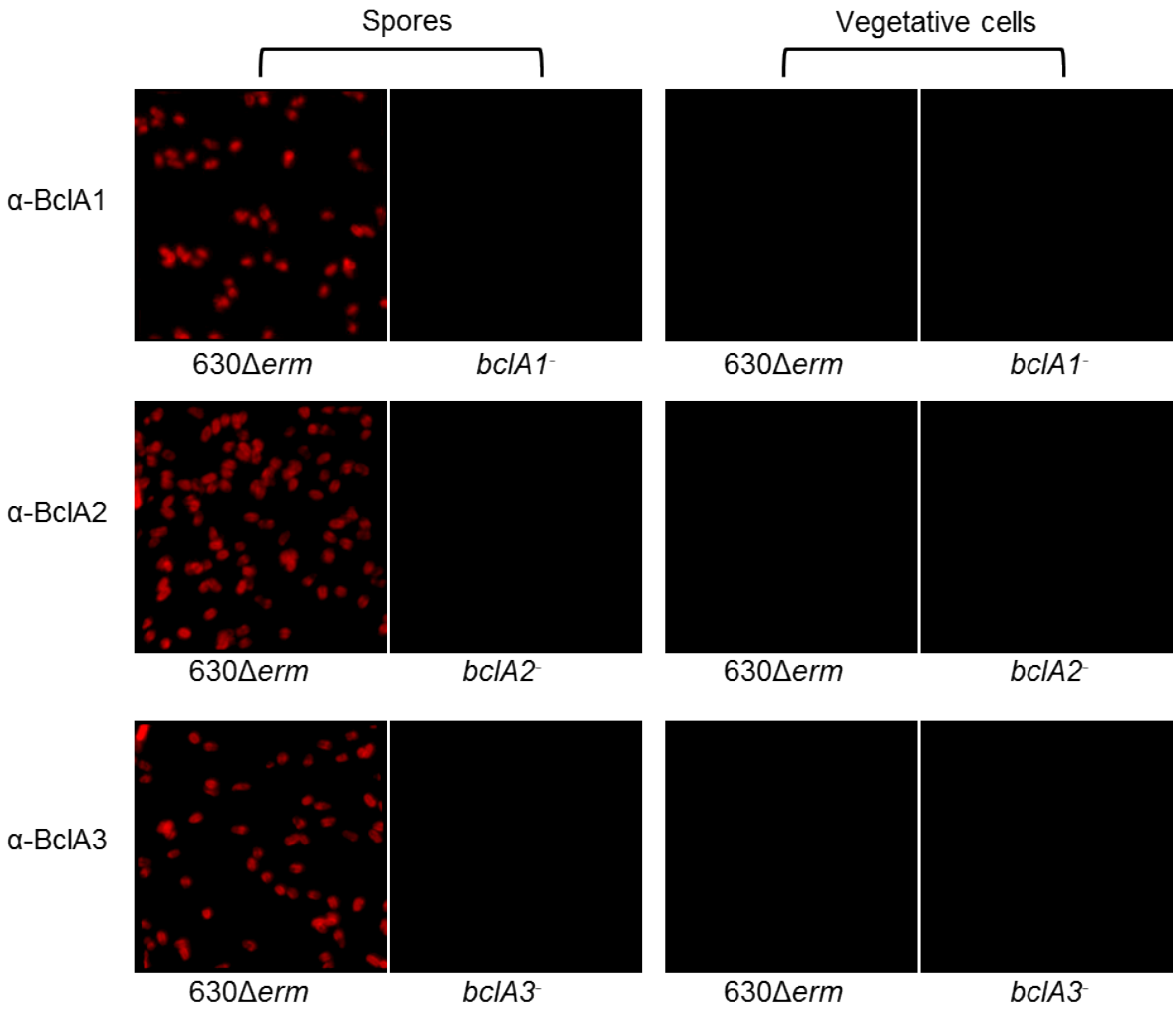


Figure 5.10. Surface display of BclA1, BclA2 and BclA3 proteins using immunofluorescence imaging of suspensions of 630D*erm*, *bclA1*$^-$, *bclA2*$^-$ and *bclA3*$^-$ spores (7-day old cultures grown on solid medium) labeled with mouse serum (1:1,000 dilution) raised against each of the three BclA proteins. An anti-mouse IgG-TRITC conjugate was used for secondary labeling. BclA1, BclA2 and BclA3 proteins were detected on both purified and non-purified 630D*erm* spores whereas the *bclA* mutants showed negative signals. Controls included vegetative cells of wild type and mutants.

5.2.3. *In vivo* characterisation of BclA1$^-$ spores

The recently described mouse model of cefoperazone pre-treatment to induce *C. difficile* infection (Theriot *et al*. 2011) was used to evaluate the progress of shedding of *C. difficile* spores. Animals were given a single dose (10^4) of mutant or wild type spores (**Figure 5.11**). Total counts (spores and vegetative cells) of *C. difficile* shed in the faeces ranged from 10^4 to 10^6 per gram although somewhat lower counts were found for the *bclA1*$^-$ mutant (**Figure 5.11B**). Mice body weights remained similar with no significant differences between groups (data not shown). Spore counts of 630Δ*erm*-dosed mice declined over time (**Figure 5.11A**). Spores were not found in the faeces from mice dosed with the *bclA3*$^-$ mutant on day 1, despite substantial levels of spores being detected on days 3, 5 and 7. Spore counts of both the *bclA2*$^-$ and *bclA3*$^-$ mutants increased after day 1 and were substantially higher (>1-log) on days 3, 5 and 7 compared to that of wild-type infected animals (**Figure 5.11A**). Surprisingly no spores were detected for the *bclA1*$^-$ mutant in the faeces post-infection (**Figure 5.11A**) in all the time points. The experiment has been repeated with similar findings.

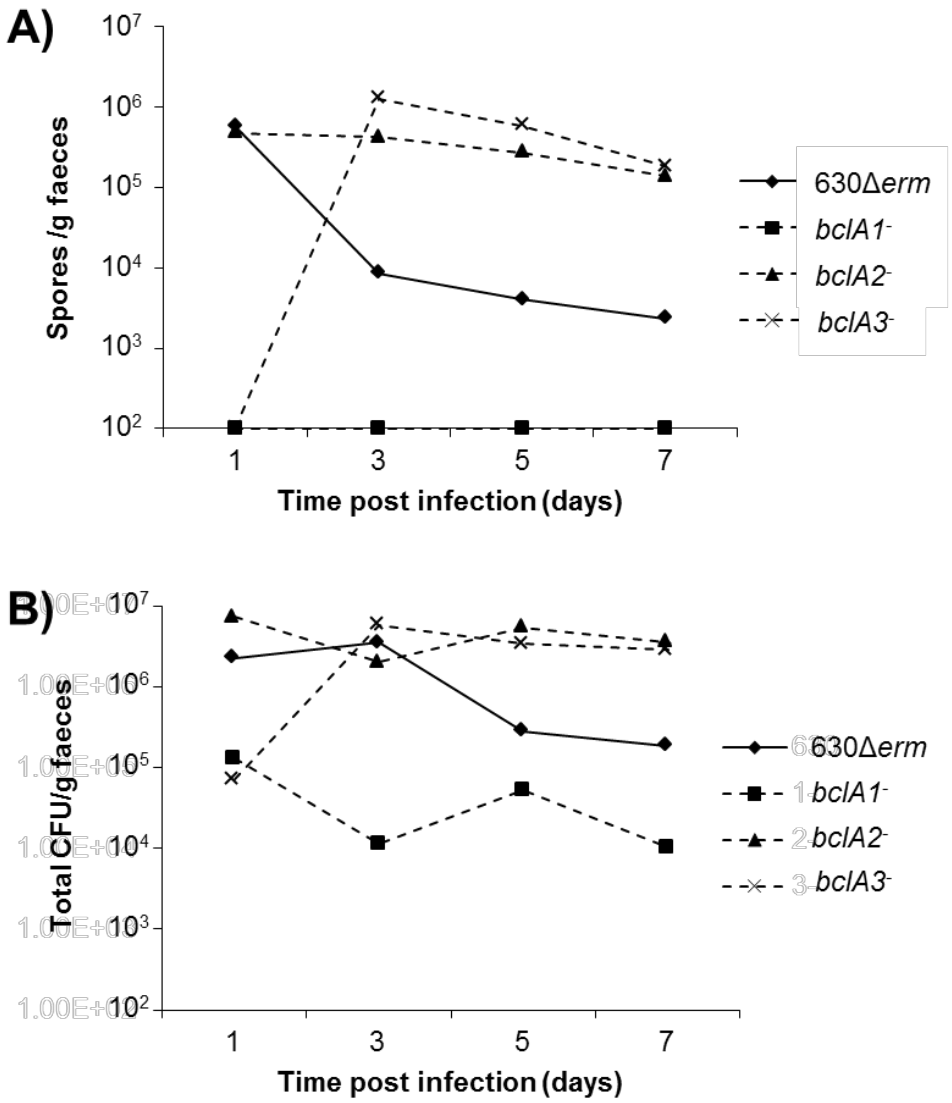


Figure 5.11. BclA⁻ strains colonisation in mice: Groups of mice (n=4) were administered a regimen of cefoperazone and then infected orally with a single dose (1×10^4) of 630Δ*erm* spores or spores of one of the three *bclA* mutants. Freshly voided faecal samples were analysed for spore counts (A) and total counts (B) on days 1, 3, 5 and 7 post-infection.

The colonisation ability of the *bclA1*$^{-}$ mutant was examined using a dose response assay. Mice were treated with clindamycin to induce susceptibility to infection, with animals given three doses (10^2, 10^3 or 10^4) of 630Δ*erm* (**Figure 5.12B**) or *bclA1*$^{-}$ mutant (**Figure 5.12C**) spores. Levels of colonisation were determined by the number of ethanol-resistant spores present in fresh faecal samples. This model of infection was used since the erythromycin-resistance cassette used in ClosTron mutants may not confer the same level of resistance to clindamycin as seen in the parental strain, depending upon its chromosomal location (N. Fairweather per. comm.).

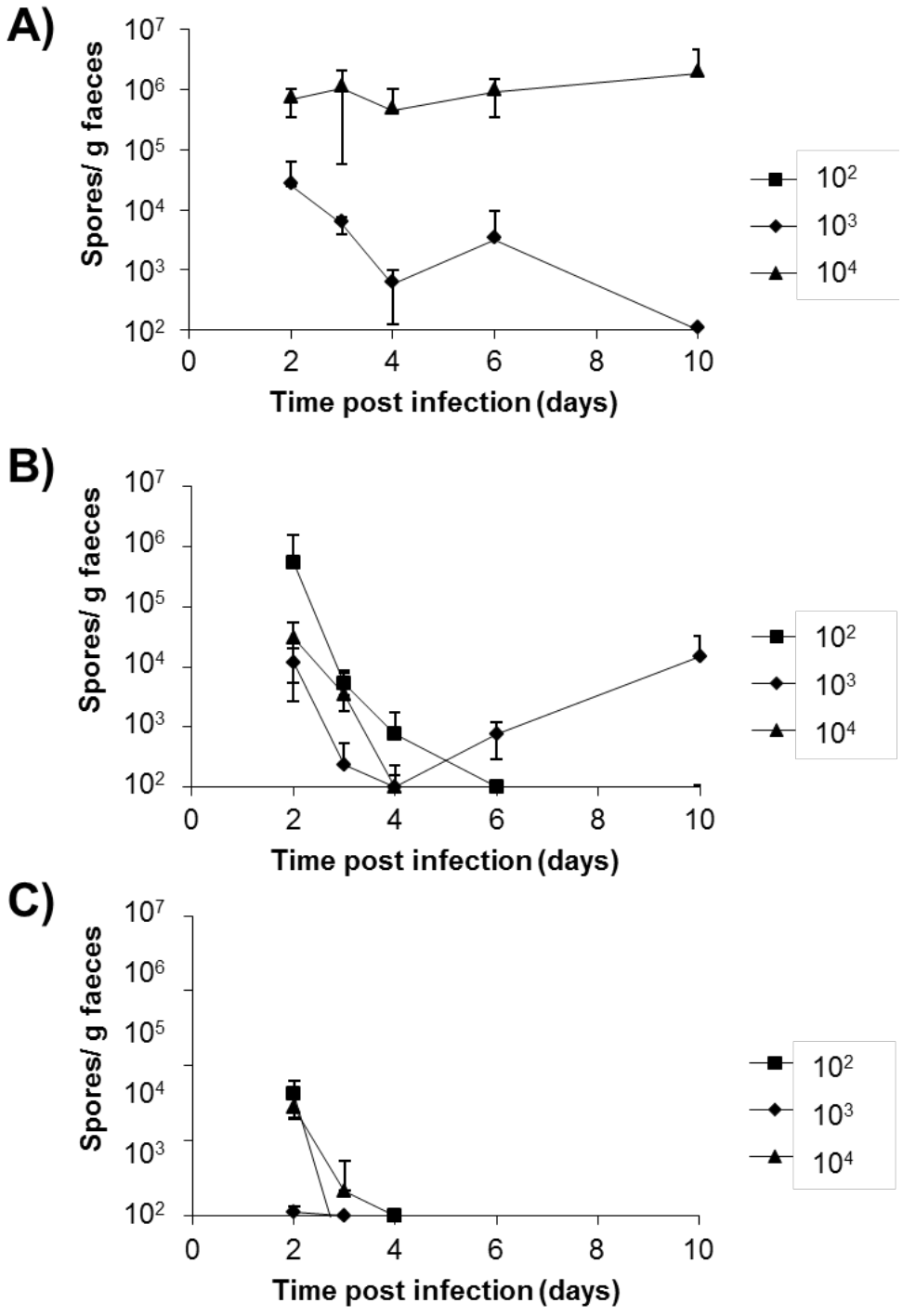


Figure 5.12. Dose-response assays in mice. Mice (n = 4) were administered a single dose of clindamycin and five days later infected with A) R20291, B) 630*Δerm* or c) *bclA1*$^-$ spores, at three different dose levels (10^2, 10^3 and 10^4). Fresh faeces were analysed for the presence of ethanol-resistant spore counts following infection. Results are shown as average counts. Where no symbols are displayed, counts were below detection limit (10^2/g) so classed as negative.

Analysis of the *bclA1* genes in the genome sequences of two 027 ribotypes, R20291 and CD196 (Stabler *et al*. 2009) revealed a stop codon at position 48 in addition to an asparagine to lysine change at position three in the ORF (**Figure 5.13**). Independent sequencing of the *bclA1* gene in R20291 confirmed that the stop codon is in place and not a sequencing error. As such, the 027 strains must each encode a BclA1 protein of approx. 6 kDa and, being significantly smaller than the one found in strain 630, would presumably lack function. Using antibodies raised against BclA1 (from strain 630) BclA1 was identified on the surface of both R20291 and CD196 spores suggesting that the truncated protein can assemble into the exosporium in these 027 strains (data not shown).

Results from this study suggest that *bclA1* deletion would impair colonisation. Therefore, to determine the infectivity of a 027 strain carrying a truncated BclA1 protein, the ability of R20291 spores to colonise mice was analysed as previously described for 630Δ*erm* and the *bclA1* mutant (**Table 5.4** and **Figure 5.12**). At an infective dose of 10^4 spores shedding was maintained till day ten, post-infection. By contrast, following the same dose of 630Δ*erm* spores shed in the faeces steadily declined to zero by day six and then increased again. The *bclA1*$^-$ mutant however was not only shed at substantially lower levels but was cleared after four days compared to six days for its isogenic parent strain, 630Δ*erm* (**Figure 5.12**). The ID_{50} for R20291 was 1 X 10^3 and therefore ten-times less infectious than 630 but more infectious than a strain completely devoid of BclA1, suggesting a correlation between the presence of an intact BclA1 protein and the susceptibility of mice to colonisation. This study indicated that i) spores lacking BclA1 are less competent at colonising the GI-tract resulting in reduced levels of faecal shedding and persistence, and ii) that at a low infective dose 630Δ*erm* was more competent at colonising the GI-tract, as evident from the lack of

shedding in animals infected with 10^2 spores of R20291 compared to those infected with the same dose of 630Δ*erm*. Interestingly, in mice dosed with 630Δ*erm* spores, following the sixth day there was a subsequent increase in counts in animals receiving the 10^3 and 10^4 doses. This may be due to recolonisation of the GI-tract by 630Δ*erm* spores shed in the faeces, as coprophagia was observed. This recolonisation was not observed for R20291-infected mice most probably for a number of reasons. First, the maximum levels of spores shed in faeces, from experience with the murine model plateaus at between 10^6-10^7/g and so any increase would not be seen for mice dosed with 10^4 spores. Second, as shown from the ID_{50} values R20291 is less infectious than 630Δ*erm* and may not be able to recolonise. Finally, for the *bclA1*$^-$ mutant, this strain clearly does not colonise efficiently so recolonisation of the GI tract would not be expected.

Strain	ID_{50}
630Δ*erm*	1 X 10^2
R20291	1 X 10^3
bclA1$^-$	> 1 X 10^4

Table 5.4. Infectivity of spores of different *C. difficile* strains in mice. Groups of mice were first treated with clindamycin followed by a 5-day interval before being given three doses (10^2, 10^3 or 10^4) of spores followed by determination of ethanol-resistant spores counted in fresh fecal samples (cfu data are shown in Figure 5.11). Colonisation was defined as animals carrying >10^3 spores/g feces at 48h post-infection. Using the Reed-Munch equation the dose of spores required to infect 50% of mice (ID_{50}) was determined.

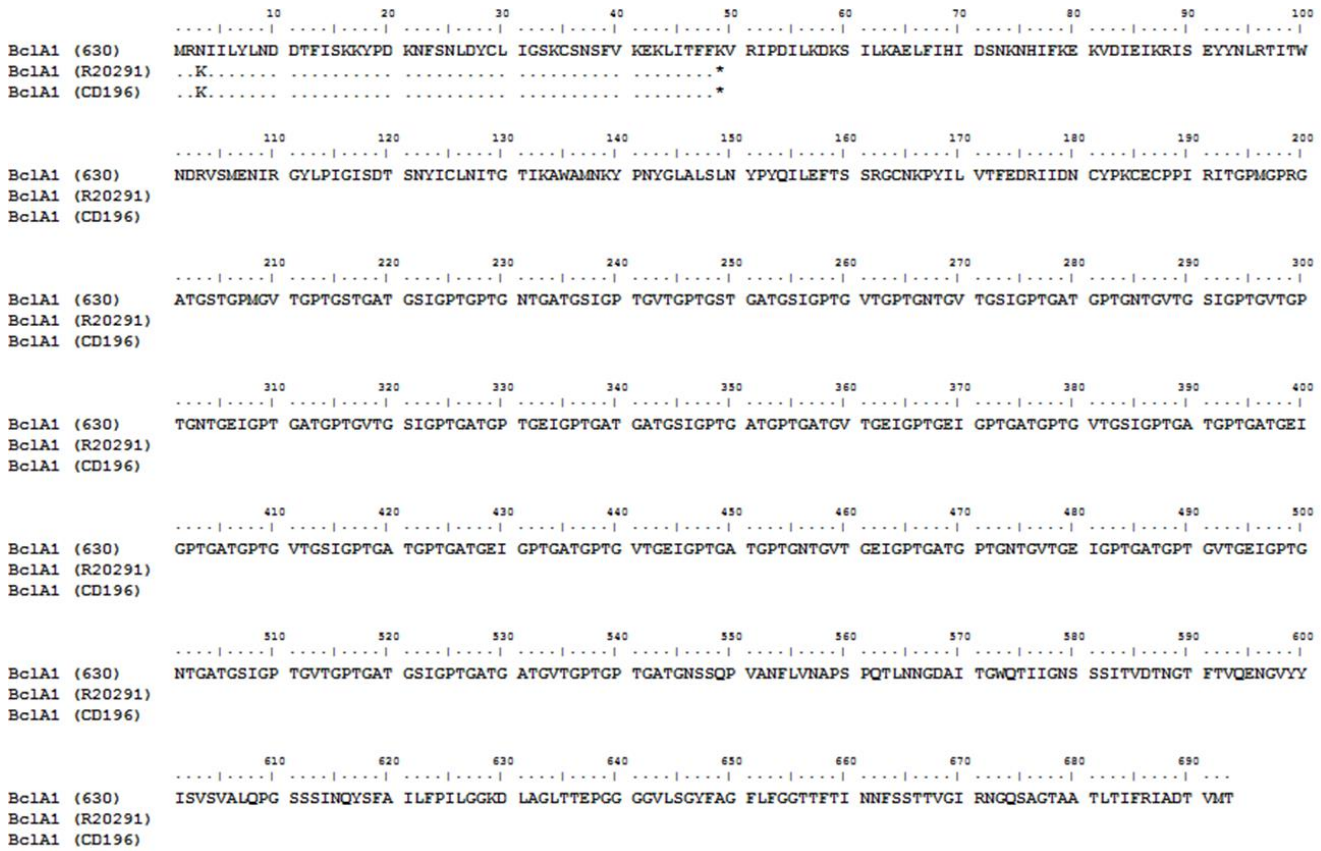


Figure 5.13. BclA1 polypeptides in *C. difficile* 630, R20291 and CD196 strains. * = stop codon present at position 49 in the *bclA1* sequence of the R20291 and CD196 '027' strains (substitution of A145 with T in nucleotide sequence). The R20291 and CD196 sequences are available on NCBI.

Hamsters provide a more sensitive model for *C. difficile* infection. Having demonstrated the difference in ability to colonise the GI tract between the parental 630Δ*erm* strain and the *bclA1*$^{-}$ mutant, the hamster model was used to evaluate the infectivity of *bclA1*$^{-}$ mutant spores. In a preliminary study groups of three hamsters were dosed with 10^2, 10^3 or 10^4 spores of 630Δ*erm* or *bclA1*$^{-}$ spores (**Figure 5.14A**). Significant differences were observed in survival times between wild type and mutant (10^2, p=0.003; 10^3, *p*=0.008; 10^4, *p*= 0.0003) as well as a dose-dependent response. For example, using an infective dose of 100 630Δ*erm* spores the clinical end point was reached in approximately 40h while with the same dose of *bclA1*$^{-}$ mutant spores this was delayed till about 47h.

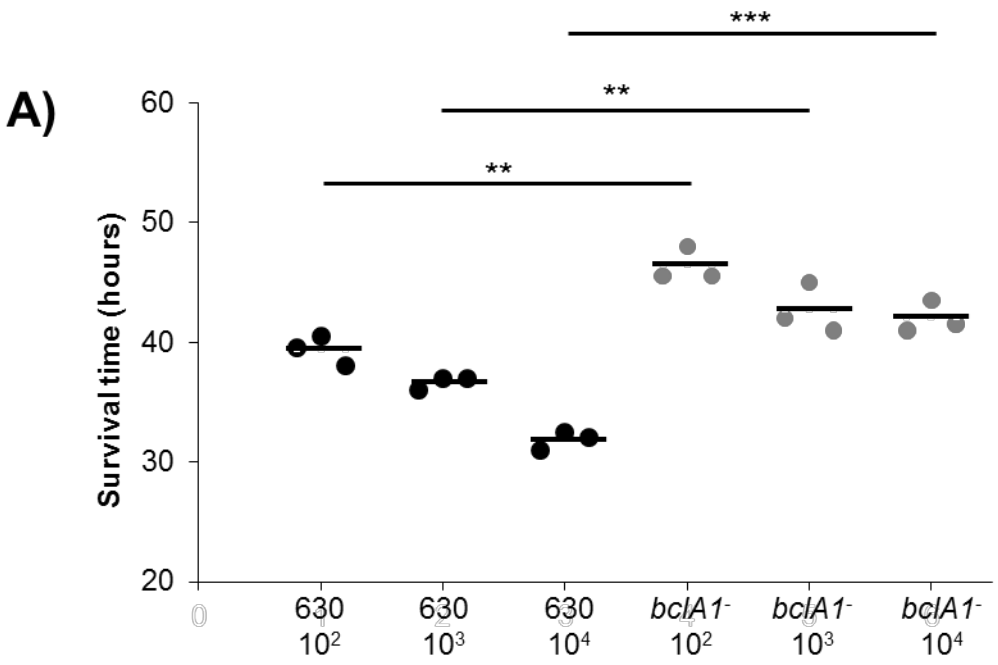


Figure 5.14. Hamster colonisation. A) Survival time for hamsters infected with spores of strain 630Δ*erm* or *bclA1*$^{-}$. Doses of 10^2, 10^3 or 10^4 spores were used to infect hamsters (n=3) by oral gavage. 630Δ*erm* infected hamsters are represented by black symbols and *bclA1*$^{-}$ infected hamsters with grey symbols. Mean survival time for each group shown by short black line. ** p value < 0.01; *** p value < 0.001. (Figure 5.14 continued overleaf)

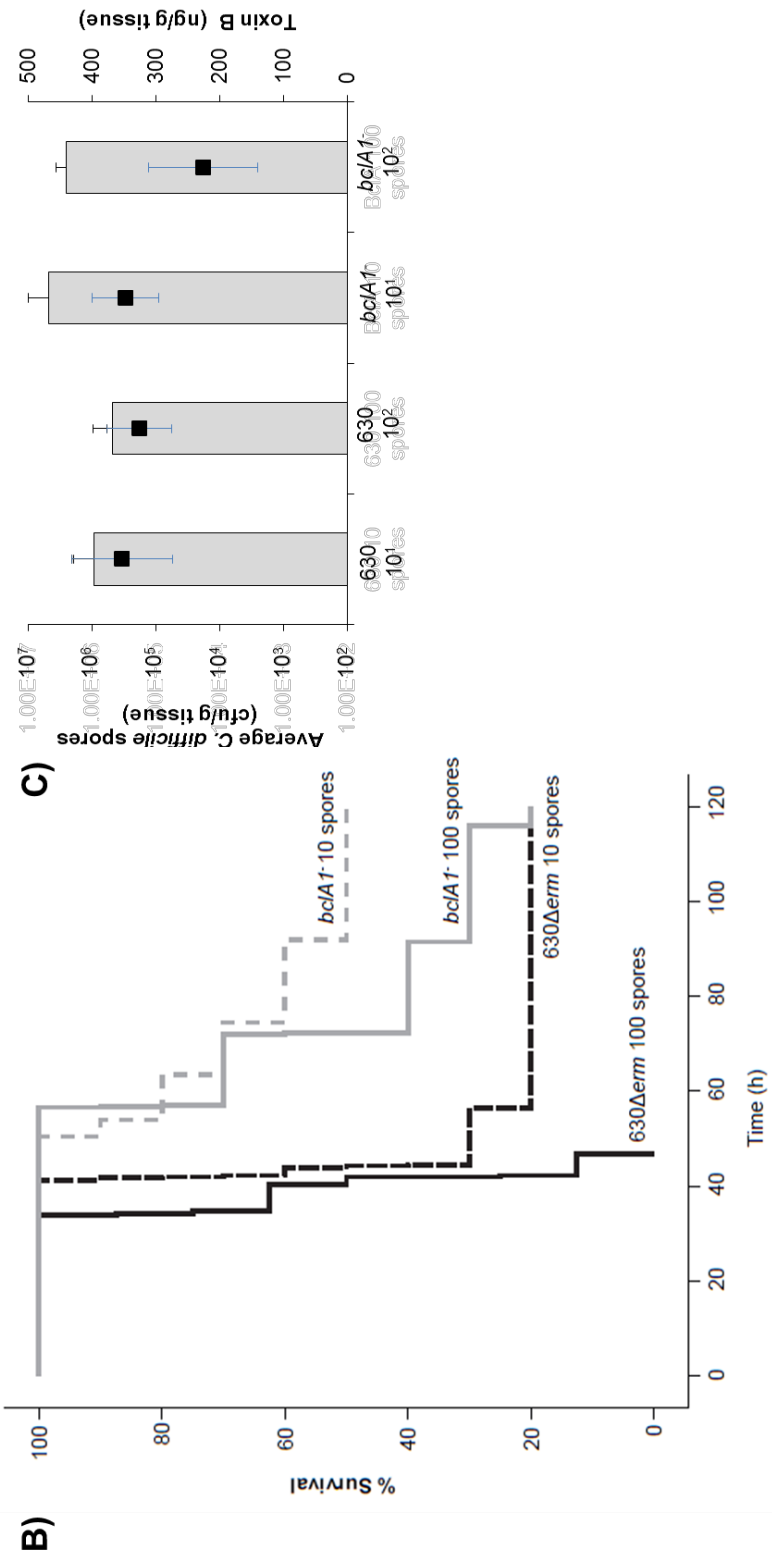


Figure 5.14. (cont.) Hamster colonisation. B) Kaplan Meier survival plot of hamsters (n=10) infected with doses of 10 or 10^2 spores of 630Δ*erm* (black lines) or *bclA1*⁻ spores (grey lines).
C) Caecum tissue excised from infected hamsters (B) was evaluated for average counts of spores (columns; cfu/g) and toxin B (ng/g; internal black squares). All samples were taken from caecum post-death at the clinical end point of infection.

The preliminary work was then expanded, using ten hamsters per group and two doses, 10 and 10^2 spores, of both the 630Δ*erm* and *bclA1*$^-$ mutant strain (**Figure 5.14B**). A dose of 10^2 spores or 630Δ*erm* resulted in no surviving infected animals while a lower dose of 10 spores resulted in the survival of two animals. By contrast, the *bclA1*$^-$ mutant was clearly less infective with 50% survival following a dose of 10 spores and 20% survival using 100 spores. The calculated ID_{50} for 630Δ*erm* spores was 2.37 X 10^1 and for the *bclA1*$^-$ mutant 2.56 X 10^2 demonstrating that the *bclA1*$^-$ mutant was ten-times less infective. Animals that were infected by either 630Δ*erm* or *bclA1*$^-$ were shown to have similar levels of *C. difficile* spores in the caecum (**Figure 5.14C**) although levels were somewhat higher with *bclA1*$^-$ infected animals possibly reflecting the ability of this mutant to germinate more efficiently and proliferate. Toxin B levels in caecum samples were measured and levels were found to be similar in all groups of infected animals (**Figure 5.14C**). In surviving animals no viable counts of *C. difficile* or toxin B could be detected in caeca. This result supports the murine study demonstrating that *bclA1*$^-$ mutant strains although able to produce toxins are clearly less infectious than the wild type.

It was possible that the low infectivity of the *bclA1*$^-$ mutant might have arisen if toxin production was reduced or delayed *in vivo*. Several pieces of evidence would suggest this unlikely: first, the *bclA1* mutation was complemented *in trans* (**Figure 5.15**); second, based on the morphogenesis of the spore, it could be predicted that the *bclA1* gene could be expressed in the late phase of spore formation, while toxin production is associated with the stationary phase of cell growth (Rupnik *et al*. 2009) and should occur before *bclA1* expression. Preliminary qPCR data (not shown) demonstrated that *tcdA* and *tcdB* are expressed during stationary phase and the early stages of spore formation, while the *bclA1*-

3 genes are expressed at the terminal stages of sporulation (approx. 9h following the onset of development). To check if there was any relationship between *bclA1* expression and toxin production, mice were infected eight days post clindamycin treatment with a high dose (10^5/mouse) of 630*Δerm* or *bclA1*$^-$ spores sufficient to cause infection in most of the mice (see **Table 5.4**). At 24 and 36 hours post infection the total CFU of *C. difficile* and toxin A and B levels were determined in caeca. As shown in **Figure 5.16,** the total CFU in mice infected with 630*Δerm* or *bclA1*$^-$ spores were equivalent at both time points, similarly no differences were observed between toxin A and B levels in the caeca and in the ratio between the two toxins.

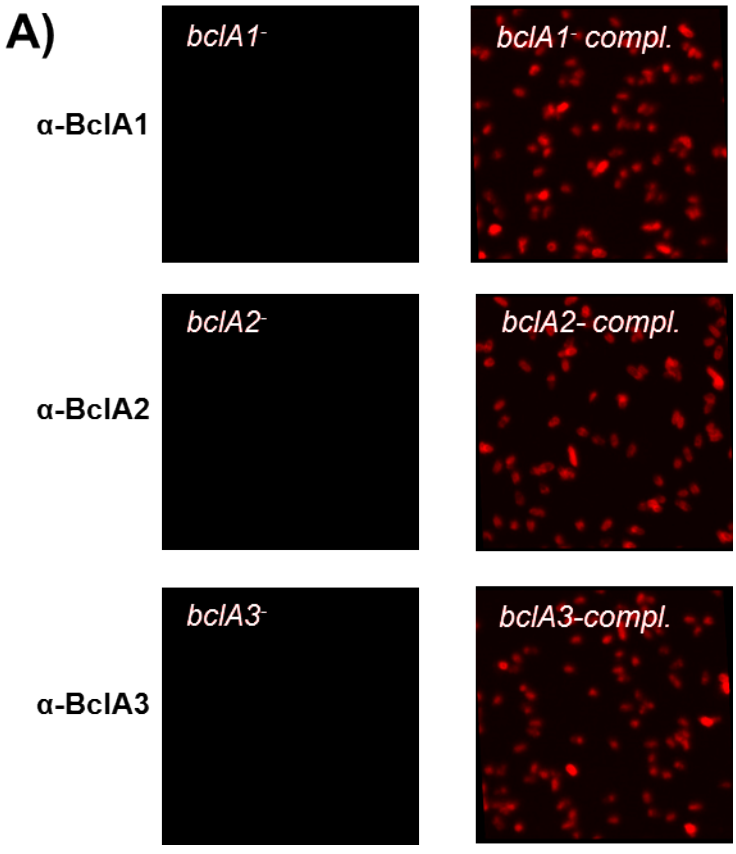


Figure 5.15. Complementation analysis of *bclA* mutants. A) pRPF185 plasmids carrying the complete *bclA1*, *bclA2* or *bclA3* genes were introduced into the *bclA1*, *bclA2* or *bclA3* mutants by conjugation. Spores (purified or unpurified) were prepared and expression of the respective BclA proteins visualised by immunofluorescence microscopy using polyclonal antibodies as shown (right column) and compared to the mutants alone (left column). (Figure 5.15 continued overleaf)

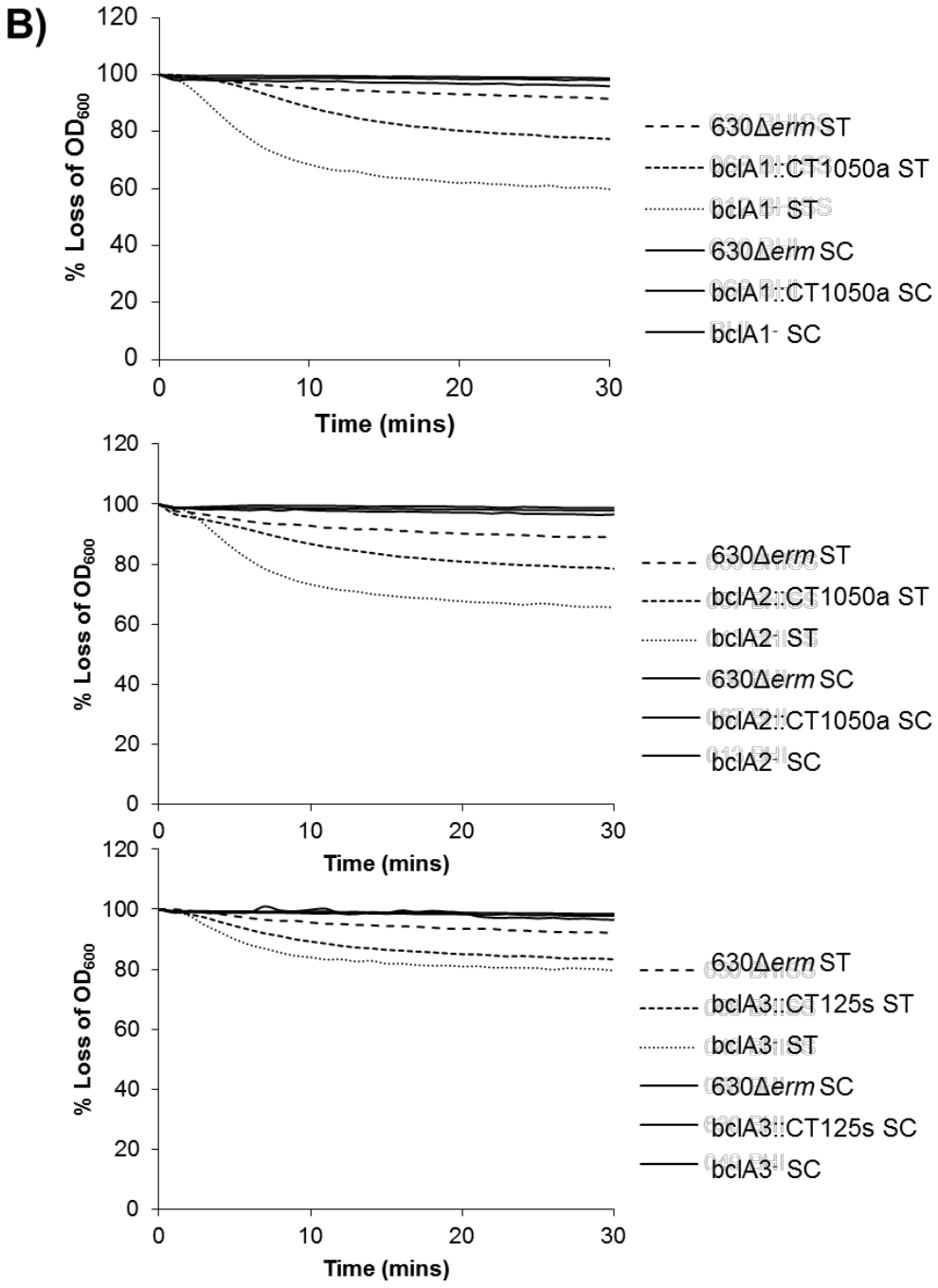


Figure 5.15. (cont.) Complementation analysis of *bclA* mutants. B) Germination (measured through decrease of OD in spore suspensions) studies using Histodenz-purified suspensions of spores (630Δ*erm*), *bclA* mutants or complemented mutants (::CT1050a or CT125s) in the presence of germinant (ST) or inhibitor (SC).

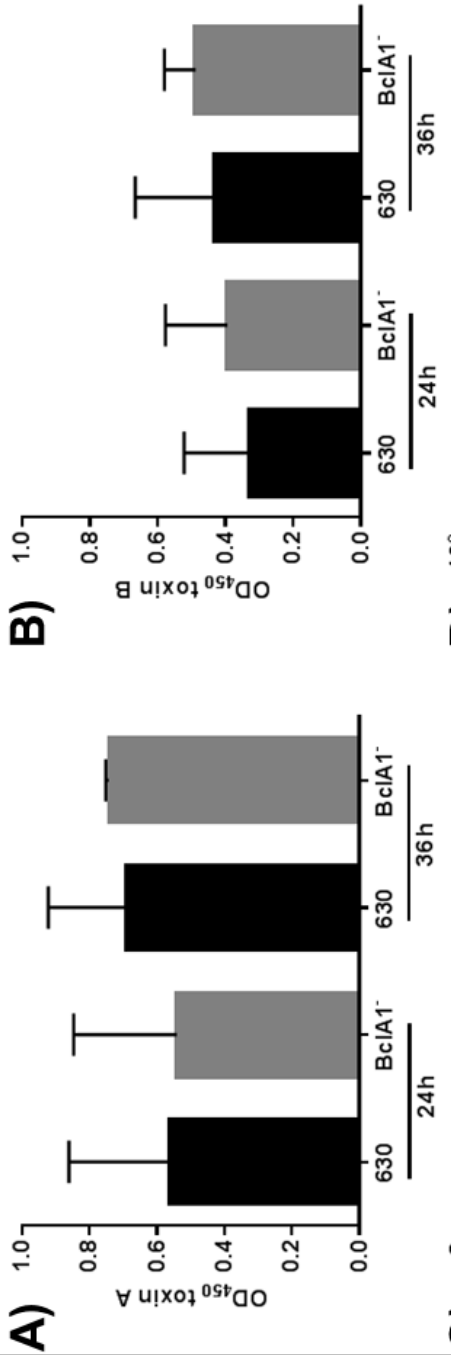


Figure 5.16. Caecal levels of toxins A and B. Levels of toxins A (A) and B (B) at different time points (24 or 36 hours) in the caeca of mice infected with 1×10^5 spores/mouse of *C. difficile* 630Δ*erm* wild type strain or *bclA1*$^-$ mutant; C) Ratio between toxin A and toxin B levels in infected mice; D) Total *C. difficile* CFU counts (cfu/g) in caecum tissues excised from infected mice.

5.3. Discussion

The results from this work show that BclA proteins contribute significantly to the ability of *C. difficile* spores to colonise and persist within the mammalian gut. Removal of this protein also has substantial effects on the properties demonstrated in *in vitro* assays. The implications of these results with regard to the pathogenesis of *C. difficile* will be discussed.

5.3.1. The role of the *C. difficile* exosporium

The exosporium is poorly defined in *C. difficile* and images of this 'sac-like' outer layer vary from a well defined thick, electron dense laminated structure (Lawley *et al.* 2009*b*) to more diffuse layers that are easily removed from the underlying spore coat (Permpoonpattana *et al.* 2011*b*; Permpoonpattana *et al.* 2013; Escobar-Cortés *et al.* 2013). Most probably the exosporium of *C. difficile* is particularly fragile, at least under the conditions commonly used in the laboratory to prepare spores, during which the exosporial layer can easily be removed. This makes study of the exosporial proteins difficult, with the processes used to produce pure suspensions of spores also resulting in removal of the exosporium. Less harsh methods of purification such as use of Histodenz or a sucrose gradient allow the outer layers of the spores to remain more intact. The study of spores with more intact outer layers is beneficial to understanding the pathogenesis of this bacterium as a more accurate representation of the normal infectious cycle. Further analysis of other exosporial proteins is required to assign potential functions of the exosporium. A recent study analysed proteins from the outer layers of *C. difficile* spores in order to identify proteins that could be exploited for detection assays or control methods (Abhyankar *et al*. 2013). However

spores used in this analysis were purified using proteinase K and sarcosyl, so presence of exosporial proteins in the analysis is unlikely.

One of the major immunodominant proteins found in the exosporium of *B. anthracis* and *B. cereus* is the BclA protein (Sylvestre *et al.* 2002; Steichen *et al.* 2003; Todd *et al.* 2003; Redmond *et al.* 2004). Filaments of the BclA protein form the hairy nap which is characteristic of the exosporia of the *Bacillus anthracis/thuringiensis* family of spores (Kailas *et al*. 2011) but in the case of *C. difficile* these hair-like filaments have yet to be observed. *C. difficile* carries three *bclA* genes whose products share similarity with the BclA proteins of *B. anthracis* and *B. cereus*. However, the composition of these proteins differ significantly especially with regard to the absence of the N-terminal (targeting the exosporium) and C-terminal (oligomerisation) domains. Variability also exists between strains of *C. difficile*, highlighted by the observation that the BclA1 protein of *C. difficile* is severely truncated in two 027 strains. Evidence from antibody labelling showing that the *C. difficile* BclA proteins reside in the outermost layers of the spore and most probably the putative exosporium is supported by a recent paper also focusing on the BclA proteins of *C. difficile* (Pizarro-Guajardo *et al*. 2013). Whether the three BclA proteins form homo- or hetero-oligomers will need to be established.

Insertional mutagenesis of the three genes also revealed noticeable defects in the spore coat. First, in two mutants, *bclA1* and *bclA2*, aberrations in the spore coat were clearly evident and presumably assembly of the outer coat or exosporium is defective in these mutants emphasizing that both proteins are likely major exosporial proteins. Sheaths of ribbon like material are seen in association with spores in both *bclA1*$^{-}$ and *bclA2*$^{-}$ mutant strains. Empty sac-like structures of this material are also seen, raising the possibility that the BclA proteins act as an anchor for the exosporium. Therefore, in the absence of BclA,

the exosporium while remaining intact, is not as strongly associated with the spore. Second, spores of all three mutants had significantly reduced hydrophobicity. Reduced hydrophobicity was also apparent in spores that had been sonicated, an approach that has been shown elsewhere to remove the exosporium (Permpoonpattana *et al.* 2011*b*; Permpoonpattana *et al.* 2013; Escobar-Cortés *et al.* 2013). In *B. anthracis, bclA* mutants also have a much-reduced hydrophobicity where the exosporium is thought to provide a water repellent shield, reducing its ability to interact with the host matrix (Brahmbhatt *et al.* 2007*b*). Third, all three *bclA* mutants showed increased germination rates, a characteristic also found in the *B. anthracis bclA* mutant and presumably a result of a defective exosporium allowing access of germinants to receptors situated in the innermost spore membranes (Brahmbhatt *et al*. 2007*b*; Carr *et al.* 2010). Sonication of *C. difficile* 630 spores also resulted in increased germination rates, highlighting the potential role of the exosporium in control of germination. As the outer layer of the spore, the exosporium serves to interact and control interactions with the environment. Preventing germination in hostile environments will be beneficial to the bacterium. The fragility of the exosporium when treated with purification enzymes could be replicated in passage through the stomach and GI tract, with removal of the exosporium allowing germination of the spore once in an environment in which it can survive.

5.3.2. Effect on infectivity of spores

In vivo infection studies in mice revealed that the *bclA1* and *bclA3* mutants had impaired colonisation efficiencies although this was most striking with the *bclA1* mutant that completely failed to colonise the mouse GI-tract. This highlights that removal of the BclA

proteins in the 630 strain severely compromises the ability of spores to colonise the gut effectively. This is likely to be attributable to the effect on the exosporium of removing a significant component of the structure. The effect of a compromised exosporium has several facets. Firstly, properties that aid potential interactions with the host are altered. Hydrophobicity is reduced and although no host/spore specific interactions with specific receptors have been described, with a damaged exosporium the spores are at a significant disadvantage. Secondly, removal of the exosporium leaves the spore outer layers more exposed. Germinants have greater access to receptors on the spore, so germination can occur at a faster rate and in environments that may otherwise not encourage germination. Increased levels of germination could explain why few spores were detected in mice infected with BclA1$^-$ spores in the colonisation assay.

In *B. anthracis* BclA has been shown to play no significant role in virulence, with a *bclA* mutant having no effect on pathogenicity in mice or in guinea pigs (Bozue *et al*. 2007*a*). Mutant and wild type strains also have similar LD_{50} values (Brahmbhatt *et al.* 2007*b*). This is in marked contrast to *C. difficile* where results from this chapter show at least one BclA protein, BclA1, to be involved in the initial stages of colonisation and infection. In mice and in hamster models of infection spores devoid of BclA1 were up to 2-logs less infective (i.e. by ID_{50}) than isogenic wild-type spores. Hamsters that were colonised by the BclA1 mutant strain took longer to succumb to fatal disease, and fewer were colonised than in groups of hamsters infected with the wild type 630 strain. This suggests then that BclA1 could be involved in the initial stages of host colonisation and that this event must be mediated by the spore, an event occurring before spore germination. This also highlights the importance of the spore being able to colonise and persist in the GI tract environment, implying that without certain proteins, the spores are less effective at initial colonisation.

Even more intriguing was the observation that two 027 strains carried truncated BclA1 proteins and that one of them, R20291, a so-called 'hypervirulent' strain, was actually less infective in a mouse model of infection than the 630 strain. This observation is suggestive of a relationship between animal susceptibility and the presence of an intact BclA1 protein in the *C. difficile* spore. Spores of strains carrying a full length BclA1 protein (i.e. 630) appear able to colonise the murine GI tract better than those carrying a defective or truncated *bclA1* gene. Interestingly there is already published work that supports this. For example, only 100 spores of 630 were required for 100% colonisation in hamsters but using the same dose lower levels of infection were found with a variety of B1 strains (Razaq *et al*. 2007). Similarly, a larger dose of 10^4 spores of R20291 has been shown to produce complete infection in hamsters (Buckley *et al.* 2011). There is now evidence showing that hamsters are more susceptible to non-toxigenic strains of *C. difficile* than toxigenic strains (e.g. M68 and B1-7) (Buckley *et al*. 2013). The variation between strains is interesting and should be a key factor in studies looking further at spore proteins in particular. In 630, the BclA1 protein is clearly an important component of the exosporium, but as other strains of *C. difficile* lack this protein there must be alternative mechanisms that contribute to virulence.

It has been proposed that hypervirulent 027 strains may have acquired additional virulence genes based on the considerable genetic differences between the epidemic and non-epidemic strains (Stabler *et al*. 2009). However, data presented here suggests that in terms of initial colonisation the hypervirulent R20291 strain is actually less effective, that is, animals are less susceptible. This then raises some interesting and provocative questions. For example, whether animals including humans are actually less susceptible to

'hypervirulent' strains yet once colonisation occurs the severity of disease is much greater due to characteristics of the vegetative phase of this pathogen. In many ways this resembles the situation of influenza where seasonal flu strains are typically highly infective but of low severity compared to the low infectivity-high severity nature of H5N1 strains. If what happens in humans mirrors that in mice then the virulence of R20291 must arise not due to its infectivity but rather, due to some other factor affecting the severity of infection, e.g. levels of toxin production, increased persistence or faster germination. For the 027 'hypervirulent' strains increased toxin production and biofilm formation (Dawson et al. 2012; Dapa & Unnikrishnan 2013) have been identified as potential virulence factors. However, the presence of an intact BclA1 protein would correlate with the susceptibility of the host to infection and this implies that BclA1 may interact with a specific host target. Comparisons between diverse strains carry inherent risks and the presence of additional factors cannot be ruled out. A more detailed and extensive study of the *bclA1* gene in clinically relevant strains coupled with analysis of infection stages will be required to address this but at this point it is clear that BclA1 plays a key role in the initial stages of infection and host susceptibility.

Recent studies utilising genomic sequencing to track infections have shown that infections stem from diverse sources, not just transference between patients in hospitals as traditionally thought. The current thought is that *C. difficile* is acquired primarily from the environment but disease only manifests when colonic microflora is disrupted. The mechanisms that allow strains to persist in the GI tract until a niche is available for proliferation are unknown. Understanding this part of the infection cycle would provide valuable information in being able to prevent or treat infections before severe disease is

established. How persistence at low levels in the gut varies between strains will be of direct interest in understanding 'hypervirulent' strains.

5.4. Conclusion

This work uses a genetic approach to removing the BclA proteins from *C. difficile* 630 spores. Both *in vitro* and *in vivo* studies have been utilised to show that the three BclA proteins are integral components of the exosporium whose removal severely destabilises this outermost layer of the spore. The disruption to the exosporium layer allows increased access of germinants and reduces surface hydrophobicity. Infectivity of spores lacking BclA1 is also decreased, introducing this glycoprotein as a potential virulence factor in the 630 strain. This encourages the hypothesis that initial colonisation is an important step in the infection cycle, and that colonisation is not a definite occurrence, especially considering the wide amount of variation that exists between strains of *C. difficile*.

This work has led to the identification of the BclA1 protein as a potential colonisation factor in *C. difficile* infection. This presents this glycoprotein as a potentially important antigen that could be used in candidate vaccines that address colonisation as an issue.

The results of this study encourage further work regarding variation in spore properties between strains and the make up of the exosporium. Identifying proteins of this layer will contribute to ascertaining an increased understanding of its role in the infection cycle.

Chapter 6

General Discussion

This thesis covered two main areas of study, the primary focus being the investigation of alternative treatments for CDI in order to reduce reliance on antibiotics. The secondary topic focused on the role of a specific spore associated protein BclA1. This protein was shown to play a role in initial colonisation of *C. difficile,* increasing knowledge with regard to the role of spores in this infection. As the mechanisms behind infection and persistence of this pathogen in the host are elucidated, more information is then available for design of novel treatments, linking to reducing antibiotic usage.

6.1. Treatment for *C. difficile*

Standard treatments for CDI rely on the use of specific antibiotics. Whilst treatment with either vancomycin or metronidazole can treat the infection, relapses will occur in around 20% of cases (Barbut *et al*. 2000), highlighting a key problem with antibiotic treatment. The work of this thesis addressed two alternative approaches to treatment. Firstly, there is currently no licensed vaccine for prevention of CDI. In this thesis, a *B. subtilis* spore based vaccine (PP108) that was previously demonstrated to provide protection from fatal disease in a hamster model (Permpoonpattana *et al*. 2011*a*) was used in further study with a murine model. In the previous study, this vaccine was delivered orally in order to induce a mucosal response. In this thesis sublingual delivery was investigated as an alternative

method of immunisation and proved to be successful in both production of an immune response and protection from symptoms of disease. Secondly, the use of a *B. subtilis* strain was investigated as a potential probiotic treatment for CDI. Pure suspensions of *B. subtilis* spores given as an oral treatment were able to increase survival rates in a murine model of infection.

6.1.1. Vaccines

Development of a vaccine that would protect against *C. difficile* infection is important as it would provide an extra tool with which this infection could be controlled. Previous research has shown that a spore based vaccine (PP108) expressing a toxin A antigen provided protection against challenge in a hamster model of CDI, also protecting against relapse of infection. This work demonstrated, in agreement with other studies (Kim *et al*. 1987; Kyne *et al.* 2001), that antibodies against toxin A are sufficient to protect against a strain that produces both toxin A and toxin B, as antibodies against toxin A cross reacts with toxin B (Permpoonpattana *et al.* 2011*a*). This vaccine was delivered orally with a focus on producing a mucosal immune response. Both mucosal and systemic responses were elicited, with antigen specific IgA detected in faeces and antigen specific IgG detected in sera. This approach represents a deviation from the norm in *C. difficile* vaccine research, where a distinct focus is placed on parenteral vaccines and production of a systemic response. The PP108 vaccine was developed based on the idea that inducing a mucosal response will be a significant factor in successful protection against CDI. This hypothesis is based on several factors, firstly that IgA produced at mucosal surfaces in the colon have been shown to prevent binding of toxin A (Kelly *et al*. 1992). Secondly, producing a local response as well as a systemic response provides two levels of protection against the

disease. The hypotheses of the original PP108 study were further investigated in this thesis, with alternative methods of delivering the vaccine and a new challenge model utilised. Sublingual delivery was used as a novel method for immunisation, this route of interest for several reasons: Oral routes of delivery tend to use high levels of antigens due to inaccurate delivery to mucosal surfaces and the risk of enzymes and pH damaging antigens. Oral immunisations are also associated with development of tolerance to the antigen. Sublingual dosing potentially avoids the issue of oral tolerance and reduces the amount of antigen required to generate an immune response. Use of the sublingual route allows the antigen direct contact with the oral mucosa where the antigen can be absorbed (Harris & Robinson 1992). Use of this route also reduces exposure of the antigen to potentially damaging conditions such as the low pH of the stomach. This thesis showed that in the murine model, both oral and sublingual routes for vaccine delivery demonstrated protection against severe symptoms of CDI.

Research in this thesis showed that a spore based vaccine delivered via mucosal routes can initiate an immune response at both local and systemic level. This outcome demonstrates that both delivery route and the antigen used are successful and effective. The novel sublingual route was the most successful in terms of level of response and also protection rate in challenge studies. Mucosal routes for immunisations represent an easier approach to vaccination than use of parenteral routes. Eliminating the use of needles is a key advantage of the sublingual and oral dosing routes (Levine & Sztein 2004). This factor alone makes the spore based vaccine platform a promising step in vaccine development. Use of sublingual dosing addresses the issue of dysphagia, so even patients with difficulties swallowing can receive this vaccine.

Coupled with the ease of dosing, the PP108 vaccine candidate also provides a flexible platform for vaccine delivery, allowing adaptations to be introduced through the use of alternative or multiple antigens delivered in the same inoculum. Multivalent approaches to inducing protection are used in other diseases, such as seasonal influenza vaccinations (CDC 2013*b*) and experimental anthrax treatments (Hahn *et al.* 2006). The nature of the PP108 vaccine means that the antigen is expressed by the bacterium on the spore surface, thereby giving the benefit of reduced costs associated with protein production. The amount of antigen required for use in the vaccination is also reduced when using sublingual dosing, further increasing the economic benefits of this vaccine strategy.

The protection shown in the murine model of CDI was not as clear cut as previously demonstrated in the hamster model. It should also be noted that in order to induce disease in the murine model, a different strain was employed from the original study. The strain used for murine infections was *C. difficile* R20291, a 027 'hypervirulent' strain. In the murine model utilised in this work, the R20291 strain will cause obvious symptoms of disease, but not fatal infection as the 630 strain does in hamsters. This difference between the models highlights two important factors in *C. difficile* research. Firstly, differences between animal models are significant. There are several variations of the murine model for *C. difficile* infection (Chen *et al*. 2008; Theriot *et al*. 2011; Lawley & Young 2013) and it should be considered that variations in the study model also contribute to variations in results. The difference in strains used is also important given variation in virulence, ideally treatments should be tested against multiple strains to represent the real life situation where strains from multiple ribotypes can cause disease (Bauer *et al*. 2011). A successful *C. difficile* vaccine should protect against multiple strains, especially clinically relevant and 'hypervirulent' strains. Use of cultured lab strains such as 630 provides a solid basis for

information, but as further study of virulence factors increases awareness of variation between strains (Lanis *et al.* 2013), vaccine protocols should also reflect this. Use of antigens common and homologous between strains of *C. difficile* is key to producing complete protection against this infection. Genetic analysis tools are useful for this and also in demonstrating the potential of this pathogen. The potential in *C. difficile* for evolution of virulence was seen clearly in the prevalence of the 027 ribotype in outbreaks of the early 2000's across North America and Europe (Pépin *et al*. 2004). Genome sequencing of the *C. difficile* strain 630 revealed a high proportion of mobile genetic elements across the genome demonstrating the capability of this organism to acquire traits by genetic transfer which could add to virulence properties (Sebaihia *et al*. 2006). Potential vaccine candidates using specific toxin antigens are effective at neutralising toxins, but if virulence of strains increases then use of alternative antigens may need to be addressed. A study using CD1342, a PaLoc negative strain of *C. difficile,* reported signs of pathology in the hamster gut despite lack of production of toxins (Buckley *et al*. 2013). This appearance of pathology in the absence of major virulence factors highlights the complexity of this disease and the challenge behind development of a successful vaccine.

A significant consideration in the production of vaccines is the antigen used in producing a protective response. Toxin fragments or toxoids, representing the major virulence factors of *C. difficile,* are the favoured antigens for inducing protection against CDI. A key finding from the study in this thesis was that in groups showing protection from the symptoms of CDI, there were still high levels of *C. difficile* detectable in faecal samples. A reduction in severity of infection is a positive result in a potential treatment for CDI, but does not address the issue of this infection in its entirety. Understanding transmission and spread of cases is essential in developing better control strategies for this disease, something that is

complicated by limited knowledge surrounding reservoirs of infection and asymptomatic carriers. A vaccine that protects against symptoms without inhibiting the spread of the disease through asymptomatic carriers may have diminished efficacy in reducing cases of disease. Without a factor preventing spread and controlling prevalence of this pathogen, evolution of virulence factors could increase the virulence of this pathogen beyond the protection of the vaccination. Evolution of virulence has already been seen in certain clades of *C. difficile*, with the appearance of the 027 ribotype that caused severe outbreaks across North America and Europe in the early 2000s. Strains from the 027 ribotype have been reported as having increased virulence compared to historic strains. Factors that contribute to increased virulence are under debate, although increased virulence has been linked to higher toxin production and increased sporulation ability (Merrigan *et al*. 2010). As *C. difficile* remains prevalent in the environment it will be subjected to evolutionary pressure that could drive further developments in virulence. Therefore in order to address this issue the PP108 vaccine could be improved significantly by use of an antigen that prevents colonisation of the pathogen, providing a higher level of protection against infection than just neutralisation of toxins. Preventing colonisation by *C. difficile* has a twofold advantage. Firstly, this opportunistic pathogen cannot cause disease if it cannot colonise the gut. Secondly, by preventing colonisation by a non-commensal organism, the normal gut flora has an increased chance of returning to a healthy state without the GI tract environment being significantly altered by the colonisation of this pathogen. To protect against colonisation, an antigen from the spore or vegetative cell is required for the basis of the immune response.

Studies in the literature have shown various surface layer proteins (SLPs) are able to elicit immune responses, with potential key proteins identified as Cwp66, FliD and FliC (Drudy *et*

al. 2004; Péchiné *et al.* 2005; Wright *et al.* 2008). Immunisations using these as antigens has shown no real success, with one such study using anti-SLP sera to passively immunise hamsters against disease (O'Brien *et al*. 2005). This approach extended the survival time of the animals, but failed to protect from fatal outcome of infection. Another study, this time using the flagella cap protein FliD to immunise mice, reported decreases but not prevention of colonisation in immunised mice, also concluding that a mucosal response was likely necessary for an effective immune response (Pechine *et al*. 2007). The observation that levels of colonisation can be reduced suggests that use of cell associated antigens is a feasible strategy for vaccination. It is possible that a multivalent approach is needed, incorporating both toxin and colonisation factors into a vaccine to produce several levels of protection. Multivalent vaccines are also used in influenza vaccines to protect against multiple strains, whereas this approach would target different stages of the infection. *In vitro* assays have been used to show the ability of spores to bind to epithelial cells (Paredes-Sabja & Sarker 2012) and it is generally accepted that spores are the primary mode of transmission for this infection (Deakin *et al*. 2012). Therefore a spore based antigen could show more potential for protecting against colonisation than a cellular antigen. With some proteins of the spore showing enzymatic activity (Permpoonpattana *et al*. 2013), they also have the potential to cause inflammatory responses, and as such could make ideal candidates as drug or vaccine targets.

The advanced progress of Sanofi Adventis' candidate *C. difficile* vaccine in clinical trials means that a vaccine for *C. difficile* should be available in the near future. However, this does not render all other investigations into vaccination strategies redundant. The work in this thesis demonstrates use of alternative methods for delivering a vaccine to a broader market and the successful generation of protection from disease symptoms. The

mechanism which allows generation of both systemic and mucosal immune responses is highly advantageous. The platform technology could also be applied to other infections, so contributing to the wider field of infection control, as well as *C. difficile.*

6.1.2. Probiotics

Probiotics are poorly described as a medical treatment for *C. difficile* and other GI tract conditions. Perception of probiotic use is greatly overshadowed by simplified marketing campaigns aimed at the general public which feature terms such as 'good' and 'bad' bacteria without any focus on the science behind a product. The potential for use of probiotics in clinical situations does attract attention however, especially with increased interest into the role the microflora plays in health and development. This is especially relevant to treatment of *C. difficile* where the state of the gut microflora is directly linked to risk of developing symptoms of *C. difficile* infection. Only a limited number of studies have produced findings powerful enough to provide definitive data on the efficacy of probiotics in the treatment or prevention of *C. difficile*. The issue is further complicated by the wide range of strains and products used in studies, often without clear definition of the strain. Recent changes in legislation have provided an incentive for investment in producing scientific evidence for probiotic based health claims rather than relying on anecdotal evidence (Saxelin 2008). Information from probiotic studies can also contribute to our understanding of how the microbiome can contribute to disease and how manipulation of the microbiome can be exploited for treatments.

Commonly used probiotics in association with GI tract disorders are *Lactobacilli spp*. and the yeast *Saccharomyces boulardii*. The work of this thesis took a new approach, using a

Bacillus subtilis strain, novel due to the spore forming ability of the species. The use of spores in a probiotic product has several benefits. Spores are heat stable and resistant to environmental extremes so packing and storage requirements of the product are simpler than products using vegetative forms of probiotic strains. Additionally, the resistance properties of spores mean that bacteria are less susceptible to damage when in transit through the GI tract. *B. subtilis* spores are able to germinate in the gut so spores act as a transit vehicle for delivery of the vegetative forms. Heat inactivated spores demonstrate no beneficial effect in the murine model tested, implying that germination of spores and ability for vegetative growth is essential for the success of this strain. The *B. subtilis* PXN21 strain was also shown to transiently colonise the murine gut with presence diminishing as normal microflora restored balance. Recovery of the normal microflora is an important factor when using treatments that could affect the structure or make-up of the flora. An issue not addressed by this study was the lasting effects of probiotic use and whether any shifts in the flora occurred that could be attributed to use of the probiotics. How the gut flora is affected by probiotic use in the long term is important as the state and structure of the microflora is reported to have implications for many conditions, not just in susceptibility to CDI.

In this current research, a study was performed demonstrating the benefits of the *B. subtilis* PXN21 strain in mice, which provides a useful preliminary model for infection studies. However, for clinical application these probiotics would be used in humans. Human subjects do not have uniform genetics, microflora or medical case histories, introducing a level of complexity that our study model cannot address. Trials in humans would show the real effectiveness of this treatment. The murine model used in this work allows controlled

preliminary work to be carried out, appraising the potential of a bacterial strain. Limitations are common in existing probiotic studies, which is understandable given the complex nature of CDI, the range of patients that are potentially vulnerable and general regulations in the use of human subjects. If potentially useful bacterial strains and mechanisms of probiotic action can be elucidated using models and studies as shown in this thesis, human trials could be planned according to information about the strain. This would enable studies in humans that carry more weight and significance, furthering our ability to use microbes to manipulate health status.

The basis of the protection given by probiotics doses of *B. subtilis* spores is difficult to attribute to one specific mechanism. Results from this thesis suggest that interactions of the probiotics aid defence of the mucosal barrier that protects the cells lining the gut. It is clear that this treatment prevents disease by protecting against toxin mediated damage rather than acting by preventing colonisation of this pathogen. Suppression of symptoms is a clear benefit in a potential treatment although an ideal treatment would also eliminate the infection. The *B. subtilis* PXN21 strain has the potential for use as a supplement to traditional antibiotic treatments, attenuating symptoms while antibiotics are used to resolve the infection. Eradication of the infection has to be a priority alongside reducing the symptoms of disease. Use of a probiotic strain as bacterial therapy during the course of antibiotic treatment could also contribute to repopulation of the gut and accelerated recovery of the microflora after infection. It should be noted that use of a single strain will not provide the benefits of a healthy, complex microflora, emphasising the case for reducing and monitoring antibiotic usage and effects.

Epidemiological evidence, numbers of relapsing cases and severe infections contribute to the complexities of managing *C. difficile* for healthcare providers. Treatments cannot rely exclusively on antibiotics, as currently available antibiotics do not produce adequate results; the relapse figures are a clear example of this (Barbut *et al*. 2000). One of the most successful treatment options for prevention of relapses is the use of faecal transplant therapy (Rohlke & Surawicz 2010; Brandt *et al*. 2012), this approach treats the disrupted microflora as a cause of susceptibility to infection rather than treating just the infection. Antibiotics that can eradicate infection from the gut are important, but are also principal risk factors for CDI. Bacteriotherapy addresses the risk associated with antibiotics by replacing microflora and preventing proliferation of *C. difficile* in the GI tract. 'Synthetic stools' which give the benfits of faecal transplants without the drawbacks, are being developed (Lawley *et al*. 2012; Petrof *et al*. 2013). This approach fits into the definition of probiotics: 'Live microorganisms which when administered in adequate amounts confer a health benefit on the host' (Araya *et al*. 2002). Whilst on a small scale, studies like the work in this thesis that assess the potential of individual bacterial strains are useful. Identification of strains with particular attributes is necessary to create mixtures of bacterial strains that can be used to replace or modify the gut microflora. Treatments targeting repopulation of the gut could have wide ranging applications. Restoration of gut homeostasis and a healthy condition would prove beneficial to not just infections of *C. difficile* but also other conditions related to dysbiosis such as colitis and IBD.

This work has presented a method of probiotic treatment that reduces symptoms of CDI through use of *B. subtilis* PXN21 spores. The strain is one used in a commercial probiotic mix, with no reported safety issues. Regulations on probiotic constituents have become

more stringent over the past few years, so more than anecdotal evidence of safety is required. However, the use of the *B. subtilis* PXN21 strain in attenuation of CDI symptoms has been demonstrated, highlighting the potential of this strain.

Understanding the role that the microbiome plays in susceptibility to *C. difficile* will facilitate the use of bacteriotherapy and of particular bacterial strains as probiotics. Research associated with the Human Microbiome Project (Turnbaugh *et al*. 2007) will be particularly useful in elucidating the interactions and complex communities required for particular gut functions. Through information gathered in the study of probiotics, key bacterial strains can be identified that hold potential for the manipulation of the gut microflora to re-establish healthy conditions.

6.2. Role of the BclA spore coat protein

The second aspect of this thesis evaluated the role that the BclA spore associated protein plays in infection. The data that resulted from this study is of interest for several reasons. Firstly, the BclA1 protein is identified as an important protein for colonisation of the pathogen in the infection cycle. Secondly, the state of the spore coat alters properties of spores. This is important for studies that utilise purified spores in experimental work as the method used to produce the spores could potentially affect results.

The formation of spores is a key process in the survival and infection process for this bacterium, allowing persistence of the infection both in the gut and in the environment. Producing consistent data about spores and understanding the role they play is important. In this work, a non-ionic density gradient medium (Histodenz, Sigma) was used in spore purification in order to keep surface layers of the spore as intact as possible. Use of enzymes and sonication for purification of spores is well established for *Bacillus* spores (Harwood & Cutting 1990) and adaptations of these methods have been successful in generating pure suspensions of *C. difficile* spores (Lawley *et al*. 2009*b*). Use of these methods degrades proteins and structures associated with the spore however, reducing the similarity between wild type and laboratory produced purified spores. *C. difficile* is described as having an exosporium layer, which is removed by harsh purification methods (Escobar-Cortés *et al*. 2013). This layer is likely to play an important role in how the spore can interact with the surrounding environment, so it is important that studies of spores take this into account when designing spore purification protocols. The number of predicted spore coat genes in the sequenced genomes far exceeds those that have been identified and studied (Sebaihia *et al.* 2006; Permpoonpattana *et al*. 2013). However, it is important that when the surface layers of spores are studied, they represent as close as possible the wild type form of the spore.

The work of this thesis addressed the importance of the BclA proteins and previous work from the Cutting group at Royal Holloway has also investigated enzymatic properties of some spore coat proteins (Permpoonpattana *et al*. 2013). The spore coat however, is a complex structure made up of at least 50 proteins (Abhyankar *et al*. 2013). The analysis of the spore coat by Abhyankar and colleagues uses spores purified using proteinase K, so does not address the composition of the exosporium. Further study is required before our

working knowledge of the spore surface layers is complete. Genetic and molecular tools give us the ability to identify and work with individual proteins but ideally, the spore as a complete structure needs to be assessed, as it is unlikely that infection will depend on a single protein. Variation between strains belonging to the same ribotype has been demonstrated in other characteristics such as germination studies (Heeg et al. 2012). The potential for significant variation in spore coat layers between different strains should also be considered therefore.

The state of the spore coat has also been demonstrated to be important for transmission. Work on a transmission model of *C. difficile* showed that spores from faecal samples were more effective at transmitting disease than purified spores (Lawley *et al*. 2010). This has implications for all studies assessing the properties of spores, as the purification methods used will affect how much of the spore coat is present and in what state. The potential for differences between strains should also be considered here. The results of this thesis show clear differences in germination rate, hydrophobicity and the colonisation ability of spores lacking BclA proteins. EM images showed aberrations to the spore surface and empty structures in suspensions of BclA mutant spores. These images and alteration of properties suggest that BclA plays a role in attachment of the exosporium. Removal of this layer results in increased germination rates and a reduced ability to colonise an animal host. The role of the BclA proteins has also been assessed in a recent study (Pizarro-Guajardo *et al*. 2013) but a different approach was taken. Pizarro-Guajardo and colleagues used wild type spores that were treated with collagenase to remove the BclA proteins instead of using mutants in these particular genes. This variation in methodology can account for differences in conclusions and again highlights how different approaches can result in

conflicting results. Clearly the outer layers of the spore structure are important and further study of spores will enable additional properties to be elucidated.

The interest in the BclA protein is due to parallels drawn to work with *B. anthracis* where this protein is a key component of the exosporium (Sylvestre *et al*. 2002). The BclA protein of *B. anthracis* was reported as an immunodominant antigen on the spore surface (Steichen *et al*. 2003). Earlier, discussion focused on the need for successful vaccines to express more than just an immune response against toxins, but also address the need for preventing colonisation. Based on studies from *B. anthracis*, the BclA proteins identified in *C. difficile* become interesting candidates for antigens to be used in a vaccine that could potentially prevent colonisation of *C. difficile.* There is little information in the literature about immunogenicity of *C. difficile* spore associated proteins, although the BclA protein has been reported as having limited immunogenicity (Pizarro-Guajardo *et al.* 2013). This however should not rule out the possibility of spore associated proteins being useful in this context.

Colonisation of individuals with *C. difficile* should be an important consideration in any control measures for this pathogen. Recent research shows that spread of infection in hospitals is actually overestimated, with only around a quarter of cases that can be attributed to contraction within hospitals (Walker *et al*. 2012). This study raises several issues, the main question being if infections are not passed between patients in hospitals, where do they indeed come from? The study did not investigate colonisation of asymptomatic individuals, which ignores a potential source of infection. Screening of all

patients and visitors would be a huge undertaking, but could identify currently undetected sources of infections. For example, nurses, doctors and visitors all have contact with patients and could all potentially be carriers of *C. difficile*. The transmission study encourages a fresh view on *C. difficile* infection and its transmission, with infections not just spread between symptomatic individuals or on inanimate objects and surfaces, but disseminating through less obvious routes of transmission. It is also feasible that patients who develop symptoms of CDI in hospital were already colonised before entry to hospital.

The increase in community associated cases of CDI highlights an aspect of *C. difficile* epidemiology that would benefit from further study. With little known about reservoirs of infection, it is difficult to design comprehensive control measures. A vaccine that focuses on reduction or prevention of colonisation would reduce the issue of asymptomatic carriers, as this reservoir of infection would be reduced if the vaccine was widely used. The work of this thesis has demonstrated an effective, easily applicable method for mucosal delivery of antigens, inducing a protective response local to the site of infection. Coupled with the finding that the BclA1 protein is important for initial colonisation and persistence of *C difficile* infection, a new strategy for vaccination is apparent. The spore based vaccine delivery platform could be engineered to deliver both toxin and spore coat protein antigens to the relevant surfaces in a multivalent strategy. This would protect against symptoms of the disease mediated by toxins, but also produce an immune response against initial colonisation of this pathogen. A similar approach has been investigated previously, using a cell associated antigen (Pechine *et al*. 2007) that resulted in reduced colonisation of *C. difficile*. This is a promising result and strengthens the plausibility of the approach. Spores of *C. difficile* are identified as persistence factors, so it is proposed that use of a spore

rather than cell associated antigen will be a more successful approach to preventing colonisation by vaccination.

The role of spores in *C. difficile* infection is important. With further study regarding the role spores play in transmitting and persistence of the infection, this information can be exploited for development of new treatments and control programs.

The BclA protein of *C. difficile* 630 spores is demonstrated to be of importance for colonisation in animal models. The exosporium and spore coat of *C. difficile* contain many other proteins that could be investigated for similar properties. As comprehensive study of the toxins of *C. difficile* has contributed to elucidating virulence mechanisms, continued study of the spore surface layers will contribute to understanding the ability for persistence of this organism.

6.3. Conclusions

Clostridium difficile, whilst only emerging as a significant pathogen in the last 30 years, is a serious problem in developed countries, not just as a nosocomial infection but also increasingly within communities. The results of this thesis contribute to the development of potential treatment strategies for CDI that could reduce reliance on antibiotics, as well as addressing the role of the BclA spore protein in infection. An increased understanding of the complex infection process of *C. difficile* enhances our ability to produce effective treatments against CDI. Alternative treatment strategies such as vaccination and use of bacteriotherapy could reduce reliance on antibiotics and bring infections under control.

A key example of how investigating the processes used by *C. difficile* for infectious capability can benefit development of treatments is demonstrated in the work of this thesis. The BclA1 protein of *C. difficile* 630 was identified as a potential colonisation factor. Coupled with research also carried out into alternative methods for delivery a candidate vaccine, this presents an interesting avenue for future work. The vaccine platform is adaptable and could be used to develop a vaccine with BclA as an antigen that has potential to prevent colonisation of *C. difficile*; an approach that is currently not employed in potential vaccines for *C. difficile.* This approach would address issues such as reservoirs of infection where little is currently understood, enabling enhanced control of disease to be established.

BIBLIOGRAPHY

Abhyankar, W., Hossain, A. H., Djajasaputra, A., Permpoonpattana, P., Ter Beek, A. S., Dekker, H. L., … de Koster, C. G. (2013). In Pursuit of Protein Targets: Proteomic characterization of Bacterial Spore Outer Layers. *Journal of Proteome Research*, *12*(10), 4507–4521.

Alkan, S. S., Decker, T., von Gabain, A., Foglia, G., Shah, S., Luxemburger, C., & Pietrobon, P. J. F. (2012). *Clostridium difficile*: Development of a novel candidate vaccine. *Vaccine*, *30*(29), 4307–4309.

Allen, S. J., Wareham, K., Wang, D., Bradley, C., Hutchings, H., Harris, W., … Mack, D. (2013). Lactobacilli and bifidobacteria in the prevention of antibiotic-associated diarrhoea and *Clostridium difficile* diarrhoea in older inpatients (PLACIDE): a randomised, double-blind, placebo-controlled, multicentre trial. *The Lancet*, *382*(9900), 1249–1257.

Araya, M., Morelli, L., Reid, G., Sanders, M. E., & Stanton, C. (2002). *Joint FAO/WHO Working Group Report on Guidelines for the Evaluation of Probiotics in Food.* (p. ftp://ftp.fao.org/es/esn/food/wgreport2.pdf.). London, Ontario.

Arnon, S. S., Schechter, R., Inglesby, T. V., Henderson, D. A., Bartlett, J. G., ... Tonat, K. (2001). Botulinum Toxin as a Biological Weapon: Medical and Public Health Management. *JAMA*, *285*(8), 1059-1070.

Aronoff, D. M.(2013). *Clostridium novyi, sordellii*, and *tetani*: Mechanisms of disease. *Anaerobe, 24,* 98-101

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., … Bork, P. (2011). Enterotypes of the human gut microbiome. *Nature*, *473*(7346), 174–80.

Asp, N.-G., & Bryngelsson, S. (2008). Health claims in Europe: new legislation and PASSCLAIM for substantiation. *The Journal of Nutrition*, *138*(6), 1210S–5S.

Azizi, A., Kumar, A., Diaz-Mitoma, F., & Mestecky, J. (2010). Enhancing oral vaccine potency by targeting intestinal M cells. *PLoS Pathogens*, *6*(11), e1001147.

Bäckhed, F., Fraser, C. M., Ringel, Y., Sanders, M. E., Sartor, R. B., Sherman, P. M., … Finlay, B. B. (2012). Defining a healthy human gut microbiome: current concepts, future directions, and clinical applications. *Cell Host & Microbe*, *12*(5), 611–22.

Baines, S. D., Freeman, J., & Wilcox, M. H. (2009). Tolevamer is not efficacious in the neutralization of cytotoxin in a human gut model of *Clostridium difficile* infection. *Antimicrobial Agents and Chemotherapy*, *53*(5), 2202–4.

Bakke, H., Setek, T. N., Huynh, P. N., Haugen, I. L., Høiby, E. A., Holst, J., … Haneberg, B. (2004). Immunisation schedules for non-replicating nasal vaccines can be made simple by allowing time for development of immunological memory. *Vaccine*, *22*(17), 2278–2284.

Barbut, F., Richard, A., Hamadi, K., Chomette, V., Burghoffer, B., & Petit, J. C. (2000). Epidemiology of recurrences or reinfections of *Clostridium difficile*-associated diarrhea. *Journal of Clinical Microbiology*, *38*(6), 2386–8.

Barnes, A. G. C., Cerovic, V., Hobson, P. S., & Klavinskis, L. S. (2007). *Bacillus subtilis* spores: a novel microparticle adjuvant which can instruct a balanced Th1 and Th2 immune response to specific antigen. *European Journal of Immunology*, *37*(6), 1538–47.

Bartlett, J. G. (2008). Historical perspectives on studies of *Clostridium difficile* and *C. difficile* infection. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *46 Suppl 1*(Supplement 1), S4–11.

Bauer, M. P., Notermans, D. W., van Benthem, B. H., Brazier, J. S., Wilcox, M. H., Rupnik, M., … Kuijper, E. J. (2011). *Clostridium difficile* infection in Europe: a hospital-based survey. *The Lancet*, *377*(9759), 63–73.

Best, E. L., Fawley, W. N., Parnell, P., & Wilcox, M. H. (2010). The potential for airborne dispersal of *Clostridium difficile* from symptomatic patients. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *50*(11), 1450–7.

Bhorade, S. M., Christenson, J., Pohlman, A. S., Arnow, P. M., & Hall, J. B. (1999). The incidence of and clinical variables associated with vancomycin-resistant enterococcal colonization in mechanically ventilated patients. *Chest*, *115*(4), 1085–91.

Bien, J., Palagani, V., & Bozko, P. (2013). The intestinal microbiota dysbiosis and *Clostridium difficile* infection: is there a relationship with inflammatory bowel disease? *Therapeutic Advances in Gastroenterology*, *6*(1), 53–68.

Björkstén, B., Sepp, E., Julge, K., Voor, T., & Mikelsaar, M. (2001). Allergy development and the intestinal microflora during the first year of life. *The Journal of Allergy and Clinical Immunology*, *108*(4), 516–20.

Boydston, J. A., Chen, P., Steichen, C. T., & Turnbough, C. L. (2005). Orientation within the exosporium and structural stability of the collagen-like glycoprotein BclA of *Bacillus anthracis*. *Journal of Bacteriology*, *187*(15), 5310–7.

Boyle, R. J., Bath-Hextall, F. J., Leonardi-Bee, J., Murrell, D. F., & Tang, M. L. (2008). Probiotics for treating eczema. *The Cochrane Database of Systematic Reviews*, (4), CD006135.

Bozue, J., Cote, C. K., Moody, K. L., & Welkos, S. L. (2007a). Fully virulent *Bacillus anthracis* does not require the immunodominant protein BclA for pathogenesis. *Infection and Immunity*, *75*(1), 508–11.

Bozue, J., Moody, K. L., Cote, C. K., Stiles, B. G., Friedlander, A. M., Welkos, S. L., & Hale, M. L. (2007b). *Bacillus anthracis* spores of the bclA mutant exhibit increased adherence to epithelial cells, fibroblasts, and endothelial cells but not to macrophages. *Infection and Immunity*, *75*(9), 4498–505.

Bratt, H., Rottiers, P., Hommes, D. W., Huyghbaert, N., Remaut, E., Remon, J-P., ...Steidler, L. (2006). A Phase I trial with transgenic bacteria expressing Interleukin-10 in Crohn's Disease. *Clinical gastroenterology and hepatology*, *4(*6), 754-759

Brahmbhatt, T. N., Darnell, S. C., Carvalho, H. M., Sanz, P., Kang, T. J., Bull, R. L., … O'Brien, A. D. (2007a). Recombinant exosporium protein BclA of *Bacillus anthracis* is effective as a booster for mice primed with suboptimal amounts of protective antigen. *Infection and Immunity*, *75*(11), 5240–7.

Brahmbhatt, T. N., Janes, B. K., Stibitz, E. S., Darnell, S. C., Sanz, P., Rasmussen, S. B., & O'Brien, A. D. (2007b). *Bacillus anthracis* exosporium protein BclA affects spore germination, interaction with extracellular matrix proteins, and hydrophobicity. *Infection and Immunity*, *75*(11), 5233–9.

Brandt, L. J., Aroniadis, O. C., Mellow, M., Kanatzar, A., Kelly, C., Park, T., … Surawicz, C. (2012). Long-term follow-up of colonoscopic fecal microbiota transplant for recurrent *Clostridium difficile* infection. *The American Journal of Gastroenterology*, *107*(7), 1079–87.

Braunlin, W., Xu, Q., Hook, P., Fitzpatrick, R., Klinger, J. D., Burrier, R., & Kurtz, C. B. (2004). Toxin binding of tolevamer, a polyanionic drug that protects against antibiotic-associated diarrhea. *Biophysical Journal*, *87*(1), 534–9.

Brazier, J. S. (1998). The epidemiology and typing of *Clostridium difficile*. *The Journal of Antimicrobial Chemotherapy*, *41 Suppl C*, 47–57.

Brown, K. A., Khanafer, N., Daneman, N., & Fisman, D. N. (2013). Meta-analysis of antibiotics and the risk of community-associated *Clostridium difficile* infection. *Antimicrobial Agents and Chemotherapy*, *57*(5), 2326–32.

Bruggemann, H., Baumer, S., Fricke, W. F., Wiezer, A., Liesegang, H., Decker, I., … Gottschalk, G. (2003). The genome sequence of *Clostridium tetani*, the causative agent of tetanus disease. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(3), 1316–21.

Brynestad, S., & Granum, P. E. (2002). *Clostridium perfringens* and foodborne infections. *International Journal of Food Microbiology*, *74*(3), 195–202.

Buckley, A. M., Spencer, J., Candlish, D., Irvine, J. J., & Douce, G. R. (2011). Infection of hamsters with the UK *Clostridium difficile* ribotype 027 outbreak strain R20291. *Journal of Medical Microbiology*, *60*(Pt 8), 1174–80.

Buckley, A. M., Spencer, J., Maclellan, L. M., Candlish, D., Irvine, J. J., & Douce, G. R. (2013). Susceptibility of Hamsters to *Clostridium difficile* Isolates of Differing Toxinotype. *PloS One*, *8*(5), e64121.

Budde, I., Steil, L., Scharf, C., Völker, U., & Bremer, E. (2006). Adaptation of *Bacillus subtilis* to growth at low temperature: a combined transcriptomic and proteomic appraisal. *Microbiology (Reading, England)*, *152*(Pt 3), 831–53.

Bulla, L. A., St Julian, G., Rhodes, R. A., & Hesseltine, C. W. (1969). Scanning electron and phase-contrast microscopy of bacterial spores. *Applied Microbiology*, *18*(3), 490–5.

Burns, D. A., Heeg, D., Cartman, S. T., & Minton, N. P. (2011). Reconsidering the sporulation characteristics of hypervirulent *Clostridium difficile* BI/NAP1/027. *PloS One*, *6*(9), e24894.

Calabi, E., Calabi, F., Phillips, A. D., & Fairweather, N. F. (2002). Binding of *Clostridium difficile* surface layer proteins to gastrointestinal tissues. *Infection and Immunity*, *70*(10), 5770–8.

Carr, K. A., Lybarger, S. R., Anderson, E. C., Janes, B. K., & Hanna, P. C. (2010). The role of *Bacillus anthracis* germinant receptors in germination and virulence. *Molecular Microbiology*, *75*(2), 365–75.

Carroll, K. C., & Bartlett, J. G. (2011). Biology of *Clostridium difficile*: implications for epidemiology and diagnosis. *Annual Review of Microbiology*. 65, 501-21.

Castagliuolo, I., Keates, A. C., Wang, C. C., Pasha, A., Valenick, L., Kelly, C. P., … Pothoulakis, C. (1998). *Clostridium difficile* toxin A stimulates macrophage-inflammatory protein-2 production in rat intestinal epithelial cells. *Journal of Immunology , 160*(12), 6039–45.

Castagliuolo, I., LaMont, J., Nikulasson, S., & Pothoulakis, C. (1996). *Saccharomyces boulardii* protease inhibits *Clostridium difficile* toxin A effects in the rat ileum. *Infection and Immunity*, *64*(12), 5225–5232.

Cazorla, F. M., Romero, D., Pérez-García, A., Lugtenberg, B. J. J., Vicente, A. de, & Bloemberg, G. (2007). Isolation and characterization of antagonistic *Bacillus subtilis* strains from the avocado rhizoplane displaying biocontrol activity. *Journal of Applied Microbiology*, *103*(5), 1950–9.

CDC. (2013a). *Antibiotic Resistance Threats in the United States, 2013*. Retrived September 20, 2013 from http://www.cdc.gov/drugresistance/threat-report-2013/

CDC. (2013b). *CDC - Quadrivalent Influenza Vaccine | Seasonal Influenza (Flu).2013-201.* Retrieved September 27, 2013, from http://www.cdc.gov/vaccines/hcp/vis/vis-statements/flulive.html

CDC. (2013c). Vaccine Information Statement: Live, Intranasal Influenza - Vaccines - CDC. Retrieved September 27, 2013, from http://www.cdc.gov/vaccines/hcp/vis/vis-statements/flulive.html

Cebra, J. J. (1999). Influences of microbiota on intestinal immune system development. *The American Journal of Clinical Nutrition*, *69*(5), 1046S–1051S.

Cerutti, A., Chen, K., & Chorny, A. (2011). Immunoglobulin responses at the mucosal interface. *Annual Review of Immunology*, *29*, 273–93.

Chang, J. Y., Antonopoulos, D. A., Kalra, A., Tonelli, A., Khalife, W. T., Schmidt, T. M., & Young, V. B. (2008). Decreased diversity of the fecal microbiome in recurrent *Clostridium difficile*-associated diarrhea. *The Journal of Infectious Diseases*, *197*(3), 435–8.

Chen, X., Gerding, D. N., & Kelly, C. (2008). A mouse model of *Clostridium difficile*-associated disease. *Gastroenterology*, *135*, 1984–1992.

Chen, Y., Cao, S., Chai, Y., Clardy, J., Kolter, R., Guo, J., & Losick, R. (2012). A *Bacillus subtilis* sensor kinase involved in triggering biofilm formation on the roots of tomato plants. *Molecular Microbiology*, *85*(3), 418–30.

Chen, Y., Yan, F., Chai, Y., Liu, H., Kolter, R., Losick, R., & Guo, J. (2013). Biocontrol of tomato wilt disease by *Bacillus subtilis* isolates from natural environments depends on conserved genes mediating biofilm formation. *Environmental Microbiology*, *15*(3), 848–64.

Cherington, M. (1998). Clinical spectrum of botulism. *Muscle & Nerve*, *21*(6), 701–10.

Chitnis, A. S., Holzbauer, S. M., Belflower, R. M., Winston, L. G., Bamberg, W. M., Lyons, C., … Lessa, F. C. (2013). Epidemiology of Community-Associated *Clostridium difficile* Infection, 2009 Through 2011. *JAMA Internal Medicine*, *173*(14), 1359–67.

Cuburu, N., Kweon, M.-N., Song, J.-H., Hervouet, C., Luci, C., Sun, J.-B., … Czerkinsky, C. (2007). Sublingual immunization induces broad-based systemic and mucosal immune responses in mice. *Vaccine*, *25*(51), 8598–610.

Curry, S. R., Marsh, J. W., Muto, C. A., O'Leary, M. M., Pasculle, A. W., & Harrison, L. H. (2007). tcdC genotypes associated with severe TcdC truncation in an epidemic clone and other strains of *Clostridium difficile*. *Journal of Clinical Microbiology*, *45*(1), 215–21.

Cutting, S. M. (2011). *Bacillus* probiotics. *Food Microbiology*, *28*(2), 214–20.

D'Souza, A. L. (2002). Probiotics in prevention of antibiotic associated diarrhoea: meta-analysis. *BMJ*, *324*(7350), 1361–1361.

Dapa, T., & Unnikrishnan, M. (2013). Biofilm formation by *Clostridium difficile*. *Gut Microbes*, *4*(5).

Daubenspeck, J. M., Zeng, H., Chen, P., Dong, S., Steichen, C. T., Krishna, N. R., … Turnbough, C. L. (2004). Novel oligosaccharide side chains of the collagen-like region of BclA, the major glycoprotein of the *Bacillus anthracis* exosporium. *The Journal of Biological Chemistry*, *279*(30), 30945–53.

Davies, A. H., Roberts, A. K., Shone, C. C., & Acharya, K. R. (2011). Super toxins from a super bug: structure and function of *Clostridium difficile* toxins. *The Biochemical Journal*, *436*(3), 517–26.

Dawson, L. F., Valiente, E., Faulds-Pain, A., Donahue, E. H., & Wren, B. W. (2012). Characterisation of *Clostridium difficile* biofilm formation, a role for Spo0A. *PloS One*, *7*(12), e50527.

De Vrese, M., Stegelmann, A., Richter, B., Fenselau, S., Laue, C., & Schrezenmeir, J. (2001). Probiotics--compensation for lactase insufficiency. *American Journal of Clinical Nutrition, 73*(2), 421S–429.

Deakin, L. J., Clare, S., Fagan, R. P., Dawson, L. F., Pickard, D. J., West, M. R., … Lawley, T. D. (2012). The *Clostridium difficile* spo0A gene is a persistence and transmission factor. *Infection and Immunity*, *80*(8), 2704–11.

Denève, C., Janoir, C., Poilane, I., Fantinato, C., & Collignon, A. (2009). New trends in *Clostridium difficile* virulence and pathogenesis. *International Journal of Antimicrobial Agents*, *33 (Supplement 1)*, S24–8.

Di Giacinto, C., Marinaro, M., Sanchez, M., Strober, W., & Boirivant, M. (2005). Probiotics Ameliorate Recurrent Th1-Mediated Murine Colitis by Inducing IL-10 and IL-10-Dependent TGF Bearing Regulatory Cells. *The Journal of Immunology*, *174*(6), 3237–3246.

Driks, A. (2002). Maximum shields: the assembly and function of the bacterial spore coat. *Trends in Microbiology*, *10*(6), 251–254.

Driks, A., Roels, S., Beall, B., Moran, C. P., & Losick, R. (1994). Subcellular localization of proteins involved in the assembly of the spore coat of *Bacillus subtilis*. *Genes & Development*, *8*(2), 234–44.

Drudy, D., Calabi, E., Kyne, L., Sougioultzis, S., Kelly, E., Fairweather, N., & Kelly, C. P. (2004). Human antibody response to surface layer proteins in *Clostridium difficile* infection. *FEMS Immunology and Medical Microbiology*, *41*(3), 237–42.

Drudy, D., Harnedy, N., Fanning, S., O'Mahony, R., & Kyne, L. (2007). Isolation and characterisation of toxin A-negative, toxin B-positive *Clostridium difficile* in Dublin, Ireland. *Clinical Microbiology and Infection : The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *13*(3), 298–304.

Drudy, D., Kyne, L., O'Mahony, R., & Fanning, S. (2007). gyrA mutations in fluoroquinolone-resistant *Clostridium difficile* PCR-027. *Emerging Infectious Diseases*, *13*(3), 504–5.

Duc, L. H., Hong, H. A., Atkins, H. S., Flick-Smith, H. C., Durrani, Z., Rijpkema, S., … Cutting, S. M. (2007). Immunization against anthrax using *Bacillus subtilis* spores expressing the anthrax protective antigen. *Vaccine*, *25*(2), 346–355.

Duc, L. H., Hong, H. A., Uyen, N. Q., & Cutting, S. M. (2004). Intracellular fate and immunogenicity of *Bacillus subtilis* spores. *Vaccine*, *22*(15), 1873–1885.

Duchmann, R., Kaiser, I., Hermann, E., Mayet, W., Ewe, K., & Meyer zum Büschenfelde, K. H. (1995). Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). *Clinical and Experimental Immunology*, *102*(3), 448–55.

Dupuy, B., Govind, R., Antunes, A., & Matamouros, S. (2008). *Clostridium difficile* toxin synthesis is negatively regulated by TcdC. *Journal of Medical Microbiology*, *57*(Pt 6), 685–9.

Durham, S. R., Yang, W. H., Pedersen, M. R., Johansen, N., & Rak, S. (2006). Sublingual immunotherapy with once-daily grass allergen tablets: A randomized controlled trial in seasonal allergic rhinoconjunctivitis. *Journal of Allergy and Clinical Immunology*, *117*(4), 802–809.

Escobar-Cortés, K., Barra-Carrasco, J., & Paredes-Sabja, D. (2013). Proteases and sonication specifically remove the exosporium layer of spores of *Clostridium difficile* strain 630. *Journal of Microbiological Methods*, *93*(1), 25–31.

Eyre, D. W., Cule, M. L., Wilson, D. J., Griffiths, D., Vaughan, A., O'Connor, L., … Walker, A. S. (2013). Diverse sources of *Clostridium difficile* infection identified on whole-genome sequencing. *The New England Journal of Medicine*, *369*(13), 1195–205.

Fagarasan, S., & Honjo, T. (2003). Intestinal IgA synthesis: regulation of front-line body defences. *Nature Reviews. Immunology*, *3*(1), 63–72.

Faille, C., Lequette, Y., Ronse, A., Slomianny, C., Garénaux, E., & Guerardel, Y. (2010). Morphology and physico-chemical properties of *Bacillus* spores surrounded or not with an exosporium. *International Journal of Food Microbiology*, *143*(3), 125–135.

Figueroa, I., Johnson, S., Sambol, S. P., Goldstein, E. J. C., Citron, D. M., & Gerding, D. N. (2012). Relapse versus reinfection: recurrent *Clostridium difficile* infection following treatment with fidaxomicin or vancomycin. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *55 (Supplement 2)*, S104–9.

Francis, M. B., Allen, C. A., Shrestha, R., & Sorg, J. A. (2013). Bile acid recognition by the *Clostridium difficile* germinant receptor, CspC, is important for establishing infection. *PLoS Pathogens*, *9*(5), e1003356.

Fridkin, S. K., Edwards, J. R., Courval, J. M., Hill, H., Tenover, F. C., Lawton, R., … McGowan, J. E. (2001). The effect of vancomycin and third-generation cephalosporins on prevalence of vancomycin-resistant enterococci in 126 U.S. adult intensive care units. *Annals of Internal Medicine*, *135*(3), 175–83.

Gao, X. W., Mubasher, M., Fang, C. Y., Reifer, C., & Miller, L. E. (2010). Dose-response efficacy of a proprietary probiotic formula of *Lactobacillus acidophilus* CL1285 and *Lactobacillus casei* LBC80R for antibiotic-associated diarrhea and *Clostridium difficile*-associated diarrhea prophylaxis in adult patients. *The American Journal of Gastroenterology*, *105*(7), 1636–41.

Garey, K. W., Jiang, Z.-D., Yadav, Y., Mullins, B., Wong, K., & Dupont, H. L. (2008). Peripartum *Clostridium difficile* infection: case series and review of the literature. *American Journal of Obstetrics and Gynecology*, *199*(4), 332–337.

Garey, K. W., Sethi, S., Yadav, Y., & DuPont, H. L. (2008). Meta-analysis to assess risk factors for recurrent *Clostridium difficile* infection. *The Journal of Hospital Infection*, *70*(4), 298–304.

Gerding, D. N. (2012). *Clostridium difficile* infection prevention: biotherapeutics, immunologics, and vaccines. *Discovery Medicine*, *13*(68), 75–83.

Gerding, D. N., Muto, C. A., & Owens, R. C. (2008a). Measures to control and prevent *Clostridium difficile* infection. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *46* (Supplement 1), S43–9.

Gerding, D. N., Muto, C. A., & Owens, R. C. (2008b). Treatment of *Clostridium difficile* infection. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *46* (Supplement 1), S32–42.

Giannasca, P. J., & Warny, M. (2004). Active and passive immunization against *Clostridium difficile* diarrhea and colitis. *Vaccine*, *22*(7), 848–56.

Goldstein, E. J. C., Babakhani, F., & Citron, D. M. (2012). Antimicrobial activities of fidaxomicin. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *55* (Supplement 2), S143–8.

Gough, E., Shaikh, H., & Manges, A. R. (2011). Systematic review of intestinal microbiota transplantation (fecal bacteriotherapy) for recurrent *Clostridium difficile* infection. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *53*(10), 994–1002.

Goulding, D., Thompson, H., Emerson, J., Fairweather, N. F., Dougan, G., & Douce, G. R. (2009). Distinctive profiles of infection and pathology in hamsters infected with *Clostridium difficile* strains 630 and B1. *Infection and Immunity*, *77*(12), 5478–85.

Grönlund, M. M., Lehtonen, O. P., Eerola, E., & Kero, P. (1999). Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. *Journal of Pediatric Gastroenterology and Nutrition*, *28*(1), 19–25.

Grubeck-Loebenstein, B., Della Bella, S., Iorio, A. M., Michel, J.-P., Pawelec, G., & Solana, R. (2009). Immunosenescence and vaccine failure in the elderly. *Aging Clinical and Experimental Research*, *21*(3), 201–9.

Guarner, F., & Malagelada, J.-R. (2003). Gut flora in health and disease. *The Lancet*, *361*(9356), 512–519.

Gurwith, M. J., Rabin, H. R., & Love, K. (1977). Diarrhea Associated with Clindamycin and Ampicillin Therapy: Preliminary Results of a Cooperative Study. *Journal of Infectious Diseases*, *135*(Supplement), S104–S110.

Hahn, U. K., Boehm, R., & Beyer, W. (2006). DNA vaccination against anthrax in mice—combination of anti-spore and anti-toxin components. *Vaccine*, *24*(21), 4569–4571.

Hall, A. (1990). The cellular functions of small GTP-binding proteins. *Science (New York, N.Y.)*, *249*(4969), 635–40.

Hall, I. C., & O'Toole, E. (1935). Intestinal flora in newborn infants with a description of a new pathogenic anaerobe, *Bacillus difficilis*. *American Journal of Diseases of Children*, *49*(2), 390.

Harris, D., & Robinson, J. R. (1992). Drug delivery via the mucous membranes of the oral cavity. *Journal of Pharmaceutical Sciences*, *81*(1), 1–10.

Hart, A. L., Lammers, K., Brigidi, P., Vitali, B., Rizzello, F., Gionchetti, P., … Stagg, A. J. (2004). Modulation of human dendritic cell phenotype and function by probiotic bacteria. *Gut*, *53*(11), 1602–9.

Harwood, C. R., & Cutting, S. M. (1990). *Molecular Biological Methods for Bacillus (Modern Microbiological Methods)*. Wiley-Blackwell.

He, M., Miyajima, F., Roberts, P., Ellison, L., Pickard, D. J., Martin, M. J., … Lawley, T. D. (2012). Emergence and global spread of epidemic healthcare-associated *Clostridium difficile. Nature Genetics*, *43*, 109–113.

Heap, J. T., Kuehne, S. A., Ehsaan, M., Cartman, S. T., Cooksley, C. M., Scott, J. C., & Minton, N. P. (2010). The ClosTron: Mutagenesis in *Clostridium* refined and streamlined. *Journal of Microbiological Methods*, *80*(1), 49–55.

Heap, J. T., Pennington, O. J., Cartman, S. T., Carter, G. P., & Minton, N. P. (2007). The ClosTron: a universal gene knock-out system for the genus *Clostridium*. *Journal of Microbiological Methods*, *70*(3), 452–64.

Hecht, G., Pothoulakis, C., LaMont, J. T., & Madara, J. L. (1988). *Clostridium difficile* toxin A perturbs cytoskeletal structure and tight junction permeability of cultured human intestinal epithelial monolayers. *The Journal of Clinical Investigation*, *82*(5), 1516–24.

Heeg, D., Burns, D. A., Cartman, S. T., & Minton, N. P. (2012). Spores of *Clostridium difficile* clinical isolates display a diverse germination response to bile salts. *PLoS ONE*, *7*(2), e32381.

Henriques, A. O., & Moran, C. P. (2007). Structure, assembly, and function of the spore surface layers. *Annual Review of Microbiology*, *61*, 555–88.

Heslop, O. D., Roye-Green, K., Coard, K., & Mulvey, M. R. (2013). A unique strain of community-acquired *Clostridium difficile* in severe complicated infection and death of a young adult. *BMC Infectious Diseases*, *13*(1), 299.

Hinkson, P. L., Dinardo, C., DeCiero, D., Klinger, J. D., & Barker, R. H. (2008). Tolevamer, an anionic polymer, neutralizes toxins produced by the BI/027 strains of *Clostridium difficile*. *Antimicrobial Agents and Chemotherapy*, *52*(6), 2190–5.

Hoa, T. T., Duc, L. H., Isticato, R., Baccigalupi, L., Ricca, E., Van, P. H., & Cutting, S. M. (2001). Fate and Dissemination of *Bacillus subtilis* Spores in a Murine Model. *Applied and Environmental Microbiology*, *67*(9), 3819–3823.

Holmgren, J. (1991). Mucosal immunity and vaccination. *FEMS Microbiology Immunology*, *4*(1), 1–9.

Holmgren, J., & Czerkinsky, C. (2005). Mucosal immunity and vaccines. *Nature Medicine*, *11*(4 Suppl), S45–53.

Hong, H. A., Duc, L. H., & Cutting, S. M. (2005). The use of bacterial spore formers as probiotics. *FEMS Microbiology Reviews*, *29*(4), 813–35.

Hong, H. A., To, E., Fakhry, S., Baccigalupi, L., Ricca, E., & Cutting, S. M. (2009). Defining the natural habitat of Bacillus spore-formers. *Research in Microbiology*, *160*(6), 375–379.

Hosoi, T., & Kiuchi, K. (2004). Production and Probiotic Effects of Natto. In E. Ricca, A. O. Henriques, & S. M. Cutting (Eds.), *Bacterial Spore Formers: Probiotics and Emerging Applications* (1st ed., pp. 143–154). Poole: Horizon Bioscience.

HSE. (2007). *The SACGM Compendium of guidance - Part 1: Introduction to the legislation and general health and safety issues - part1.pdf*.

Huang, J.-M., Hong, H. A., Van Tong, H., Hoang, T. H., Brisson, A., & Cutting, S. M. (2010). Mucosal delivery of antigens using adsorption to bacterial spores. *Vaccine*, *28*(4), 1021–30.

Huang, J.-M., La Ragione, R. M., Nunez, A., & Cutting, S. M. (2008). Immunostimulatory activity of *Bacillus* spores. *FEMS Immunology and Medical Microbiology*, *53*(2), 195–203.

Hull, H. F., Birmingham, M. E., Melgaard, B., & Lee, J. W. (1997). Progress toward Global Polio Eradication. *Journal of Infectious Diseases*, *175*(Supplement 1), S4–S9.

Human Microbiome Project Consortium. (2012). Structure, function and diversity of the healthy human microbiome. *Nature*, *486*(7402), 207–14.

Hundsberger, T., Braun, V., Weidmann, M., Leukel, P., Sauerborn, M., & Eichel-Streiber, C. (1997). Transcription Analysis of the Genes tcdA-E of the Pathogenicity Locus of *Clostridium difficile*. *European Journal of Biochemistry*, *244*(3), 735–742.

Islam, J., Cohen, J., Rajkumar, C., & Llewelyn, M. J. (2012). Probiotics for the prevention and treatment of *Clostridium difficile* in older patients. *Age and Ageing*, *41*(6), 706–11.

Isticato, R., Cangiano, G., Tran, H. O. A. T., Ciabattini, A., Medaglini, D., Oggioni, M. R., … Ricca, E. (2001). Surface Display of Recombinant Proteins on *Bacillus subtilis* Spores, *183*(21), 6294–6301.

Janezic, S., & Rupnik, M. (2010). Molecular typing methods for *Clostridium difficile*: pulsed-field gel electrophoresis and PCR ribotyping. *Methods in Molecular Biology (Clifton, N.J.)*, *646*, 55–65.

Janoir, C., Péchiné, S., Grosdidier, C., & Collignon, A. (2007). Cwp84, a surface-associated protein of *Clostridium difficile*, is a cysteine protease with degrading activity on extracellular matrix proteins. *Journal of Bacteriology*, *189*(20), 7174–80.

Jarchum, I., Liu, M., Lipuma, L., & Pamer, E. G. (2011). Toll-like receptor 5 stimulation protects mice from acute *Clostridium difficile* colitis. *Infection and Immunity*, *79*(4), 1498–503.

Jarvis, W. R. (1996). The epidemiology of colonisation. *Infection control and hospital epidemiology*, *17*(1), 47-52.

Jhung, M. A., Thompson, A. D., Killgore, G. E., Zukowski, W. E., Songer, G., Warny, M., … Limbago, B. M. (2008). Toxinotype V *Clostridium difficile* in humans and food animals. *Emerging Infectious Diseases*, *14*(7), 1039–45.

Jiang, Z.-D., DuPont, H. L., Garey, K., Price, M., Graham, G., Okhuysen, P., … LaRocco, M. (2006). A common polymorphism in the interleukin 8 gene promoter is associated with *Clostridium difficile* diarrhea. *The American Journal of Gastroenterology*, *101*(5), 1112–6.

Johnson, S. (2009). Recurrent *Clostridium difficile* infection: a review of risk factors, treatments, and outcomes. *The Journal of Infection*, *58*(6), 403–10.

Johnson, S., Gerding, D. N., & Janoff, E. N. (1992). Systemic and mucosal antibody responses to toxin A in patients infected with *Clostridium difficile*. *The Journal of Infectious Diseases*, *166*(6), 1287–94.

Johnson, S., Sambol, S. P., Brazier, J. S., Delmée, M., Avesani, V., Merrigan, M. M., & Gerding, D. N. (2003). International typing study of toxin A-negative, toxin B-positive *Clostridium difficile* variants. *Journal of Clinical Microbiology*, *41*(4), 1543–7.

Johnson, S., Sypura, W. D., Gerding, D. N., Ewing, S. L., & Janoff, E. N. (1995). Selective neutralization of a bacterial enterotoxin by serum immunoglobulin A in response to mucosal disease. *Infection and Immunity*, *63*(8), 3166–73.

Johnston, P. F., Gerding, D. N., & Knight, K. L. (2013). Protection from *Clostridium difficile* infection in CD4 T cell and polymeric immunoglobulin receptor deficient mice. *Infection and Immunity*, *82*(2), 522-31.

Jones, D. T., & Woods, D. R. (1986). Acetone-butanol fermentation revisited. *Microbiological Reviews*, *50*(4), 484–524.

Just, I., Selzer, J., Wilm, M., von Eichel-Streiber, C., Mann, M., & Aktories, K. (1995). Glucosylation of Rho proteins by *Clostridium difficile* toxin B. *Nature*, *375*(6531), 500–3.

Just, I., Wilm, M., Selzer, J., Rex, G., von Eichel-Streiber, C., Mann, M., & Aktories, K. (1995). The enterotoxin from *Clostridium difficile* (ToxA) monoglucosylates the Rho proteins. *The Journal of Biological Chemistry*, *270*(23), 13932–6.

Kailas, L., Terry, C., Abbott, N., Taylor, R., Mullin, N., Tzokov, S. B., … Bullough, P. A. (2011). Surface architecture of endospores of the *Bacillus cereus/anthracis/thuringiensis* family at the subnanometer scale. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(38), 16014–9.

Kaisho, T., & Akira, S. (2002). Toll-like receptors as adjuvant receptors. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1589*(1), 1–13.

Kelly, C. P., Pothoulakis, C., Orellana, J., & LaMont, J. T. (1992). Human colonic aspirates containing immunoglobulin A antibody to *Clostridium difficile* toxin A inhibit toxin A-receptor binding. *Gastroenterology*, *102*(1), 35–40.

Kennedy, M. J., Reader, S. L., & Swierczynski, L. M. (1994). Preservation records of micro-organisms: evidence of the tenacity of life. *Microbiology*, *140* (Pt 1), 2513–29.

Kew, O. (2012). Reaching the last one per cent: progress and challenges in global polio eradication. *Current Opinion in Virology*, *2*(2), 188–198.

Khanna, S., Pardi, D. S., Aronson, S. L., Kammer, P. P., Orenstein, R., St Sauver, J. L., … Zinsmeister, A. R. (2012). The epidemiology of community-acquired *Clostridium difficile* infection: a population-based study. *The American Journal of Gastroenterology*, *107*(1), 89–95.

Kim, P. H., Iaconis, J. P., & Rolfe, R. D. (1987). Immunization of adult hamsters against *Clostridium difficile*-associated ileocecitis and transfer of protection to infant hamsters. *Infection and Immunity*, *55*(12), 2984–92.

Kink, J. A., & Williams, J. A. (1998). Antibodies to recombinant *Clostridium difficile* toxins A and B are an effective treatment and prevent relapse of *C. difficile*-associated disease in a hamster model of infection. *Infection and Immunity*, *66*(5), 2018–25.

Koinange, W., Rogowski, J. J., & Metselaar, D. (1973). Poliomyelitis: epidemiology and prophylaxis- Nationwide vaccination campaign with the help of lay volunteers. *Bulletin of the World Health Organization*, *48*(5), 543–5.

Kuehne, S. A, Cartman, S. T., Heap, J. T., Kelly, M. L., Cockayne, A., & Minton, N. P. (2010). The role of toxin A and toxin B in *Clostridium difficile* infection. *Nature*, *467*(7316), 711–3.

Kunkel, E. J., & Butcher, E. C. (2003). Plasma-cell homing. *Nature Reviews. Immunology*, *3*(10), 822–9.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., … Danchin, A. (1997). The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature*, *390*(6657), 249–56.

Kweon, M.-N. (2011). Sublingual mucosa: A new vaccination route for systemic and mucosal immunity. *Cytokine*, *54*(1), 1–5.

Kweon, M.-N., Yamamoto, M., Watanabe, F., Tamura, S., Van Ginkel, F. W., Miyauchi, A., … Kiyono, H. (2002). A nontoxic chimeric enterotoxin adjuvant induces protective immunity in both mucosal and systemic compartments with reduced IgE antibodies. *The Journal of Infectious Diseases*, *186*(9), 1261–9.

Kyne, L., Hamel, M. B., Polavaram, R., & Kelly, C. P. (2002). Health care costs and mortality associated with nosocomial diarrhea due to *Clostridium difficile*. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *34*(3), 346–53.

Kyne, L., Warny, M., Qamar, A., & Kelly, C. P. (2001). Association between antibody response to toxin A and protection against recurrent *Clostridium difficile* diarrhoea. *The Lancet*, *357*(9251), 189–193.

Lahtinen, S. J. (2012). Probiotic viability - does it matter? *Microbial Ecology in Health and Disease*, *23*, 18567.

Lanis, J. M., Barua, S., & Ballard, J. D. (2010). Variations in TcdB activity and the hypervirulence of emerging strains of *Clostridium difficile*. *PLoS Pathogens*, *6*(8), e1001061.

Lanis, J. M., Heinlen, L. D., James, J. A., & Ballard, J. D. (2013). *Clostridium difficile* 027/BI/NAP1 encodes a hypertoxic and antigenically variable form of TcdB. *PLoS Pathogens*, *9*(8), e1003523.

Lawley, T. D., Clare, S., Walker, A. W., Goulding, D., Stabler, R. A., Croucher, N., … Dougan, G. (2009a). Antibiotic treatment of *Clostridium difficile* carrier mice triggers a supershedder state, spore-mediated transmission, and severe disease in immunocompromised hosts. *Infection and Immunity*, *77*(9), 3661–9.

Lawley, T. D., Croucher, N. J., Yu, L., Clare, S., Sebaihia, M., Goulding, D., … Dougan, G. (2009b). Proteomic and genomic characterization of highly infectious *Clostridium difficile* 630 spores. *Journal of Bacteriology*, *191*(17), 5377–86.

Lawley, T. D., Clare, S., Deakin, L. J., Goulding, D., Yen, J. L., Raisen, C., … Dougan, G. (2010). Use of purified *Clostridium difficile* spores to facilitate evaluation of health care disinfection regimens. *Applied and Environmental Microbiology*, *76*(20), 6895–900.

Lawley, T. D., Clare, S., Walker, A. W., Stares, M. D., Connor, T. R., Raisen, C., … Dougan, G. (2012). Targeted Restoration of the Intestinal Microbiota with a Simple, Defined Bacteriotherapy Resolves Relapsing *Clostridium difficile* Disease in Mice. *PLoS Pathogens*, *8*(10), e1002995.

Lawley, T. D., & Young, V. B. (2013). Murine models to study *Clostridium difficile* infection and transmission. *Anaerobe*, *24*, 94–7.

Lee, J., Jang, Y.-S., Choi, S. J., Im, J. A., Song, H., Cho, J. H., … Lee, S. Y. (2012). Metabolic engineering of *Clostridium acetobutylicum* ATCC 824 for isopropanol-butanol-ethanol fermentation. *Applied and Environmental Microbiology*, *78*(5), 1416–23.

Lee, J., Seto, D., & Bielory, L. (2008). Meta-analysis of clinical trials of probiotics for prevention and treatment of pediatric atopic dermatitis. *The Journal of Allergy and Clinical Immunology*, *121*(1), 116–121.e11.

Lee, S., Belitsky, B. R., Brown, D. W., Brinker, J. P., Kerstein, K. O., Herrmann, J. E., … Tzipori, S. (2010). Efficacy, heat stability and safety of intranasally administered *Bacillus subtilis* spore or vegetative cell vaccines expressing tetanus toxin fragment C. *Vaccine*, *28*(41), 6658–65.

Lequette, Y., Garénaux, E., Tauveron, G., Dumez, S., Perchat, S., Slomianny, C., … Faille, C. (2011). Role played by exosporium glycoproteins in the surface properties of *Bacillus cereus* spores and in their adhesion to stainless steel. *Applied and Environmental Microbiology*, *77*(14), 4905–11.

Levine, M. M., & Sztein, M. B. (2004). Vaccine development strategies for improving immunization: the role of modern immunology. *Nature Immunology*, *5*(5), 460–4.

Li, K., Bihan, M., Yooseph, S., & Methé, B. A. (2012). Analyses of the microbial diversity across the human microbiome. *PloS One*, *7*(6), e32118.

Lima, K. M., dos Santos, S. A., Rodrigues, J. M., & Silva, C. L. (2004). Vaccine adjuvant: it makes the difference. *Vaccine*, *22*(19), 2374–2379.

Loo, V. G., Bourgault, A. M., Poirier, L., Lamothe, F., Michaud, S., Turgeon, N., … Dascal, A. (2011). Host and Pathogen Factors for *Clostridium difficile* Infection and Colonization. *New England Journal of Medicine*, *365*(18), 1693-703.

Louie, T. J., Miller, M. A., Mullane, K. M., Weiss, K., Lentnek, A., Golan, Y., … Shue, Y.-K. (2011). Fidaxomicin versus Vancomycin for *Clostridium difficile* Infection. *New England Journal of Medicine*, *364*(5), 422–431.

Lusk, R. H., Fekety, F. R., Silva, J., Bodendorfer, T., Devine, B. J., Kawanishi, H., … Siskin, S. B. (1977). Gastrointestinal Side Effects of Clindamycin and Ampicillin Therapy. *Journal of Infectious Diseases*, *135*(Supplement), S111–S119.

Lyras, D., O'Connor, J. R., Howarth, P. M., Sambol, S. P., Carter, G. P., Phumoonna, T., … Rood, J. I. (2009). Toxin B is essential for virulence of *Clostridium difficile*. *Nature*, *458*(7242), 1176–9.

Mantis, N., Rol, N., & Corthésy, B. (2011). Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal immunology,4*(6), 603-11.

Mauriello, E. M. F., Cangiano, G., Maurano, F., Saggese, V., De Felice, M., Rossi, M., & Ricca, E. (2007). Germination-independent induction of cellular immune response by *Bacillus subtilis* spores displaying the C fragment of the tetanus toxin. *Vaccine*, *25*(5), 788–93.

Mauriello, E. M. F., Duc, L. H., Isticato, R., Cangiano, G., Hong, H. A., Felice, M. De, … Cutting, S. M. (2004). Display of heterologous antigens on the *Bacillus subtilis* spore coat using CotC as a fusion partner. *Vaccine*, *22*(9), 1177–1187.

McFarland, L. (2002). Breaking the Cycle: Treatment Strategies for 163 Cases of Recurrent *Clostridium difficile* Disease. *American Journal of Gastroenterology*, *97*(7), 1796–75.

McFarland, L. V. (2006). Meta-analysis of probiotics for the prevention of antibiotic associated diarrhea and the treatment of *Clostridium difficile* disease. *The American Journal of Gastroenterology*, *101*(4), 812–22.

McFarland, L. V. (2009). Evidence-based review of probiotics for antibiotic-associated diarrhea and *Clostridium difficile* infections. *Anaerobe*, *15*(6), 274–80.

McKenney, P. T., Driks, A., Eskandarian, H. A., Grabowski, P., Guberman, J., Wang, K. H., … Eichenberger, P. (2010). A distance-weighted interaction map reveals a previously uncharacterized layer of the *Bacillus subtilis* spore coat. *Current Biology*, *20*(10), 934–8.

Merrigan, M., Venugopal, A., Mallozzi, M., Roxas, B., Viswanathan, V. K., Johnson, S., … Vedantam, G. (2010). Human hypervirulent *Clostridium difficile* strains exhibit increased sporulation as well as robust toxin production. *Journal of Bacteriology*, *192*(19), 4904–11.

Miller, M. (2009). The fascination with probiotics for *Clostridium difficile* infection: Lack of evidence for prophylactic or therapeutic efficacy. *Anaerobe*, *15*(6), 281–284.

Moingeon, P., Batard, T., Fadel, R., Frati, F., Sieber, J., & Van Overtvelt, L. (2006). Immune mechanisms of allergen-specific sublingual immunotherapy. *Allergy*, *61*(2), 151–65.

Muñoz, P., Bouza, E., Cuenca-Estrella, M., Eiros, J. M., Pérez, M. J., Sánchez-Somolinos, M., … Peláez, T. (2005). *Saccharomyces cerevisiae* fungemia: an emerging infectious disease. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *40*(11), 1625–34.

Mutsch, M., Zhou, W., Rhodes, P., Bopp, M., Chen, R. T., Linder, T., … Steffen, R. (2004). Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. *The New England Journal of Medicine*, *350*(9), 896–903.

Nagórska, K., Bikowski, M., & Obuchowski, M. (2007). Multicellular behaviour and production of a wide variety of toxic substances support usage of *Bacillus subtilis* as a powerful biocontrol agent. *Acta Biochimica Polonica*, *54*(3), 495–508.

Neu, H. C. (1992). The Crisis in Antibiotic Resistance. *Science*, *257*(5073), 1064–1073.

Neutra, M. R., & Kozlowski, P. A. (2006). Mucosal vaccines: the promise and the challenge. *Nature Reviews. Immunology*, *6*(2), 148–58.

Nguyen, V. A. T., Huynh, H. A., Hoang, T. Van, Ninh, N. T., Pham, A. T. H., Nguyen, H. A., … Cutting, S. M. (2013). Killed *Bacillus subtilis* spores expressing streptavidin: a novel carrier of drugs to target cancer cells. *Journal of Drug Targeting*, *21*(6), 528–41.

Nicholson, W. L., Munakata, N., Horneck, G., Melosh, H. J., & Setlow, P. (2000). Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiology and Molecular Biology Reviews*, *64*(3), 548–72.

Noirey, N., Rougier, N., André, C., Schmitt, D., & Vincent, C. (2000). Langerhans-like dendritic cells generated from cord blood progenitors internalize pollen allergens by macropinocytosis, and part of the molecules are processed and can activate autologous naive T lymphocytes. *Journal of Allergy and Clinical Immunology*, *105*(6), 1194–1201.

O'Brien, J. B., McCabe, M. S., Athié-Morales, V., McDonald, G. S. A., Ní Eidhin, D. B., & Kelleher, D. P. (2005). Passive immunisation of hamsters against *Clostridium difficile* infection using antibodies to surface layer proteins. *FEMS Microbiology Letters*, *246*(2), 199–205.

O'Hara, A. M., & Shanahan, F. (2006). The gut flora as a forgotten organ. *EMBO Reports*, *7*(7), 688–93.

Oggioni, M. R., Pozzi, G., Valensin, P. E., Galieni, P., & Bigazzi, C. (1998). Recurrent Septicemia in an Immunocompromised Patient Due to Probiotic Strains of *Bacillus subtilis*. *J. Clin. Microbiol.*, *36*(1), 325–326.

Ogra, P. L., Faden, H., & Welliver, R. C. (2001). Vaccination strategies for mucosal immune responses. *Clinical Microbiology Reviews*, *14*(2), 430–45.

Ongena, M., & Jacques, P. (2008). *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. *Trends in Microbiology*, *16*(3), 115–25.

ONS. (2013). *Deaths Involving Clostridium difficile, England and Wales, 2012*. Retrived December 1, 2014, from http://www.ons.gov.uk/ons/rel/subnational-health2/deaths-involving-clostridium-difficile/2012/stb-deaths-involving-clostridium-difficile-2012.html

Ozaki, E., Kato, H., Kita, H., Karasawa, T., Maegawa, T., Koino, Y., … Nakamura, S. (2004). *Clostridium difficile* colonization in healthy adults: transient colonization and correlation with enterococcal colonization. *Journal of Medical Microbiology*, *53*(2), 167–172.

Pandey, A., & Palni, L. M. S. (1997). *Bacillus* species: The dominant bacteria of the rhizosphere of established tea bushes. *Microbiological Research*, *152*(4), 359–365.

Paredes, C. J., Alsaker, K. V, & Papoutsakis, E. T. (2005). A comparative genomic view of clostridial sporulation and physiology. *Nature Reviews. Microbiology*, *3*(12), 969–78.

Paredes-Sabja, D., & Sarker, M. R. (2012). Adherence of *Clostridium difficile* spores to Caco-2 cells in culture. *Journal of Medical Microbiology*, *61*(Pt 9), 1208–18.

Péchiné, S., Gleizes, A., Janoir, C., Gorges-Kergot, R., Barc, M.-C., Delmée, M., & Collignon, A. (2005). Immunological properties of surface proteins of *Clostridium difficile*. *Journal of Medical Microbiology*, *54*(Pt 2), 193–6.

Pechine, S., Janoir, C., Boureau, H., Gleizes, A., Tsaspis, N., Hoys, S., … Collignon, A. (2007). Diminished intestinal colonization by *Clostridium difficile* and immune response in mice after mucosal immunization with surface proteins of *Clostridium difficile*. *Vaccine*, *25*(20), 3946–3954.

Pépin, J., Valiquette, L., Alary, M.-E., Villemure, P., Pelletier, A., Forget, K., … Chouinard, D. (2004). *Clostridium difficile*-associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. *Canadian Medical Association Journal*, *171*(5), 466–72.

Permpoonpattana, P., Hong, H. A., Phetcharaburanin, J., Huang, J.-M., Cook, J., Fairweather, N. F., & Cutting, S. M. (2011a). Immunization with *Bacillus* spores expressing toxin A peptide repeats protects against infection with *Clostridium difficile* strains producing toxins A and B. *Infection and Immunity*, *79*(6), 2295–302.

Permpoonpattana, P., Tolls, E. H., Nadem, R., Tan, S., Brisson, A., & Cutting, S. M. (2011b). Surface layers of *Clostridium difficile* endospores. *Journal of Bacteriology*, *193*(23), 6461–70.

Permpoonpattana, P., Hong, H. A., Khaneja, R., & Cutting, S. M. (2012). Evaluation of *Bacillus subtilis* strains as probiotics and their potential as a food ingredient. *Beneficial Microbes*, *3*(2), 127–35.

Permpoonpattana, P., Phetcharaburanin, J., Mikelsone, A., Dembek, M., Tan, S., Brisson, M.-C., … Cutting, S. M. (2013). Functional characterization of *Clostridium difficile* spore coat proteins. *Journal of Bacteriology*, *195*(7), 1492–503.

Petit, L., Gibert, M., & Popoff, M. R. (1999). *Clostridium perfringens*: toxinotype and genotype. *Trends in Microbiology*, *7*(3), 104–110.

Petrof, E. O., Gloor, G. B., Vanner, S. J., Weese, S. J., Carter, D., Daigneault, M. C., … Allen-Vercoe, E. (2013). Stool substitute transplant therapy for the eradication of *Clostridium difficile* infection: "RePOOPulating" the gut. *Microbiome*, *1*(1), 3.

Pils, M. C., Bleich, A., Prinz, I., Fasnacht, N., Bollati-Fogolin, M., Schippers, A., … Müller, W. (2011). Commensal gut flora reduces susceptibility to experimentally induced colitis via T-cell-derived interleukin-10. *Inflammatory Bowel Diseases*, *17*(10), 2038–46.

Pizarro-Guajardo, M., Olguín-Araneda, V., Barra-Carrasco, J., Brito-Silva, C., Sarker, M. R., & Paredes-Sabja, D. (2013). Characterization of the collagen-like exosporium protein, BclA1, of *Clostridium difficile* spores. *Anaerobe*, *25,* 18-30.

Pochapin, M. (2000). The effect of probiotics on *Clostridium difficile* diarrhea. *The American Journal of Gastroenterology*, *95*(1 Suppl), S11–3.

Popham, D. L. (2002). Specialized peptidoglycan of the bacterial endospore: the inner wall of the lockbox. *Cellular and Molecular Life Sciences*, *59*(3), 426–33.

Popoff, M. R., Rubin, E. J., Gill, D. M., & Boquet, P. (1988). Actin-specific ADP-ribosyltransferase produced by a *Clostridium difficile* strain. *Infection and Immunity*, *56*(9), 2299–306.

Pothoulakis, C., & Lamont, J. T. (2001). Microbes and microbial toxins: paradigms for microbial-mucosal interactions II. The integrated response of the intestine to *Clostridium difficile* toxins. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *280*(2), G178–83.

Power, E. G. M., & Russell, A. D. (1990). Sporicidal action of alkaline glutaraldehyde: factors influencing activity and a comparison with other aldehydes. *Journal of Applied Bacteriology*, *69*(2), 261–268.

Pozzoni, P., Riva, A., Bellatorre, A. G., Amigoni, M., Redaelli, E., Ronchetti, A., … Colli, A. (2012). *Saccharomyces boulardii* for the prevention of antibiotic-associated diarrhea in adult hospitalized patients: a single-center, randomized, double-blind, placebo-controlled trial. *The American Journal of Gastroenterology*, *107*(6), 922–31.

Public Health England. (2013). *Summary Points on Clostridium difficile infection (CDI)*. Retrived September 2013,from http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1278944283388

Qureshi, N., & Blaschek, H. (2001). Recent advances in ABE fermentation: hyper-butanol producing *Clostridium beijerinckii* BA101. *Journal of Industrial Microbiology and Biotechnology*, *27*(5), 287–291.

Ramsay, A., Husband, A., Ramshaw, I., Bao, S., Matthaei, K., Koehler, G., & Kopf, M. (1994). The role of interleukin-6 in mucosal IgA antibody responses in vivo. *Science*, *264*(5158), 561–563.

Rappuoli, R. (1994). Toxin inactivation and antigen stabilization: two different uses of formaldehyde. *Vaccine*, *12*(7), 579–581.

Razaq, N., Sambol, S., Nagaro, K., Zukowski, W., Cheknis, A., Johnson, S., & Gerding, D. N. (2007). Infection of hamsters with historical and epidemic BI types of *Clostridium difficile*. *The Journal of Infectious Diseases*, *196*(12), 1813–9.

Read, T. D., Peterson, S. N., Tourasse, N., Baillie, L. W., Paulsen, I. T., Nelson, K. E., … Fraser, C. M. (2003). The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria, *423*(6935), 81–86.

Redmond, C., Baillie, L. W. J., Hibbs, S., Moir, A. J. G., & Moir, A. (2004). Identification of proteins in the exosporium of *Bacillus anthracis*. *Microbiology*, *150*(2), 355–363.

Rey, M. W., Ramaiya, P., Nelson, B. A., Brody-Karpin, S. D., Zaretsky, E. J., Tang, M., … Berka, R. M. (2004). Complete genome sequence of the industrial bacterium *Bacillus licheniformis* and comparisons with closely related *Bacillus species*. *Genome Biology*, *5*(10), R77.

Rhee, J. H., Lee, S. E., & Kim, S. Y. (2012). Mucosal vaccine adjuvants update. *Clinical and Experimental Vaccine Research*, *1*(1), 50–63.

Riggs, M. M., Sethi, A. K., Zabarsky, T. F., Eckstein, E. C., Jump, R. L. P., & Donskey, C. J. (2007). Asymptomatic carriers are a potential source for transmission of epidemic and nonepidemic *Clostridium difficile* strains among long-term care facility residents. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *45*(8), 992–8.

Rodriguez-Palacios, A., Stämpfli, H. R., Duffield, T., Peregrine, A. S., Trotz-Williams, L. A., Arroyo, L. G., … Weese, J. S. (2006). *Clostridium difficile* PCR ribotypes in calves, Canada. *Emerging Infectious Diseases*, *12*(11), 1730–6. *doi:10.3201/eid1211.051581*

Rohlke, F. B., & Surawicz, C. (2010). Fecal Flora Reconstitution for Recurrent *Clostridium difficile* Infection: Results and Methodology. *Journal of Clinical Gastroenterology*.

Round, J. L., & Mazmanian, S. K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. *Nature Reviews. Immunology*, *9*(5), 313–23.

Rowland, I., Capurso, L., Collins, K., Cummings, J., Delzenne, N., Goulet, O., … Meier, R. (2010). Current level of consensus on probiotic science: report of an expert meeting. London, 23 November 2009. *Gut Microbes*, *1*(6), 436–9.

Rupnik, M. (2010). *Clostridium difficile* toxinotyping. *Methods in Molecular Biology (Clifton, N.J.)*, *646*, 67–76.

Rupnik, M., Avesani, V., Janc, M., von Eichel-Streiber, C., & Delmee, M. (1998). A Novel Toxinotyping Scheme and Correlation of Toxinotypes with Serogroups of *Clostridium difficile* Isolates. *J. Clin. Microbiol.*, *36*(8), 2240–2247.

Rupnik, M., Wilcox, M. H., & Gerding, D. N. (2009). *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. *Nature Reviews. Microbiology*, *7*(7), 526–36.

Russell, A. D. (1990). Bacterial spores and chemical sporicidal agents. *Clinical Microbiology Reviews*, *3*(2), 99–119.

Sabin, A. B. (1985). Oral Poliovirus Vaccine: History of Its Development and Use and Current Challenge to Eliminate Poliomyelitis from the World. *Journal of Infectious Diseases*, *151*(3), 420–436.

Salcedo, J., Keates, S., Pothoulakis, C., Warny, M., Castagliuolo, I., LaMont, J. T., & Kelly, C. P. (1997). Intravenous immunoglobulin therapy for severe *Clostridium difficile* colitis. *Gut*, *41*(3), 366–370.

Salminen, S., Gibson, G. R., McCartney, A. L., & Isolauri, E. (2004). Influence of mode of delivery on gut microbiota composition in seven year old children. *Gut*, *53*(9), 1388–9.

Sambol, S. P., Tang, J. K., Merrigan, M. M., Johnson, S., & Gerding, D. N. (2001). Infection of hamsters with epidemiologically important strains of *Clostridium difficile*. *The Journal of Infectious Diseases*, *183*(12), 1760–6.

Sanders, M. E., & Marco, M. L. (2010). Food formats for effective delivery of probiotics. *Annual Review of Food Science and Technology*, *1*, 65–85.

Saulnier, D. M., Riehle, K., Mistretta, T.-A., Diaz, M.-A., Mandal, D., Raza, S., … Versalovic, J. (2011). Gastrointestinal microbiome signatures of pediatric patients with irritable bowel syndrome. *Gastroenterology*, *141*(5), 1782–91.

Savage, D. C. (1977). Microbial ecology of the gastrointestinal tract. *Annual Review of Microbiology*, *31*, 107–33.

Saxelin, M. (2008). Probiotic formulations and applications, the current probiotics market, and changes in the marketplace: a European perspective. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, *46 Suppl*(Supplement 2), S76–9.

Saxton, K., Baines, S. D., Freeman, J., O'Connor, R., & Wilcox, M. H. (2009). Effects of exposure of *Clostridium difficile* PCR ribotypes 027 and 001 to fluoroquinolones in a human gut model. *Antimicrobial Agents and Chemotherapy*, *53*(2), 412–20.

Schallmey, M., Singh, A., & Ward, O. P. (2004). Developments in the use of *Bacillus* species for industrial production. *Canadian Journal of Microbiology*, *50*(1), 1–17.

Schiffrin, E. J., & Blum, S. (2002). Interactions between the microbiota and the intestinal mucosa. *European Journal of Clinical Nutrition*, *56* (Supplement 3), S60–4.

Schwan, C., Stecher, B., Tzivelekidis, T., van Ham, M., Rohde, M., Hardt, W.-D., … Aktories, K. (2009). *Clostridium difficile* toxin CDT induces formation of microtubule-based protrusions and increases adherence of bacteria. *PLoS Pathogens*, *5*(10), e1000626.

Schyns, G., Serra, C. R., Lapointe, T., Pereira-Leal, J. B., Potot, S., Fickers, P., … Henriques, A. O. (2013). Genome of a Gut Strain of *Bacillus subtilis*. *Genome Announcements*, *1*(1), e00184–12.

Sebaihia, M., Wren, B. W., Mullany, P., Fairweather, N. F., Minton, N., Stabler, R., … Parkhill, J. (2006). The multidrug-resistant human pathogen *Clostridium difficile* has a highly mobile, mosaic genome. *Nature Genetics*, *38*(7), 779–86.

Setlow, P. (2006). Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *Journal of Applied Microbiology*, *101*(3), 514–25.

Smith, L. D., & King, E. O. (1962). Occurence of *Clostridium difficile* infections in man. *J. Bacteriol.*, *84*(1), 65–67.

Sneath, P. H. (1962). Longevity of micro-organisms. *Nature*, *195*, 643–6.

Song, J.-H., Kim, J.-I., Kwon, H.-J., Shim, D.-H., Parajuli, N., Cuburu, N., … Kweon, M.-N. (2009). CCR7-CCL19/CCL21-regulated dendritic cells are responsible for effectiveness of sublingual vaccination. *Journal of Immunology*, *182*(11), 6851–60.

Song, J.-H., Nguyen, H. H., Cuburu, N., Horimoto, T., Ko, S.-Y., Park, S.-H., … Kweon, M.-N. (2008a). Sublingual vaccination with influenza virus protects mice against lethal viral infection. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(5), 1644–9.

Song, J.-H., Nguyen, H. H., Cuburu, N., Horimoto, T., Ko, S.-Y., Park, S.-H., … Kweon, M.-N. (2008b). Sublingual vaccination with influenza virus protects mice against lethal viral infection. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(5), 1644–9.

Song, M., Hong, H. A., Huang, J.-M., Colenutt, C., Khang, D. D., Nguyen, T. V. A., … Cutting, S. M. (2012). Killed *Bacillus subtilis* spores as a mucosal adjuvant for an H5N1 vaccine. *Vaccine*, *30*(22), 3266–77.

Songer, J. G. (2010). Clostridia as agents of zoonotic disease. *Veterinary Microbiology*, *140*(3), 399–404.

Songer, J. G., & Anderson, M. A. (2006). *Clostridium difficile*: An important pathogen of food animals. *Anaerobe*, *12*(1), 1–4.

Songer, J. G., & Uzal, F. A. (2005). *Clostridial* Enteric Infections in Pigs. *Journal of Veterinary Diagnostic Investigation*, *17*(6), 528–536.

Sorg, J. A., & Sonenshein, A. L. (2008). Bile salts and glycine as cogerminants for *Clostridium difficile* spores. *Journal of Bacteriology*, *190*(7), 2505–12.

Sorg, J. A., & Sonenshein, A. L. (2009). Chenodeoxycholate is an inhibitor of *Clostridium difficile* spore germination. *Journal of Bacteriology*, *191*(3), 1115–7.

Sougioultzis, S., Kyne, L., Drudy, D., Keates, S., Maroo, S., Pothoulakis, C., … Kelly, C. P. (2005). *Clostridium difficile* toxoid vaccine in recurrent *C. difficile*-associated diarrhea. *Gastroenterology*, *128*(3), 764–770.

Spigaglia, P., & Mastrantonio, P. (2002). Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (TcdC) among *Clostridium difficile* clinical isolates. *Journal of Clinical Microbiology*, *40*(9), 3470–5.

Stabler, R. A., Gerding, D. N., Songer, J. G., Drudy, D., Brazier, J. S., Trinh, H. T., … Wren, B. W. (2006). Comparative phylogenomics of *Clostridium difficile* reveals clade specificity and microevolution of hypervirulent strains. *Journal of Bacteriology*, *188*(20), 7297–305.

Stabler, R. A., He, M., Dawson, L., Martin, M., Valiente, E., Corton, C., … Wren, B. W. (2009). Comparative genome and phenotypic analysis of *Clostridium difficile* 027 strains provides insight into the evolution of a hypervirulent bacterium. *Genome Biology*, *10*(9), R102.

Steichen, C., Chen, P., Kearney, J. F., & Turnbough, C. L. (2003). Identification of the immunodominant protein and other proteins of the *Bacillus anthracis* exosporium. *Journal of Bacteriology*, *185*(6), 1903–10.

Steidler, L., Hans, W., Schotte, L., Neirynck, S., Obermeier, F., Falk, W., Fiers, W. & Remaut, E. (2000) Treatment of murine colitis by *Lactococcus lactis* secreting Interleukin-10. *Science*, *289* (5483), 1352-1355.

Strachan, D. P. (1989). Hay fever, hygiene, and household size. *British Medical Journal*, *299*(6710), 1259–60.

Stubbe, H., Berdoz, J., Kraehenbuhl, J.-P., & Corthesy, B. (2000). Polymeric IgA Is Superior to Monomeric IgA and IgG Carrying the Same Variable Domain in Preventing *Clostridium difficile* Toxin A Damaging of T84 Monolayers. *The Journal of Immunology*, *164*(4), 1952–1960.

Surawicz, C. M., Elmer, G. W., Speelman, P., McFarland, L. V, Chinn, J., & van Belle, G. (1989). Prevention of antibiotic-associated diarrhea by *Saccharomyces boulardii:* a prospective study. *Gastroenterology*, *96*(4), 981–8.

Surawicz, C. M., McFarland, L. V, Greenberg, R. N., Rubin, M., Fekety, R., Mulligan, M. E., … Elmer, G. W. (2000). The search for a better treatment for recurrent *Clostridium difficile* disease: use of high-dose vancomycin combined with *Saccharomyces boulardii*. *Clinical Infectious Diseases*, *31*(4), 1012–7.

Swiecki, M. K., Lisanby, M. W., Shu, F., Turnbough, C. L., & Kearney, J. F. (2006). Monoclonal antibodies for *Bacillus anthracis* spore detection and functional analyses of spore germination and outgrowth. *Journal of Immunology*, *176*(10), 6076–84.

Sylvestre, P., Couture-Tosi, E., & Mock, M. (2002). A collagen-like surface glycoprotein is a structural component of the *Bacillus anthracis* exosporium. *Molecular Microbiology*, *45*(1), 169–78.

Sylvestre, P., Couture-Tosi, E., & Mock, M. (2003). Polymorphism in the collagen-like region of the *Bacillus anthracis* BclA protein leads to variation in exosporium filament length. *Journal of Bacteriology*, *185*(5), 1555–63.

Takami, H., Nakasone, K., Takaki, Y., Maeno, G., Sasaki, R., Masui, N., … Horikoshi, K. (2000). Complete genome sequence of the alkaliphilic bacterium *Bacillus halodurans* and genomic sequence comparison with *Bacillus subtilis*. *Nucleic Acids Research*, *28*(21), 4317–31.

Tan, L., Li, M., & Turnbough, C. L. (2011). An unusual mechanism of isopeptide bond formation attaches the collagenlike glycoprotein BclA to the exosporium of *Bacillus anthracis*. *mBio*, *2*(3), e00084–11.

Tedesco, F. J., Barton, R., & Alpers, D. H. (1974). Clindamycin-Associated Colitis: A Prospective Study. *Annals of Internal Medicine*, *81*(4), 429.

Tennen, R., Setlow, B., Davis, K. L., Loshon, C. A., & Setlow, P. (2000). Mechanisms of killing of spores of *Bacillus subtilis* by iodine, glutaraldehyde and nitrous acid. *Journal of Applied Microbiology*, *89*(2), 330–338.

Theriot, C. M., Koumpouras, C. C., Carlson, P. E., Bergin, I. I., Aronoff, D. M., & Young, V. B. (2011). Cefoperazone-treated mice as an experimental platform to assess differential virulence of *Clostridium difficile* strains. *Gut Microbes*, *2*(6), 326–34.

Thompson, B. M., Binkley, J. M., & Stewart, G. C. (2011). Current physical and SDS extraction methods do not efficiently remove exosporium proteins from *Bacillus anthracis* spores. *Journal of Microbiological Methods*, *85*(2), 143–8.

Thompson, B. M., Hsieh, H.-Y., Spreng, K. A., & Stewart, G. C. (2011). The co-dependence of BxpB/ExsFA and BclA for proper incorporation into the exosporium of *Bacillus anthracis. Molecular Microbiology*, *79*(3), 799–813.

Thompson, B. M., & Stewart, G. C. (2008). Targeting of the BclA and BclB proteins to the *Bacillus anthracis* spore surface. *Molecular Microbiology*, *70*(2), 421–34.

Tian, J.-H., Fuhrmann, S. R., Kluepfel-Stahl, S., Carman, R. J., Ellingsworth, L., & Flyer, D. C. (2012). A novel fusion protein containing the receptor binding domains of *Clostridium difficile* toxin A and toxin B elicits protective immunity against lethal toxin and spore challenge in preclinical efficacy models. *Vaccine*, *30*(28), 4249–58.

Todd, S. J., Moir, A. J. G., Johnson, M. J., & Moir, A. (2003). Genes of *Bacillus cereus* and *Bacillus anthracis* encoding proteins of the exosporium. *Journal of Bacteriology*, *185*(11), 3373–8.

Torres, J. F., Lyerly, D. M., Hill, J. E., & Monath, T. P. (1995). Evaluation of formalin-inactivated *Clostridium difficile* vaccines administered by parenteral and mucosal routes of immunization in hamsters. *Infection and Immunity*, *63*(12), 4619–27.

Tu, J., Zhang, G., Datta, K., Xu, C., He, Y., Zhang, Q., … Datta, S. K. (2000). Field performance of transgenic elite commercial hybrid rice expressing *Bacillus thuringiensis* delta-endotoxin. *Nature Biotechnology*, *18*(10), 1101–4.

Tucker, K. D., & Wilkins, T. D. (1991). Toxin A of *Clostridium difficile* binds to the human carbohydrate antigens I, X, and Y. *Infection and Immunity*, *59*(1), 73–8.

Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley, R. E., … Gordon, J. I. (2009). A core gut microbiome in obese and lean twins. *Nature*, *457*(7228), 480–4.

Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R., & Gordon, J. I. (2007). The human microbiome project. *Nature*, *449*(7164), 804–10.

Underwood, S., Guan, S., Vijayasubhash, V., Baines, S. D., Graham, L., Lewis, R. J., … Stephenson, K. (2009). Characterization of the sporulation initiation pathway of *Clostridium difficile* and its role in toxin production. *Journal of Bacteriology*, *191*(23), 7296–305.

Valiente, E., Dawson, L. F., Cairns, M. D., Stabler, R. A., & Wren, B. W. (2012). Emergence of new PCR ribotypes from the hypervirulent *Clostridium difficile* 027 lineage. *Journal of Medical Microbiology*, *61*(Pt 1), 49–56.

Van Ginkel, F. W., Jackson, R. J., Yoshino, N., Hagiwara, Y., Metzger, D. J., Connell, T. D., … McGhee, J. R. (2005). Enterotoxin-based mucosal adjuvants alter antigen trafficking and induce inflammatory responses in the nasal tract. *Infection and Immunity*, *73*(10), 6892–902.

Van Ginkel, F. W., Jackson, R. J., Yuki, Y., & McGhee, J. R. (2000). Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. *Journal of Immunology*, *165*(9), 4778–82.

Van Nood, E., Vrieze, A., Nieuwdorp, M., Fuentes, S., Zoetendal, E. G., de Vos, W. M., … Keller, J. J. (2013). Duodenal infusion of donor feces for recurrent *Clostridium difficile. The New England Journal of Medicine*, *368*(5), 407–15.

Ventola, H., Lehtoranta, L., Madetoja, M., Simonen-Tikka, M.-L., Maunula, L., Roivainen, M., … Holma, R. (2012). Effects of the viability of *Lactobacillus rhamnosus* GG on rotavirus infection in neonatal rats. *World Journal of Gastroenterology*, *18*(41), 5925–31.

Viscidi, R., Laughon, B. E., Yolken, R., Bo-Linn, P., Moench, T., Ryder, R. W., & Bartlett, J. G. (1983). Serum antibody response to toxins A and B of *Clostridium difficile*. *The Journal of Infectious Diseases*, *148*(1), 93–100.

Vohra, P., & Poxton, I. R. (2011). Comparison of toxin and spore production in clinically relevant strains of *Clostridium difficile*. *Microbiology (Reading, England)*, *157*(Pt 5), 1343–53.

Von Eichel-Streiber, C., Boquet, P., Sauerborn, M., & Thelestam, M. (1996). Large clostridial cytotoxins — a family of glycosyltransferases modifying small GTP-binding proteins. *Trends in Microbiology*, *4*(10), 375–382.

Vonberg, R.-P., Kuijper, E. J., Wilcox, M. H., Barbut, F., Tüll, P., Gastmeier, P., … Wiuff, C. (2008). Infection control measures to limit the spread of *Clostridium difficile*. *Clinical Microbiology and Infection : The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *14* (Supplement 5), 2–20.

Voth, D. E., & Ballard, J. D. (2005). *Clostridium difficile* toxins: mechanism of action and role in disease. *Clinical Microbiology Reviews*, *18*(2), 247–63.

Walker, A. S., Eyre, D. W., Wyllie, D. H., Dingle, K. E., Harding, R. M., O'Connor, L., … Peto, T. E. A. (2012). Characterisation of *Clostridium difficile* hospital ward-based transmission using extensive epidemiological data and molecular typing. *PLoS Medicine*, *9*(2), e1001172.

Waller, L. N., Stump, M. J., Fox, K. F., Harley, W. M., Fox, A., Stewart, G. C., & Shahgholi, M. (2005). Identification of a second collagen-like glycoprotein produced by *Bacillus anthracis* and demonstration of associated spore-specific sugars. *Journal of Bacteriology*, *187*(13), 4592–7.

Wang, H., Sun, X., Zhang, Y., Li, S., Chen, K., Shi, L., … Feng, H. (2012). A chimeric toxin vaccine protects against primary and recurrent *Clostridium difficile* infection. *Infection and Immunity*, *80*(8), 2678–88.

Warny, M., Pepin, J., Fang, A., Killgore, G., Thompson, A., Brazier, J., … McDonald, L. C. (2005). Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. *Lancet*, *366*(9491), 1079–84.

Weiner, H. L., Friedman, A., Miller, A., Khoury, S. J., Al-Sabbagh, A., Santos, L., … Hafler, D. A. (1994). Oral tolerance: immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. *Annual Review of Immunology*, *12*, 809–37.

Weiss, K. (2009). Toxin-binding treatment for *Clostridium difficile*: a review including reports of studies with tolevamer. *International Journal of Antimicrobial Agents*, *33*(1), 4–7.

Wilcox, M. H. (2004). Descriptive study of intravenous immunoglobulin for the treatment of recurrent *Clostridium difficile* diarrhoea. *The Journal of Antimicrobial Chemotherapy*, *53*(5), 882–4.

Wilcox, M. H., Mooney, L., Bendall, R., Settle, C. D., & Fawley, W. N. (2008). A case-control study of community-associated *Clostridium difficile* infection. *The Journal of Antimicrobial Chemotherapy*, *62*(2), 388–96.

Wolff, D., Brüning, T., & Gerritzen, A. (2009). Rapid detection of the *Clostridium difficile* ribotype 027 tcdC gene frame shift mutation at position 117 by real-time PCR and melt curve analysis. *European Journal of Clinical Microbiology & Infectious Diseases : Official Publication of the European Society of Clinical Microbiology*, *28*(8), 959–62.

Woodmansey, E. J., McMurdo, M. E. T., Macfarlane, G. T., & Macfarlane, S. (2004). Comparison of compositions and metabolic activities of fecal microbiotas in young adults and in antibiotic-treated and non-antibiotic-treated elderly subjects. *Applied and Environmental Microbiology*, *70*(10), 6113–22.

Woodrow, K. A., Bennett, K. M., & Lo, D. D. (2012). Mucosal vaccine design and delivery. *Annual Review of Biomedical Engineering*, *14*, 17–46.

Wright, A., Drudy, D., Kyne, L., Brown, K., & Fairweather, N. F. (2008). Immunoreactive cell wall proteins of *Clostridium difficile* identified by human sera. *Journal of Medical Microbiology*, *57*(Pt 6), 750–6.

Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y.-Y., Keilbaugh, S. A., … Lewis, J. D. (2011). Linking long-term dietary patterns with gut microbial enterotypes. *Science (New York, N.Y.)*, *334*(6052), 105–8.

Wullt, M., Hagslätt, M.-L. J., & Odenholt, I. (2003). *Lactobacillus plantarum* 299v for the treatment of recurrent *Clostridium difficile*-associated diarrhoea: a double-blind, placebo-controlled trial. *Scandinavian Journal of Infectious Diseases*, *35*(6-7), 365–7.

Xiao, S. D., Zhang, D. Z., Lu, H., Jiang, S. H., Liu, H. Y., Wang, G. S., … Wang, G. L. (2002). Multicenter randomized controlled trial of heat-killed *Lactobacillus acidophilus* LB in patients with chronic diarrhea. *Chinese Journal of Digestive Diseases*, *3*(4), 167–171.

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., … Gordon, J. I. (2012). Human gut microbiome viewed across age and geography. *Nature*, *486*(7402), 222–7.

Zhang, H., Zhang, J., & Streisand, J. B. (2002). Oral mucosal drug delivery: clinical pharmacokinetics and therapeutic applications. *Clinical Pharmacokinetics*, *41*(9), 661–80.

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The spore-associated protein BclA1 affects the susceptibility of animals to colonization and infection by *Clostridium difficile*

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Summary

The BclA protein is a major component of the outermost layer of spores of a number of bacterial species and *Clostridium difficile* carries three *bclA* genes. Using insertional mutagenesis each gene was characterized and spores devoid of these proteins had surface aberrations, reduced hydrophobicity and germinated faster than wild-type spores. Therefore the BclA proteins were likely major components of the spore surface and when absent impaired the protective shield effect of this outermost layer. Analysis of infection and colonization in mice and hamsters revealed that the 50% infectious dose (ID_{50}) of spores was significantly higher (2-logs) in the *bclA1*$^-$ mutant compared to the isogenic wild-type control, but that levels of toxins (A and B) were indistinguishable from animals dosed with wild-type spores. *bclA1*$^-$ spores germinated faster than wild-type spores yet mice were less susceptible to infection suggesting that BclA1 must play a key role in the initial (i.e. pre-spore germination) stages of infection. We also show that the ID_{50} was higher in mice infected with R20291, a 'hypervirulent' 027 strain, that carries a truncated BclA1 protein.

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Introduction

Clostridium difficile is a leading cause of nosocomial antibiotic-associated diarrhoea in industrialized countries (Rupnik *et al.*, 2009). This spore forming bacterium is able to colonize the gastro-intestinal (GI) tract of infected patients and, during antibiotic therapy, the resulting disturbance to the natural gut microflora promotes germination of *C. difficile* spores, outgrowth and proliferation of live cells (Songer and Anderson, 2006) followed by shedding of large numbers of spores in the faeces (Lawley *et al.*, 2009a). Disease is caused mainly by the production of two toxins, A (TcdA) and B (TcdB), which leads to diarrhoea and in more severe cases, pseudomembrane colitis (Rupnik *et al.*, 2009). The spore of *C. difficile* is the dormant state of this organism and the primary agent of transmission (Gerding *et al.*, 2008). This has been supported by recent studies where a mutant strain of *C. difficile*, unable to produce the Spo0A protein (a transcriptional regulatory protein essential for the initiation of sporulation) fails to persist and transmit the disease (Deakin *et al.*, 2012). Interestingly, mice infected with *C. difficile* can exist in two physiological states, a carrier state, where low levels of *C. difficile* spores are shed in the faeces and a 'supershedder' state where large numbers of spores are shed (Lawley *et al.*, 2009a). This 'supershedder' state is induced following antibiotic treatment and most closely resembles the clinical situation where patients contract *C. difficile* infection. Capable of withstanding heat, desiccation and noxious chemicals, spores transmit *C. difficile* outside of the host and therefore present a major burden to hospitals in containment and disinfection (Gerding *et al.*, 2008).

Strains producing neither toxin are completely attenuated in the hamster model of infection (Kuehne *et al.*, 2010) yet non-toxigenic strains have been found to be endowed with vaccine-strain attributes. Although toxin A and toxin B are considered the two main virulence factors, others cannot be excluded; for example, it has been shown that hamsters challenged with spores of the non-toxigenic strain CD1342 showed mild caecal pathology

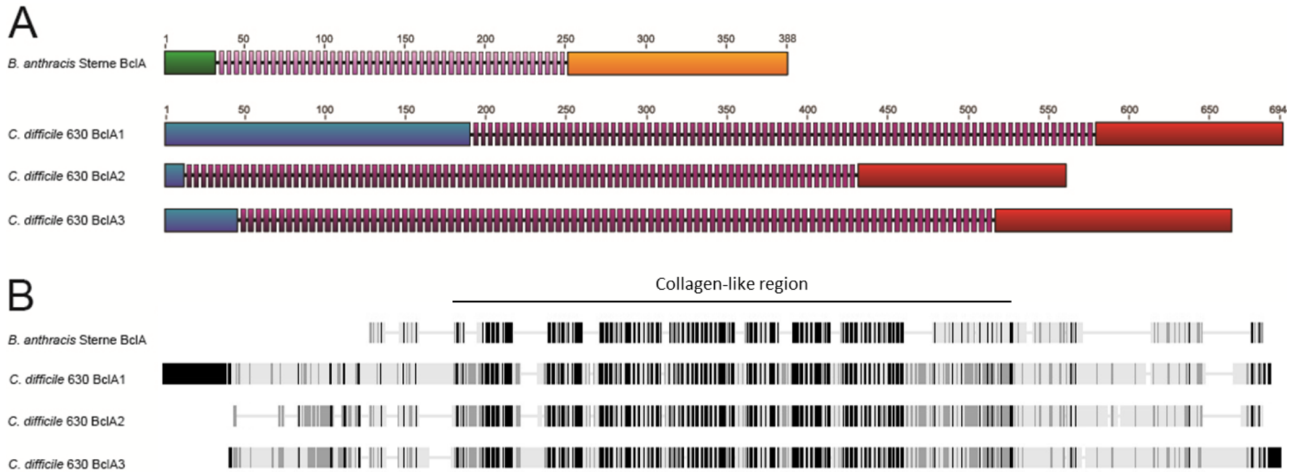

Fig. 1. A. Schematic representation of the domain structure of BclA proteins from *B. anthracis* Sterne (AY995120.1) and *C. difficile* 630 (BclA1, CAJ67154.1; BclA2, CAJ70128.1; BclA3, CAJ70248.1). The central, collagen-like region (purple) contains multiple GXX repeats and is flanked by the N-terminal (green/blue) and C-terminal (orange/red) domains. In *B. anthracis* the C-terminal domain mediates trimerization of the BclA monomers while the N-terminal domain is implicated in anchoring the proteins to the exosporial basal layer. The function of these domains in *C. difficile* remains to be confirmed.
B. Pair-wise alignment of the same BclA protein sequences from *B. anthracis* Sterne and *C. difficile* 630. Most sequence similarity is limited to the central, collagen-like region. Key: black, 100% similarity; dark grey, 80–100%; light grey, 60–80; white, < 60%. Pair-wise identity was generated using CLUSTALW and a Blosum62 scoring matrix and between all four proteins was 39.1%.

characterized by local acute epithelial cell loss, haemorrhagic congestion and neutrophil infiltration (Buckley *et al.*, 2013). Hamsters colonized with non-toxigenic strains, M3 and T7, were protected against challenge with toxigenic B1 group strains (Nagaro *et al.*, 2013), suggesting non-toxic strains can exclude toxigenic strains from colonization. However, the mechanism for how these non-toxigenic strains confer protection remains both intriguing and unclear.

The role of the spore in transmission of the disease suggests that this dormant life form may play a key role in colonization, a process better divided into three stages: establishment of infection, maintenance of infection (persistence) and spore shedding. Spores of *C. difficile* resemble those of other Gram-positive spore-formers but differ somewhat in the abundance of enzymes they carry on their surface layers including three catalases and a bifunctional peroxiredoxin-chitinase (Permpoonpattana *et al.*, 2011b; 2013). *C. difficile* spores also carry a poorly defined outer surface layer whose function has been linked to germination, adhesion and resistance properties of the spore (Henriques and Moran, 2007; Lawley *et al.*, 2009b; Escobar-Cortes *et al.*, 2013). This outermost layer of *C. difficile* spores has similarities to the exosporium of some spore formers but conflicting published data have delayed a definitive assignment. The BclA (bacillus collagen-like protein of anthracis) glycoprotein is a major component of the exosporium in some spore formers that can form hair-like filaments and carries collagen-like repeats of the amino-acid triplet GPT used for attachment to oligosaccharides (Sylvestre *et al.*, 2002; 2003; Steichen *et al.*, 2003). A second collagen-like protein, BclB has also been identified in *Bacillus anthracis* and has been linked to exosporium assembly (Waller *et al.*, 2005; Thompson and Stewart, 2008).

The genome of *C. difficile* 630 carries three *bclA* genes (*bclA1–3*) which, other than a recent paper (Pizarro-Guajardo *et al.*, 2014) showing that BclA1 is located on the spore surface, have not been characterized in any detail. To define gene function we have used insertional mutagenesis to disrupt each gene coupled with analysis of infectivity of mutants in two different animal models, mice and hamsters. We conclude first that BclA1 appears to be a critical factor in the initial stages of colonization, and second that truncation of BclA1 in some 027 strains may account in part for a reduced infectivity of these strains.

Results

The C. difficile *bclA genes*

Three genes encoding BclA-like proteins, annotated as *bclA1, bclA2* and *bclA3* are present on the genome of strain 630 (Fig. S1) and encode proteins with predicted masses of 67.8, 49.0 and 58.2 kDa respectively. Similar to the BclA proteins found in *B. anthracis* and *B. cereus*, all three *C. difficile* BclA proteins consist of an extensive, central, collagen-like region with multiple GXX repeats flanked by N- and C-terminal domains of variable length (Fig. 1A and B). Most of the triplet repeats are GPT with nearly all

Table 1. *C. difficile bclA* genes and mutations.

Gene	Locus tag[a]	Encoded protein[b]	Mutant allele[c]	Retargeted sequence[d]
bclA1	CD0332	Putative exosporium glycoprotein (83 kDa)	*bclA1::CT1050a*	ACTCCTGTCGCTCCTGTTGGACCTGTTGCT<intron>CCTGTTGGTCCTATA
bclA2	CD3230	Putative exosporium glycoprotein (67 kDa)	*bclA2::CT150a*	GCTCCATTTGCTCCTGTTGCTCCTGTCGCC<intron>CCTGTTGCTCCTGTC
bclA3	CD3349	Putative exosporium glycoprotein (79 kDa)	*bclA3::CT125s*	GTCGTGATGATTATAATAGCTGTGATTGC<intron>CATCATTGCTGTCCAC

a. As described in Sebaihia *et al*. (2006) and schematically in Fig. S1.
b. Predicted MW of full-length protein in brackets.
c. The mutant allele is shown with (a) CT designing ClosTron insertion, (b) the number showing the bp within the ORF immediately preceding the ClosTron insertion and (c) letter *a* indicating insertion in the antisense strand.
d. The 45 bp targeting sequence produced using the http://www.clostron.com algorithm and used for mutant construction. The intron insertion site within the 45-mer target sequence is shown.

containing a threonine residue, which could provide multiple potential sites for *O*-glycosylation as seen in *B. anthracis* (Daubenspeck *et al*., 2004) (Fig. S2). Bioinformatic comparison of BclA protein sequences from *B. anthracis, B. cereus* and *C. difficile* revealed that most of the similarity between the proteins is limited to the collagen-like central region since both the C- and N-terminal domains of the *C. difficile* BclA proteins seem to be distinct from those found in other species (Fig. S3). In *B. anthracis* BclA these terminal regions are implicated in trimerization of the BclA monomers and their attachment to the exosporial basal layer (Boydston *et al*., 2005; Thompson and Stewart, 2008). Notably, the *C. difficile* BclA proteins do not appear to carry at their N-termini a sequence resembling the motif (LIGPTLPPIPP) that targets the BclA and BclB proteins of *B. anthracis* to the exosporium (Tan and Turnbough, 2010; Thompson *et al*., 2011a,b).

Phenotypes of bclA mutant spores

ClosTron mutagenesis can be used to inactivate genes by using a group II intron to insert an erythromycin resistance allele within a target gene (Heap *et al*., 2009). Using this technique, the three *bclA* genes were inactivated in strain 630Δ*erm* creating the mutants *bclA1*$^-$*, bclA2*$^-$ *and bclA3*$^-$ (Table 1). In *bclA1* and *bclA2*, the erythromycin resistance cassette was inserted in the anti-sense direction, while in *bclA3*, it was in the sense direction. Mutants were examined for their sporulation and germination phenotypes in parallel with the isogenic Spo$^+$ parent strain, 630Δ*erm*. Growth and sporulation of mutants in liquid medium was essentially identical between strains with approximately 10^4–10^5 spores ml^{-1} produced after 5 days (Fig. S4). Histodenz-purified spores of all mutants showed no susceptibility to treatment with heat, ethanol and lysozyme (Table S1). Transmission electron microscopy (TEM) was used to examine the structure of wild-type and mutant spores (Fig. 2). Compared to 630Δ*erm* spores (Fig. 2A) it was clear that for the *bclA1*$^-$ and *bclA2*$^-$ mutants they carried substantial aberrations in the spore coats. In both cases sheets of coat-like material were present in the medium (Fig. 2C and E) as well as angular projections of material at the spore surface (Fig. 2B and D). The *bclA3*$^-$ mutant did not present any apparent defect compared to wild-type spores nor was any coat-like material shed into the culture medium (Fig. 2F). The hydrophobicity of spores was assessed by measurement of the optical density of the aqueous layer after mixing with hexadecane. All three *bclA* mutants were found to be significantly ($P < 0.035$) less hydrophobic than spores of the wild-type 630Δ*erm* (Fig. 3). It has been shown recently that sonication of *C. difficile* spores is efficient at removing the putative exosporium (Escobar-Cortes *et al*., 2013) and for comparison we demonstrated here that sonication of spores significantly ($P < 0.024$) reduced the surface hydrophobicity of 630Δ*erm, bclA1* and *bclA2* mutant spores. Polyclonal antibodies raised against recombinant BclA1, BclA2 and BclA3 proteins were used to confirm that each protein was (i) located on the surface of 630Δ*erm* spores, (ii) absent in vegetative cells and (iii) not present in spores of the corresponding isogenic mutant (Fig. 4).

Purified spores were assessed for their ability to germinate in BHI medium supplemented with 0.1% sodium taurocholate as a germinant (Table 2 and Fig. S5). Germination correlates to a loss in OD_{600} as spores rehydrate and become phase dark. 630Δ*erm* spores germinated relatively slowly with a 16% reduction in OD_{600} over a 30 min period. All three mutants germinated faster than the wild-type strain with the *bclA1*$^-$ and *bclA2*$^-$ mutants exhibiting the highest germination rates with 40% and 34% loss in OD_{600}, respectively, over 30 min. As a control, spore germination was conducted in parallel in the presence of the inhibitor sodium chenodeoxycholate. In the presence of this inhibitor, spores of wild-type and all *bclA* mutant strains remained stable and exhibited a maximum OD_{600} drop of 5–6% over 30 min. Germination of sonicated

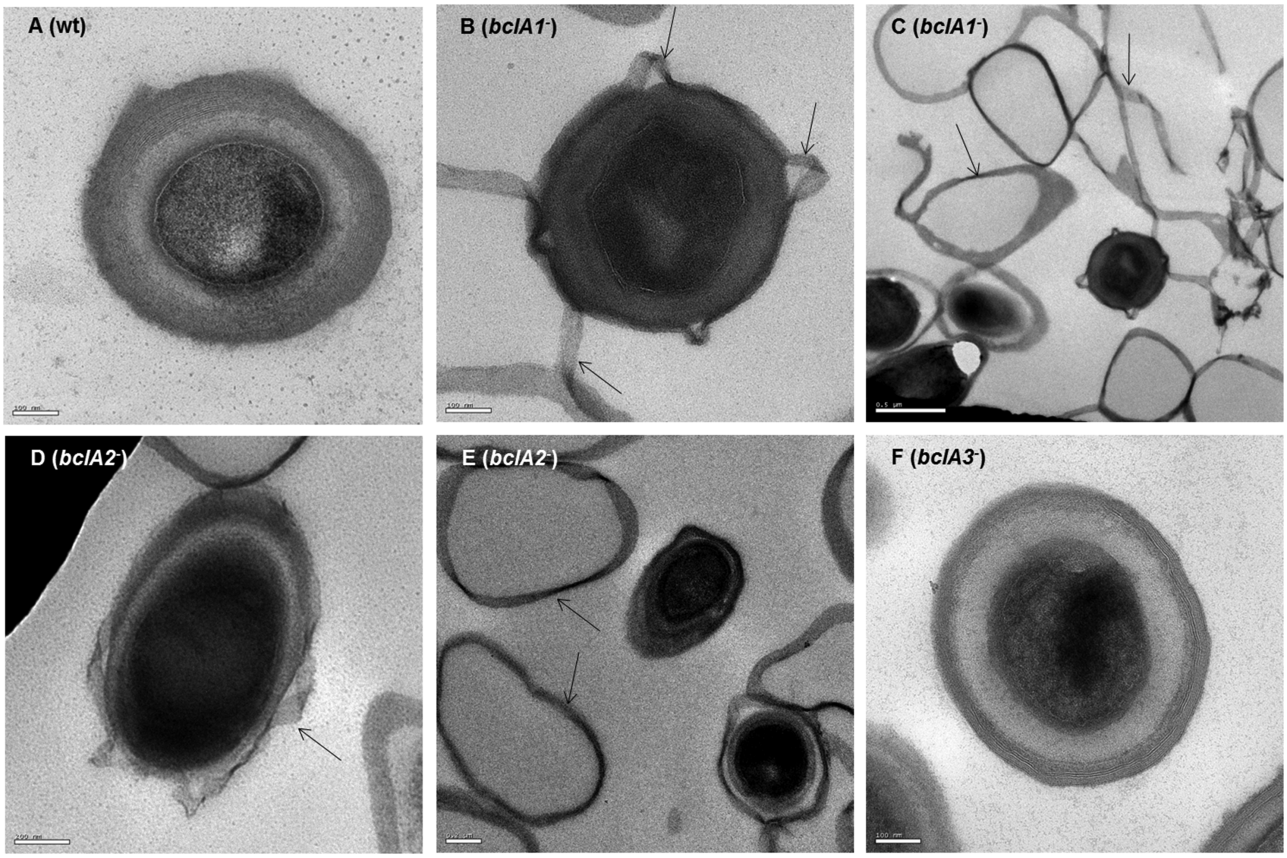

Fig. 2. Ultrastructure analysis by TEM.
A. High-magnification image showing purified 630Δ*erm* spores with a normal morphology. Bar: 100 nm.
B and C. *bclA1*⁻ purified mutant spores showing clear defects including sheet-like material on the outermost layer (arrows indicated). Bars: 100 nm (panel B) and 0.5 μm (panel C).
D and E. Ill-formed *bclA2*⁻ purified mutant spores with sheet-like material (arrows indicated). Bars: 200 nm (panel D) and 0.2 μm (panel E).
F. A *bclA3*⁻ mutant spore showing normal morphology. Bar: 100 nm.

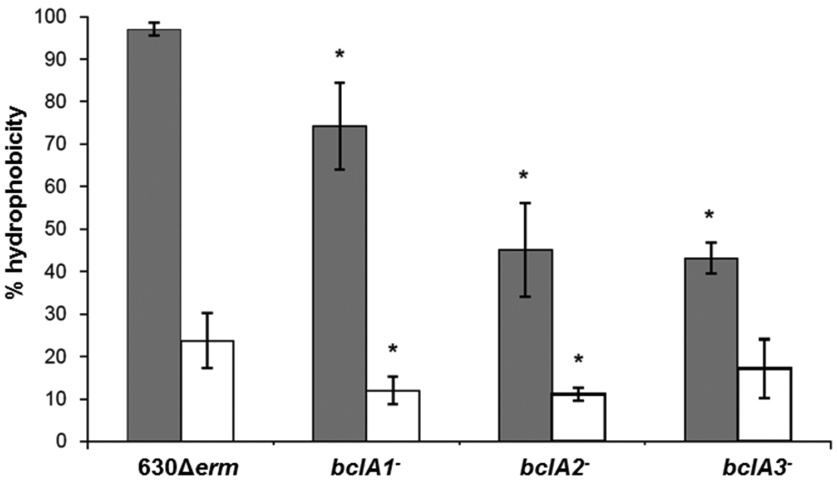

Fig. 3. Spore hydrophobicity. The SATH assay was used to calculate % hydrophobicity of Histodenz-purified spores of wild-type and mutant spores with (open column) or without sonication (grey column). The analysis was performed three times. The asterisk (*) indicates values significantly different between *bclA* mutants and 630Δ*erm* (*bclA1*⁻, 0.036; *bclA2*⁻, 0.0064; *bclA3*⁻, 0.0006) and sonicated mutant and 630Δ*erm* spores (*bclA1*⁻, 0.02; *bclA2*⁻, 0.0243).

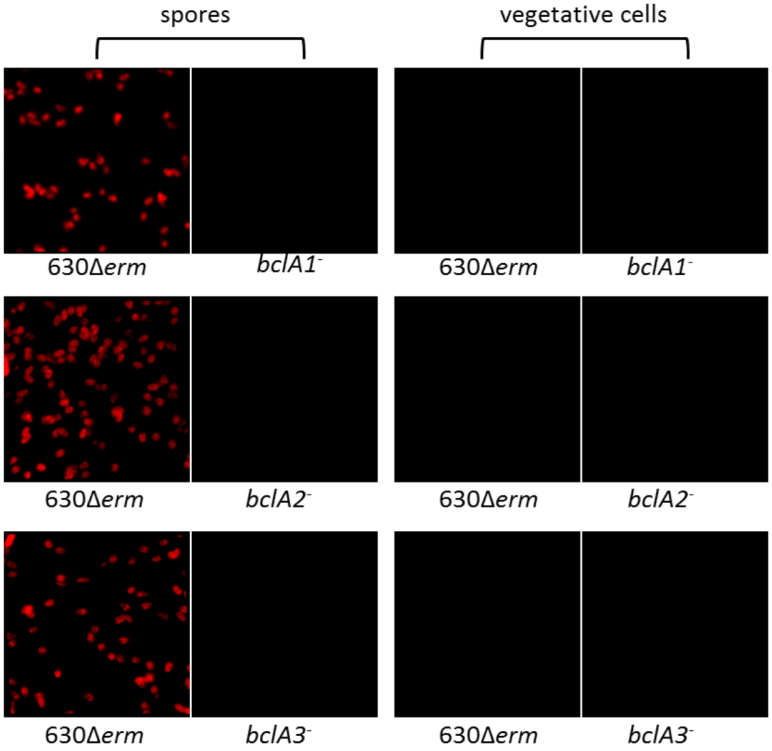

Fig. 4. Surface display of BclA proteins. Surface display of BclA1, BclA2 and BclA3 using immunofluorescence imaging of suspensions of 630Δ*erm*, *bclA1*⁻, *bclA2*⁻ and *bclA3*⁻ spores (7-day-old cultures grown on solid medium) labelled with mouse serum (1:1000 dilution) raised against each of the three BclA proteins. An anti-mouse IgG-TRITC conjugate was used for secondary labelling. BclA1, BclA2 and BclA3 proteins were detected on both purified and non-purified 630Δ*erm* spores whereas the *bclA* mutants showed negative signals. Controls included vegetative cells of wild-type and mutants.

spores of the wild-type strain, evaluated in parallel, revealed an OD_{600} drop of 29%, indicating that disruption of the spore surface layers enhanced germination (Fig. S5).

Infectivity of bclA mutants in the mouse model of infection

The recently described mouse model of cefoperazone pre-treatment to induce *C. difficile* infection (Theriot *et al.*, 2011) was used to evaluate the progress of shedding of *C. difficile* spores. Animals were given a single dose (10^4) of mutant or wild-type spores (Fig. 5). Total counts (spores and vegetative cells) of *C. difficile* shed in the faeces ranged from 10^4 to 10^6 per gram although somewhat lower counts were found for the *bclA1*⁻ mutant (Fig. 5B). Mice body weights remained similar with no significant differences between groups (data not shown). Heat-resistant spore counts of 630Δ*erm*-dosed mice declined over time (Fig. 5A). Spores were not found in the faeces from mice dosed with the *bclA3*⁻ mutant on day 1, even if substantial levels of spores were detected on days 3, 5 and 7. Spore counts of both the *bclA2*⁻ and *bclA3*⁻ mutants increased after day 1 and were substantially higher (> 1-log) on days 3, 5 and 7 if compared to that of wild-type infected animals (Fig. 5A). Surprisingly no heat-resistant spores were detected for the *bclA1*⁻ mutant in the faeces post infection (Fig. 5A) in all the time points. The experiment has been repeated with similar findings. However, using a dose–response assay (Table 3 and Fig. S6) where counts were detected following ethanol treatment spores of the *bclA1*⁻ mutant were clearly detected in the faeces albeit at lower levels than in mice infected with wild-type 630 spores. This suggested that the *bclA1*⁻ mutant spores in faecal samples were susceptible to heat treatment but not to ethanol. One explanation might be that the *bclA1*⁻ mutant, being more germination proficient than the isogenic parent strain 630 was more susceptible to heat treatment, or more likely, that heat was producing premature germination of *bclA1*⁻ mutant spores. For this reason, in subsequent analysis we used ethanol for measurement of wild-type and mutant spores.

Table 2. Germination phenotypes.

	% germination[a]	
Spores	+ Inhibitor	+ Germinant
630Δ*erm*	5.6 ± 3.3	15.6 ± 4.3
bclA1⁻	6.2 ± 2.2	40.2 ± 0.7
bclA2⁻	5.9 ± 2.0	34.4 ± 1.3
bclA3⁻	5.3 ± 2.5	20.5 ± 1.8
630Δ*erm* (sonicated)	5.6 ± 0.6	29.1 ± 0.9

a. Per cent germination was determined as % loss in OD_{600} in the presence of inhibitor (0.1% sodium chenodeoxycholate) or germinant (0.1% sodium taurocholate).

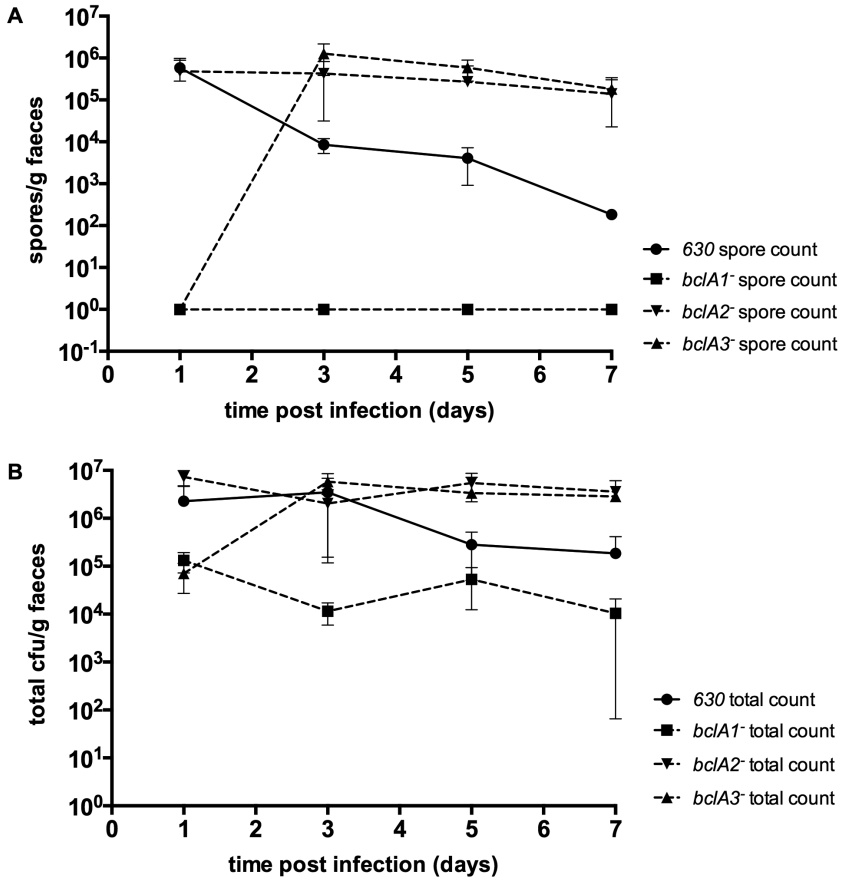

Fig. 5. Colonization of mice with *C. difficile bclA* mutants. Groups of mice ($n = 4$) were orally administered a regimen of cefoperazone and then infected orally with a single dose (1×10^4) of 630Δ*erm* spores or spores of one of the three *bclA* mutants. Freshly voided faeces was analysed for heat-resistant spore counts (panel A) and total counts (spores plus vegetative cells) (panel B) on days 1, 3, 5 and 7 post infection.

Returning to the dose response assay this showed that the number of spores required to infect 50% of mice (ID_{50}) was 2-logs higher in the *bclA1*⁻ mutant compared to the wild-type control. In contrast to 630, spores of the *bclA1*⁻ mutant were not detectable 3 and 4 days post infection (Fig. S6). Together these data show that *bclA1*⁻ mutants are less infective than wild-type strains.

Reduced colonization by a 'hypervirulent' 027 strain

Analysis of the *bclA1* genes in the genome sequences of two ribotype 027 strains, R20291 and CD196 (Stabler *et al.*, 2009) revealed a stop codon at position 48 in addition to an asparagine to lysine change at position 3 in the ORF (Fig. S7). We have independently sequenced the *bclA1* gene in R20291 and confirm that the stop codon is present and is not a sequencing error. As such, the 027 strains must each encode a BclA1 protein of approximately 6 kDa and, being significantly smaller than the one found in strain 630, would presumably lack function. Using antibodies raised against BclA1 (from strain 630) we have been able to identify BclA1 on the surface of both R20291 and CD196 spores suggesting that the truncated protein can assemble into the exosporium in these 027 strains (Fig. S8).

R20291 is a so-called 'hypervirulent' strain (Stabler *et al.*, 2009; Buckley *et al.*, 2011), is clinically relevant and would, *prima facie*, be considered more virulent than the 630 strain. Our studies suggest that *bclA1* deletion would impair colonization. Therefore, to determine the infectivity of a 027 strain carrying a truncated BclA1 protein, the ability of R20291 spores to colonize mice was analysed as previously described for 630Δ*erm* and the *bclA1* mutant (Table 3 and Fig. S6). The ID_{50} for R20291 was 1×10^3 and therefore ten-times less infectious than 630 but more infectious than a strain completely devoid of BclA1, suggesting a correlation between the presence of an intact BclA1 protein and the susceptibility of mice to colonization. Interestingly, compared to the 630Δ*erm* spores, R20291 spores (i.e. at doses $\geq 10^3$) were able to persist longer in the GI-tract and were maintained at higher levels.

BclA1 is a virulence determinant in hamsters

Hamsters provide a more acute model of *C. difficile* infection (Sambol *et al.*, 2001; Goulding *et al.*, 2009) with wild-type strains causing a rapid fulminant infection most likely due to the sensitivity of these animals to *C. difficile* toxins. Accordingly, this model was used to evaluate the infectivity of *bclA1*⁻ mutant spores. In a preliminary study, groups of

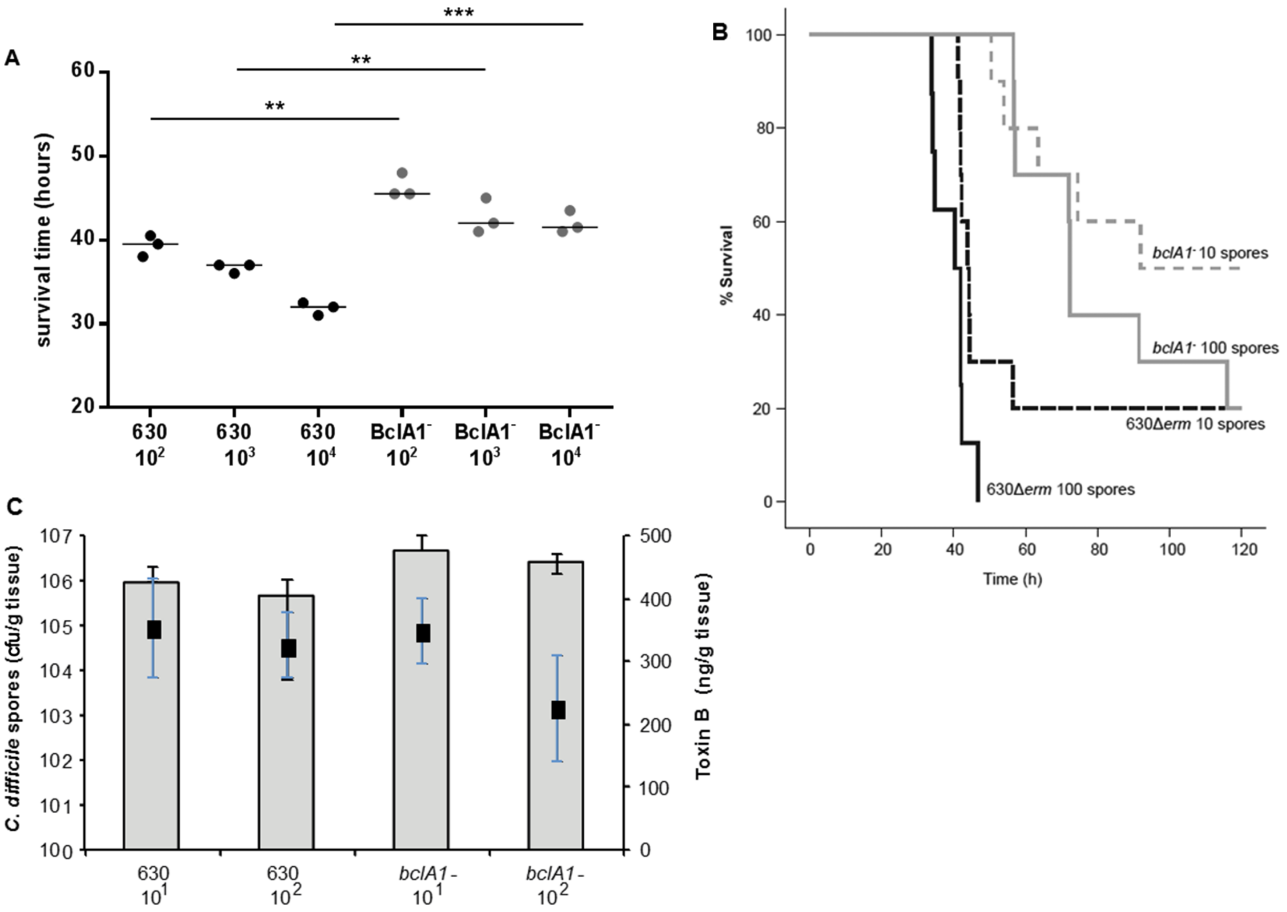

Fig. 6. Infection of hamsters with *bclA* mutants. The hamster model of infection was used to assess the virulence of the *bclA1* mutant. Animals were pre-treated by oral gavage with clindamycin followed by *C. difficile* spores.
A. Survival time for hamsters infected with spores of strain 630Δ*erm* or *bclA1*⁻. Doses of 10^2, 10^3 or 10^4 spores were used to infect hamsters ($n = 3$) by oral gavage. 630Δ*erm* black symbols and *bclA1*⁻ grey symbols. ***P* value < 0.01; ****P* value < 0.001.
B. Kaplan Meier survival plot of hamsters ($n = 10$) infected with doses of 10 or 10^2 spores of 630Δ*erm* (black lines) or *bclA1*⁻ spores.
C. Caecum tissue excised from infected hamsters (from panel A) was evaluated for average counts of ethanol-resistant spores (columns; cfu g^{-1}) and toxin B (ng g^{-1}; internal black squares). All samples were taken from caecum post death at the clinical end-point of infection.

three hamsters were dosed with 10^2, 10^3 or 10^4 of 630Δ*erm* or *bclA1*⁻ spores (Fig. 6A). Significant differences were observed in survival times between wild-type and mutant (10^2, $P = 0.003$; 10^3, $P = 0.008$; 10^4, $P = 0.0003$) as well as in the dose-dependent response. Using an infective dose of 10^2 630Δ*erm* spores the clinical end-point was reached in approximately 40 h while this was delayed until approximately 47 h with the same dose of *bclA1*⁻ mutant spores. This study was repeated using 10 hamsters per group and two doses, 10 and 10^2 spores, of either 630Δ*erm* or the *bclA1*⁻ mutant. As shown in Fig. 6B, a dose of 10^2 spores of 630Δ*erm* resulted in no survival of infected animals while a lower dose of 10 spores resulted in the survival of two animals. By contrast, the *bclA1*⁻ mutant was clearly less infective with 50% survival following a dose of 10 spores and 20% survival using 10^2 spores. The calculated ID_{50} for 630Δ*erm* spores was 2.37×10^1 and for the *bclA1*⁻ mutant 2×10^2, indicating that the *bclA1*⁻ mutant was ten-times less infective. Animals infected with either 630Δ*erm* or *bclA1*⁻ had similar levels of *C. difficile* spores in the caecum (Fig. 6C), although levels were somewhat higher in *bclA1*⁻ infected animals, possibly reflecting the ability of this

Table 3. Infectivity of spores of different *C. difficile* strains in mice.[a]

Strain	ID_{50}[b]
630Δ*erm*	1×10^2
R20291	1×10^3
bclA1⁻	$>1 \times 10^4$

a. Groups of mice were first treated with clindamycin followed by a 5-day interval before being given three doses (10^2, 10^3 or 10^4) of spores followed by determination of ethanol-resistant spores counted in fresh faecal samples (cfu data are shown in Fig. S6). Colonization was defined as animals carrying > 10^3 spores per gram of faeces at 48 h post infection.
b. Using the Reed-Munch equation (Ozanne, 1984) the dose of spores required to infect 50% of mice (ID_{50}) was determined.

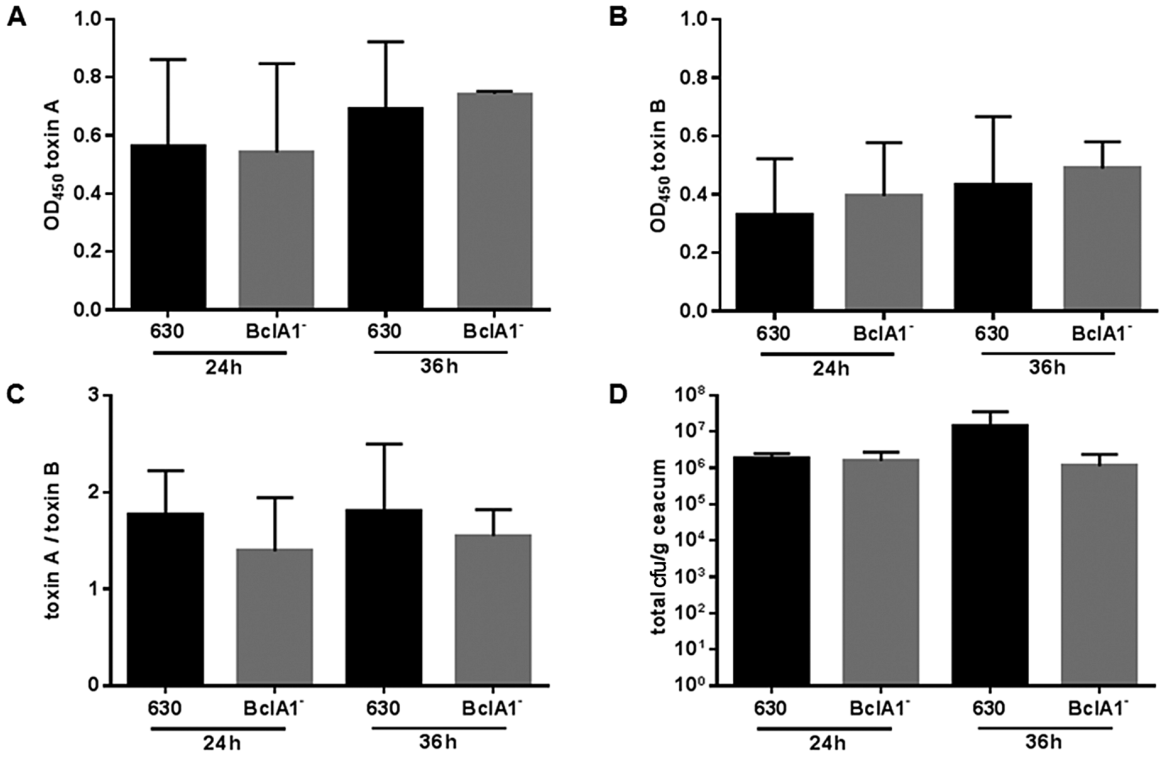

Fig. 7. Toxins A and B *in vivo* kinetics.
A and B. Levels of toxins A (panel A) and B (panel B) at different time points (24 or 36 h) in the caeca of mice infected with 1×10^5 spores per mouse of *C. difficile* 630Δ*erm* wild-type strain or *bclA1*⁻ mutant.
C. Ratio between toxin A and toxin B levels in infected mice.
D. Total *C. difficile* cfu counts (cfu g^{-1}) in caecum tissues excised from infected mice.

mutant to germinate more efficiently and proliferate. The levels of toxin B in caecum samples were measured and found to be similar in all samples, showing no significant differences (Fig. 6C). In surviving animals no viable *C. difficile* or toxin B could be detected in caeca. These data support the murine study demonstrating that *bclA1*⁻ mutant strains, although able to produce toxins, are clearly less infectious than the wild-type.

It was possible that the low infectivity of the *bclA1*⁻ mutant might have arisen if toxin production was reduced or delayed *in vivo*. This is unlikely though since based on the morphogenesis of the spore, we would predict that the *bclA1* gene would be expressed in the late phase of spore formation, while toxin production is associated with the stationary phase of vegetative cell growth (Rupnik *et al*., 2009) and should occur before *bclA1* expression. Preliminary qPCR data (not shown) demonstrated that *tcdA* and *tcdB* are expressed during stationary phase and the early stages of spore formation, while the *bclA1–3* genes are expressed at the terminal stages of sporulation (approximately 9 h following the onset of development). To rule out differences in production of toxins *in vivo* between 630Δ*erm* and the *bclA1* mutant, we infected mice 8 days post clindamycin treatment with a high dose (10^5 per mouse) of 630Δ*erm* or *bclA1*⁻ spores sufficient to cause infection in most of the mice (see Table 3). At 24 and 36 h post infection the total cfu of *C. difficile* and toxin A and B levels were determined in caeca. As shown in Fig. 7, the total cfu in mice infected with 630Δ*erm* or *bclA1*⁻ spores were equivalent at both time points and no differences were observed between toxin A and B levels in the caeca and in the ratio between the two toxins.

Discussion

The exosporium is poorly defined in *C. difficile* and images of this 'sac-like' outer layer vary from a well-defined thick, electron dense laminated structure (Lawley *et al*., 2009b) to more diffuse layers that are easily removed from the underlying spore coat (Permpoonpattana *et al*., 2011b; 2013; Escobar-Cortes *et al*., 2013). Most probably the exosporium of *C. difficile* is particularly fragile at least under the conditions commonly used in the laboratory to prepare spores so defining this structure in *C. difficile* remains elusive. One of the major immunodominant proteins found in the exosporium of *B. anthracis* and *B. cereus* is the BclA protein (Sylvestre *et al*., 2002; Steichen *et al*., 2003; Todd *et al*., 2003; Redmond *et al*., 2004). Filaments of the BclA

protein form the hairy nap which is characteristic of the exosporia of the *Bacillus anthracis/thuringiensis* family of spores (Kailas *et al*., 2011) but in the case of *C. difficile* these hair-like filaments have yet to be observed. *C. difficile* carries three *bclA* genes whose products share similarity with the BclA proteins of *B. anthracis* and *B. cereus*. However, the composition of these proteins differ significantly especially with regard to the absence of the N-terminal (targeting the exosporium) and C-terminal (oligomerization) domains. Our evidence suggests that the *C. difficile* BclA proteins reside in the outermost layers of the spore and most probably the putative exosporium. Antibodies against all three BclA proteins confirmed expression on the spore surface and mutagenesis of the three genes also revealed noticeable defects in the spore coat. First, in two mutants, *bclA1* and *bclA2*, aberrations in the spore coat were clearly evident and presumably assembly of the outer coat or exosporium is defective in these mutants emphasizing that both proteins are likely major exosporial proteins. Second, spores of all three mutants had significantly reduced hydrophobicity. Reduced hydrophobicity was also apparent in spores that had been sonicated, an approach that has been shown elsewhere to remove the exosporium (Permpoonpattana *et al*., 2011b; 2013; Escobar-Cortes *et al*., 2013). In *B. anthracis, bclA* mutants also have a much-reduced hydrophobicity where the exosporium is thought to provide a water repellant shield reducing its ability to interact with the host matrix (Brahmbhatt *et al*., 2007). Third, all three *bclA* mutants showed increased germination rates, a characteristic also found in the *B. anthracis bclA* mutant and presumably a result of a defective exosporium allowing access of germinants to receptors situated in the innermost spore membranes (Brahmbhatt *et al*., 2007; Carr *et al*., 2010). Finally, *in vivo* infection studies in mice revealed that the *bclA1* and *bclA3* mutants had impaired colonization efficiencies although this was most striking with the *bclA1* mutant that completely failed to colonize the mouse GI-tract. Thus, the three BclA proteins are integral components of the outermost layers of the spore (and most probably the exosporium) and whose removal severely destabilizes this outermost layer allowing access of germinants and reducing surface hydrophobicity.

In *B. anthracis* BclA has not been shown to play a significant role in virulence with a *bclA* mutant having no effect on pathogenicity in mice or in guinea pigs (Bozue *et al*., 2007) and with mutant and wild-type strains having similar LD_{50} values (Brahmbhatt *et al*., 2007). This is in marked contrast to our study where we show that in *C. difficile* at least one BclA protein, BclA1, is involved in the initial stages of colonization and infection. In mice and in hamster models of infection spores devoid of BclA1 were up to 2-logs less infective (i.e. by ID_{50}) than isogenic wild-type spores and showed increased times to death in hamsters. This suggests that BclA1 could be involved in the initial stages of host colonization and that this event must be mediated by the spore, an event occurring before spore germination. Even more intriguing was the observation that two 027 strains carried truncated BclA1 proteins and that one of them, R20291, a so-called 'hypervirulent' strain, was actually less infective in a mouse model of infection than its counterpart 630 suggesting a relationship of animal susceptibility to the presence of an intact BclA1 protein in the *C. difficile* spore. Spores of strains carrying a full-length BclA1 protein (i.e. 630) were more infectious than those carrying a defective or truncated *bclA1* gene. Interestingly there is already published work that would support this. For example, only 10^2 spores of 630 were required for 100% colonization in hamsters but using the same dose lower levels of infection were found with a variety of B1 strains (Razaq *et al*., 2007). Similarly, 10^4 spores of R20291 have been shown to produce complete infection in hamsters (Buckley *et al*., 2011). Finally there is now evidence showing that hamsters are more susceptible to colonization with non-toxigenic strains of *C. difficile* than with toxigenic strains (e.g. M68 and B1–7) (Buckley *et al*., 2013).

It has been proposed that hypervirulent 027 strains may have acquired additional virulence genes based on the considerable genetic differences between the epidemic and non-epidemic strains (Stabler *et al*., 2009). However, we suggest that in terms of initial colonization the hypervirulent R20291 strain is actually less effective, that is, animals are less susceptible. This then raises some interesting and provocative questions. We wonder whether animals including humans are actually less susceptible to 'hypervirulent' strains yet once colonization occurs the severity of disease is much greater. In many ways this resembles the situation of influenza where seasonal flu strains are typically highly infective but of low severity compared to the low infectivity-high severity nature of H5N1 strains. If what happens in humans mirrors that in mice then the virulence of R20291 must arise not due to its infectivity but rather, due to some other factor affecting the severity of infection, e.g. levels of toxin production, increased persistence or faster germination. For the 027 'hypervirulent strains increased toxin production and biofilm formation (Dawson *et al*., 2012; Dapa and Unnikrishnan, 2013) have been identified as potential virulence factors. However, the presence of an intact BclA1 protein would correlate with the susceptibility of the host to infection and we assume that BclA1 may interact with a specific host target. We acknowledge that comparisons between diverse and non-isogenic strains carry inherent risks and of course we cannot rule out the presence of additional factors. A more detailed and extensive study of the *bclA1* gene in clinically relevant strains coupled with analysis of infection will be required to address this but at

this stage it is clear that BclA1 plays a key role in the initial stages of infection and host susceptibility. Current thought is that *C. difficile* is acquired primarily from the environment but is it possible for hypervirulent strains to remain as latent members of the gut flora and to be rendered infectious only after their numbers reach a critical level resulting from antibiotic disturbance?

In *B. anthracis* it has recently been shown that BclA interacts with the integrin Mac-1 leading to uptake by professional phagocytes. Rhamnose residues within BclA have been shown to interact directly with CD14 molecules (Oliva *et al*., 2009). If *C. difficile* BclA1 also recognizes a specific target then it is a prime candidate for inclusion in a more robust vaccine to *C. difficile* infection. In preliminary trials we have expressed the 48-amino-acid N-terminus of BclA1 on the surface of *Bacillus subtilis* spores. This segment is that which is present in the 027 strain R20291 (Fig. S7). When combined, 50:50, with *B. subtilis* recombinant spores expressing the C-terminus of toxin A [strain PP108 as described elsewhere (Permpoonpattana *et al*., 2011a)] they were able to provide significantly greater levels of protection than PP108 alone when administered orally (data not shown). This is encouraging and suggests that BclA1 can act as a decolonization factor and could be combined with an anti-toxin-based vaccine to prevent *C. difficile* infection.

Experimental procedures

Strains

630 is a toxigenic (*tcdA*$^+$ *tcdB*$^+$) strain of *C. difficile* isolated from a patient with pseudomembranous colitis during an outbreak of *C. difficile* infection (CDI) (Wust *et al*., 1982). For ClosTron mutagenesis and mutant analysis an erythromycin-sensitive derivative 630Δ*erm* (Hussain *et al*., 2005) was used (provided by N. Minton, Univ. Nottingham, UK). R20291 is an epidemic strain of ribotype 027 isolated from Stoke Mandeville Hospital in 2006 (Stabler *et al*., 2009) and was obtained from T. Lawley (Wellcome Trust Sanger Institute, UK).

Growth of C. difficile *and preparation of spores*

Clostridium difficile was routinely grown in vegetative culture by overnight growth in TGY- medium (Paredes-Sabja *et al*., 2008). Spores of *C. difficile* were prepared by growth on SMC agar plates using an anaerobic incubator (Don Whitley, UK) as described previously (Permpoonpattana *et al*., 2011a). After growth for 7 days at 37°C spores were harvested and either washed three times with water or purified using HistoDenz as follows. Crude spore suspensions were washed five times with ice-cold sterile water, re-suspended in 500 µl of 20% HistoDenz (Sigma) and layered over 1ml of 50% HistoDenz in a 1.5 ml tube. Tubes were centrifuged at 10 000 *g* for 15 min. The spore pellet was recovered and washed three times with ice-cold sterile water. Spore purity was assessed by phase-contrast microscopy and spore yields in individual preparations were estimated by counting colony-forming units (cfu) of heat-treated (60°C, 20 min) aliquots on BHIS agar plates (Brain heart infusion supplemented with 0.1% L-cysteine and 5 mg ml^{-1} yeast extract) supplemented with 0.1% sodium taurocholate (BHISS).

ClosTron mutagenesis

Insertional mutations in the *bclA* genes were made using the ClosTron system developed at the University of Nottingham (Heap *et al*., 2007; 2009; 2010). The Perutka algorithm (Perutka *et al*., 2004) available at http://www.clostron.com was used to design 45 bp retargeting sequences for each gene (Table 1). Derivatives of plasmid pMTL007C-E2 carrying these retargeting sequences were obtained from DNA2.0 (DNA20.com, Menlo Park, USA). Using the protocols provided by Heap *et al*. (2007; 2009; 2010) plasmids were first introduced into *Escherichia coli* and then conjugated with *C. difficile* 630Δ*erm*. For each mutant five erythromycin-resistant (Erm^R) transconjugants were checked by PCR for ClosTron insertion. Genomic DNA was prepared as described (Antunes *et al*., 2011) and then three PCR reactions were performed (Fig. S9). First, PCR using the ErmRAM primers resulted in a 900 bp product confirming that the Erm^R phenotype was due to splicing of the group I intron from the group II intron following integration. Second, primers targeting the gene left and right ends of the insertion site were used to check the site of insertion. If insertion occurred a PCR product 1800bp greater than that obtained in the wild-type strain would be found. Third, PCR was made using primers flanking the gene and intron (EBS-universal) to confirm the insertion site where no product would be expected in the wild-type strain.

Complementation of bclA mutants

All three *bclA* mutants were complemented with wild-type copies of the respective genes using pRPF185 (Fagan and Fairweather, 2011). Briefly, a DNA fragment including the entire coding sequence of each gene and Shine–Dalgarno sequence was PCR amplified using KOD Hot Start polymerase (Merck) and primers listed in Table S2. The resulting fragments were cloned using SacI and BamHI sites into pRPF185 under the control of the inducible P_{tet} promoter. Plasmids were transferred into the corresponding *bclA* mutant strains by conjugation. Gene expression was induced using anhydrous tetracycline (ATc) at 500 ng ml^{-1}. To confirm that the *bclA* mutants were due to a single insertional mutation we used *in trans* complementation analysis to demonstrate that the wild-type phenotype could be restored using two methods: (i) immunofluorescence microscopy of spores to demonstrate surface expression of the BclA protein on spores of the complemented strain, and (ii) restoration of wild-type levels of germination (Fig. S10).

Germination assays

Spore germination was carried out in a 96-well plate (Greiner Bio-One) and germination of spores was measured by the

percentage change in OD_{600}. HistoDenz-purified spores at an OD_{600} of ~ 0.8–1.0 (~ 1×10^8 ml^{-1}) were pelleted by centrifugation (10 000 *g*, 1 min) and suspended in 1 ml of BHIS supplemented with 0.1% sodium taurocholate (germinant) or 0.1% sodium chenodeoxycholate (inhibitor). The initial OD_{600} was recorded and then measured at 1 min intervals over 30 min using a microplate reader (Molecular Devices, Spectramax plus). Per cent germination was determined as recorded OD_{600} at time interval/initial $OD_{600}) \times 100$. The experiment was performed three times. For preparations of sonicated spores 10 cycles of sonication were used as described elsewhere (Permpoonpattana *et al*., 2011b).

SATH (spore adhesion to hydrocarbon) assay

As described elsewhere (Huang *et al*., 2010) HistoDenz-purified spores were washed in 1 M NaCl and then suspended in 0.1 M NaCl for assay. Five hundred microlitres of spore suspension was added to 800 µl *n*-hexadecane (Sigma) and vortexed for 1 min. Samples were then incubated for 10 min at 37°C with mild agitation, vortexed (30 s) and absorbance (OD_{600}) read. Per cent hydrophobicity was determined from the absorbance of the original spore suspension (A_1) and the absorbance of the aqueous phase after incubation with hydrocarbon (A_2) using the equation: $\%H = [(A_1 - A_2)/A_1]$.

Recombinant proteins and antibody production

Escherichia coli pET28b expression vectors carrying the *bclA1, bclA2* and *bclA3* ORFs were used to express rBclA proteins. The segments of BclA used for expression were rBclA1 (Met-1 to Pro-393), rBclA2 (Met-1 to Gly-302) and rBclA3 (Thr-489 to Ala-645). High levels of expression were obtained upon IPTG induction and purification of proteins made by passage of the cell lysate through a HiTrap chelating HP column on a Pharmacia AKTA liquid chromatography system. Polyclonal antibodies were raised in BALB/c mice immunized by the intra-peritoneal route with 2 µg of purified recombinant proteins on days 1, 14 and 28. Antibodies were first purified using a Protein G HP Spin-Trap column (GE Healthcare).

Transmission electron microscopy (TEM)

Spores were processed for ultra-microtomy according to standard procedures (Hong *et al*., 2009). Briefly, spore suspensions were diluted 10× in dH_2O and washed twice by centrifugation (10 000 *g* for 10 min) to eliminate residual debris. Spore pellets were fixed for 12 h at 4°C in a mixture of 2.5% glutaraldehyde and 4% paraformaldehyde in 0.2 M cacodylate buffer (pH 7.4), then post-fixed for 1 h at RT with 1% osmium tetroxide in the same buffer. Sample pellets were dehydrated with ethanol and embedded in Epon-Araldite. Thin sections were stained successively with 5% uranyl acetate and 1% lead citrate. TEM observation was performed with a FEI CM120 operated at 120 kV.

Immunofluorescence microscopy

The procedure followed was as described in Duc *et al*. (2003) with minor modifications. Microscope coverslips were first treated with 0.01% poly-L-lysine overnight. Spores (1×10^7) were added to the slide and dried for 1 h at RT. After three washes with PBS (pH 7.4) and blocking in PBS + 2% BSA + 0.05% Tween-20 for 1.5 h, the first antibody was added (1:1000). Spores were incubated for 30 min at RT followed by three washes with PBS + 0.05% Tween-20 after which anti-mouse-TTFC sera (1:1000) was added and incubated for 30 min at RT. After six more washes the slide was viewed under a Nikon Eclipse Ti-S fluorescence microscope.

Colonization experiments

(a) Infection of mice using cefoperazone pre-treatment. The cefoperazone murine model was initially used since the erythromycin-resistance cassette used in ClosTron mutants may not confer the same level of resistance to clindamycin as seen in the parental strain, depending upon its chromosomal location although this was found in this work to be unfounded (N. Fairweather, pers. comm.). Groups ($n = 4$) of C57BL/6 mice (6–8 weeks old; female, Charles River) were administered with five doses of cefoperazone (MP Biomedicals), LLC (100 mg kg^{-1}; by intra-gastric gavage) on day 1, 3, 5, 7 and 9 using a procedure previously described (Theriot *et al*., 2011). Animals were kept in IVCs (independently ventilated cages) under sterile conditions. On day 10, mice were orogastrically (o.g.) infected with *C. difficile* 1×10^4 spores per mouse of the wild-type 630Δ*erm* strain or one of the three *bclA* mutants (one group/mutant). Fresh faeces from individually infected mice were collected on day 1, 3, 5 and 7 post challenge. Samples were reconstituted in PBS supplemented with protease inhibitor (Thermo Scientific) using a ratio of 1:5 [weight faeces (g): volume (ml)]. Total counts and spore counts of *C. difficile* were performed by plating serial dilutions on BHIS and BHISS respectively, media was supplemented with cefoxitin and cycloserine (Bioconnections, Knypersley, UK). Spore counts were determined after heat-treating (60°C, 30 min) samples, serial dilution and plating for cfu ml^{-1}.

(b) Infection of mice using clindamycin pre-treatment. On days 1 and 3 animals received a single dose of clindamycin (30 mg kg^{-1}) as described above for cefoperazone (a) and they were kept in IVCs under sterile conditions. On day 8, animals were o.g. infected with different doses (ranging from 10^2 to 10^4 spores per mouse) of *C. difficile* strains R20291, 630Δ*erm* or *bclA1*$^-$ mutant (n.b., the 630Δ*erm* and *bclA1*$^-$ mutant are sensitive to clindamycin). Spore counts in freshly voided faeces were determined after ethanol treatment (100% ethanol, 20 min) by plating as described above (a).

(c) Analysis in mice of in vivo *toxin levels and spore kinetics.* Groups of 9–10 mice were administered with clindamycin as described above (b) and housed in IVCs. On day 8, mice were o.g. infected with spores of *C. difficile* wild-type 630Δ*erm* and *bclA1* mutant strains at the dose of 1×10^5 spores per mouse. Caeca from infected mice were aseptically removed 24 or 36 h post challenge. Samples were processed as described above (a). For detection of levels of toxin A and toxin B in caecum, samples were centrifuged for 10 min (10 000 *g*; 4°C) and supernatants sterilized using 0.2 µm filters. An ELISA assay was performed following the method described below (toxin detection, e).

(d) Hamster infections. Golden Syrian Hamsters (female, aged 10 months; ~ 100 g; Charles River) housed in IVCs were dosed o.g. with clindamycin (30 mg kg^{-1}) and infected 5 days later with *C. difficile* spores of the wild-type 630Δ*erm* strain or *bclA1*⁻ mutant at doses of either 10^2, 10^3 or 10^4 spores per hamster. Hamsters were then monitored for signs of disease progression and, based on severity of symptoms, culled upon reaching the clinical end-point. Caecum samples were examined for toxin B by ELISA as described below. Toxin cytotoxicity assays using HT29 cells was assessed as described previously (Permpoonpattana *et al.*, 2011a). Spore counts in caeca was performed as described above (b). Statistical significance between groups was calculated using a Student's *t*-test.

(e) Toxin detection. Toxins were extracted using a protease inhibitor buffer as described previously (Permpoonpattana *et al.*, 2011a) and detected by a capture ELISA method. ELISA plates (Greiner, high binding) were coated with rabbit polyclonal antibodies against toxin A or toxin B (Meridian Life Science; 1 μg ml^{-1} in PBS buffer). Plates were blocked with 2% BSA (1 h, 30°C), 10 μl of samples and 2 μl of reference toxin A or toxin B (Ab Serotec) were added to plates and incubated at 30°C for 3 h. Monoclonal antibodies against toxin A (1/500) and toxin B (1/500) were used for detection (1 h, 30°C). HRP-conjugated anti-mouse IgG was added as secondary antibody (1 h, RT). Plates were developed with TMB (Sigma). The sensitivity of the assays for both toxin A and B is 7 ng ml^{-1}.

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References

Antunes, A., Martin-Verstraete, I., and Dupuy, B. (2011) CcpA-mediated repression of *Clostridium difficile* toxin gene expression. *Mol Microbiol* **79:** 882–899.

Boydston, J.A., Chen, P., Steichen, C.T., and Turnbough, C.L., Jr (2005) Orientation within the exosporium and structural stability of the collagen-like glycoprotein BclA of *Bacillus anthracis*. *J Bacteriol* **187:** 5310–5317.

Bozue, J., Cote, C.K., Moody, K.L., and Welkos, S.L. (2007) Fully virulent *Bacillus anthracis* does not require the immunodominant protein BclA for pathogenesis. *Infect Immun* **75:** 508–511.

Brahmbhatt, T.N., Janes, B.K., Stibitz, E.S., Darnell, S.C., Sanz, P., Rasmussen, S.B., and O'Brien, A.D. (2007) *Bacillus anthracis* exosporium protein BclA affects spore germination, interaction with extracellular matrix proteins, and hydrophobicity. *Infect Immun* **75:** 5233–5239.

Buckley, A.M., Spencer, J., Candlish, D., Irvine, J.J., and Douce, G.R. (2011) Infection of hamsters with the UK *Clostridium difficile* ribotype 027 outbreak strain R20291. *J Med Microbiol* **60:** 1174–1180.

Buckley, A.M., Spencer, J., Maclellan, L.M., Candlish, D., Irvine, J.J., and Douce, G.R. (2013) Susceptibility of hamsters to *Clostridium difficile* isolates of differing toxinotype. *PLoS ONE* **8:** e64121.

Carr, K.A., Lybarger, S.R., Anderson, E.C., Janes, B.K., and Hanna, P.C. (2010) The role of *Bacillus anthracis* germinant receptors in germination and virulence. *Mol Microbiol* **75:** 365–375.

Dapa, T., and Unnikrishnan, M. (2013) Biofilm formation by *Clostridium difficile*. *Gut Microbes* **4:** 397–402.

Daubenspeck, J.M., Zeng, H., Chen, P., Dong, S., Steichen, C.T., Krishna, N.R., *et al.* (2004) Novel oligosaccharide side chains of the collagen-like region of BclA, the major glycoprotein of the *Bacillus anthracis* exosporium. *J Biol Chem* **279:** 30945–30953.

Dawson, L.F., Valiente, E., Faulds-Pain, A., Donahue, E.H., and Wren, B.W. (2012) Characterisation of *Clostridium difficile* biofilm formation, a role for Spo0A. *PLoS ONE* **7:** e50527.

Deakin, L.J., Clare, S., Fagan, R.P., Dawson, L.F., Pickard, D.J., West, M.R., *et al.* (2012) The *Clostridium difficile spo0A* gene is a persistence and transmission factor. *Infect Immun* **80:** 2704–2711.

Duc, L.H., Hong, H.A., Fairweather, N., Ricca, E., and Cutting, S.M. (2003) Bacterial spores as vaccine vehicles. *Infect Immun* **71:** 2810–2818.

Escobar-Cortes, K., Barra-Carrasco, J., and Paredes-Sabja, D. (2013) Proteases and sonication specifically remove the exosporium layer of spores of *Clostridium difficile* strain 630. *J Microbiol Methods* **93:** 25–31.

Fagan, R.P., and Fairweather, N.F. (2011) *Clostridium difficile* has two parallel and essential Sec secretion systems. *J Biol Chem* **286:** 27483–27493.

Gerding, D.N., Muto, C.A., and Owens, R.C., Jr (2008) Measures to control and prevent *Clostridium difficile* infection. *Clin Infect Dis* **46** (Suppl. 1): S43–S49.

Goulding, D., Thompson, H., Emerson, J., Fairweather, N.F., Dougan, G., and Douce, G.R. (2009) Distinctive profiles of infection and pathology in hamsters infected with *Clostridium difficile* strains 630 and B1. *Infect Immun* **77:** 5478–5485.

Heap, J.T., Pennington, O.J., Cartman, S.T., Carter, G.P., and Minton, N.P. (2007) The ClosTron: a universal gene knockout system for the genus *Clostridium*. *J Microbiol Methods* **70:** 452–464.

Heap, J.T., Pennington, O.J., Cartman, S.T., and Minton, N.P. (2009) A modular system for *Clostridium* shuttle plasmids. *J Microbiol Methods* **78:** 79–85.

Heap, J.T., Kuehne, S.A., Ehsaan, M., Cartman, S.T., Cooksley, C.M., Scott, J.C., and Minton, N.P. (2010) The ClosTron: mutagenesis in *Clostridium* refined and streamlined. *J Microbiol Methods* **80:** 49–55.

Henriques, A.O., and Moran, C.P., Jr (2007) Structure, assembly, and function of the spore surface layers. *Annu Rev Microbiol* **61:** 555–588.

Hong, H.A., Khaneja, R., Tam, N.M., Cazzato, A., Tan, S., Urdaci, M., *et al.* (2009) *Bacillus subtilis* isolated from the human gastrointestinal tract. *Res Microbiol* **160:** 134–143.

Huang, J.M., Hong, H.A., Van Tong, H., Hoang, T.H., Brisson, A., and Cutting, S.M. (2010) Mucosal delivery of antigens using adsorption to bacterial spores. *Vaccine* **28:** 1021–1030.

Hussain, H.A., Roberts, A.P., and Mullany, P. (2005) Generation of an erythromycin-sensitive derivative of *Clostridium difficile* strain 630 (630Derm) and demonstration that the conjugative transposon Tn916DE enters the genome of this strain at multiple sites. *J Med Microbiol* **54:** 137–141.

Kailas, L., Terry, C., Abbott, N., Taylor, R., Mullin, N., Tzokov, S.B., *et al*. (2011) Surface architecture of endospores of the *Bacillus cereus/anthracis/thuringiensis* family at the subnanometer scale. *Proc Natl Acad Sci USA* **108:** 16014–16019.

Kuehne, S.A., Cartman, S.T., Heap, J.T., Kelly, M.L., Cockayne, A., and Minton, N.P. (2010) The role of toxin A and toxin B in *Clostridium difficile* infection. *Nature* **467:** 711–713.

Lawley, T.D., Clare, S., Walker, A.W., Goulding, D., Stabler, R.A., Croucher, N., *et al*. (2009a) Antibiotic treatment of *Clostridium difficile* carrier mice triggers a supershedder state, spore-mediated transmission, and severe disease in immunocompromised hosts. *Infect Immun* **77:** 3661–3669.

Lawley, T.D., Croucher, N.J., Yu, L., Clare, S., Sebaihia, M., Goulding, D., *et al*. (2009b) Proteomic and genomic characterization of highly infectious *Clostridium difficile* 630 spores. *J Bacteriol* **191:** 5377–5386.

Nagaro, K.J., Phillips, S.T., Cheknis, A.K., Sambol, S.P., Zukowski, W.E., Johnson, S., and Gerding, D.N. (2013) Nontoxigenic *Clostridium difficile* protects hamsters against challenge with historic and epidemic strains of toxigenic BI/NAP1/027 *C. difficile*. *Antimicrob Agents Chemother* **57:** 5266–5270.

Oliva, C., Turnbough, C.L., Jr, and Kearney, J.F. (2009) CD14-Mac-1 interactions in *Bacillus anthracis* spore internalization by macrophages. *Proc Natl Acad Sci USA* **106:** 13957–13962.

Ozanne, G. (1984) Estimation of endpoints in biological systems. *Comput Biol Med* **14:** 377–384.

Paredes-Sabja, D., Bond, C., Carman, R.J., Setlow, P., and Sarker, M.R. (2008) Germination of spores of *Clostridium difficile* strains, including isolates from a hospital outbreak of *Clostridium difficile*-associated disease (CDAD). *Microbiology* **154:** 2241–2250.

Permpoonpattana, P., Hong, H.A., Phetcharaburanin, J., Huang, J.M., Cook, J., Fairweather, N.F., and Cutting, S.M. (2011a) Immunization with *Bacillus* spores expressing toxin A peptide repeats protects against infection with *Clostridium difficile* strains producing toxins A and B. *Infect Immun* **79:** 2295–2302.

Permpoonpattana, P., Tolls, E.H., Nadem, R., Tan, S., Brisson, A., and Cutting, S.M. (2011b) Surface layers of *Clostridium difficile* endospores. *J Bacteriol* **193:** 6461–6470.

Permpoonpattana, P., Phetcharaburanin, J., Mikelsone, A., Dembek, M., Tan, S., Brisson, M.C., *et al*. (2013) Functional characterization of *Clostridium difficile* spore coat proteins. *J Bacteriol* **195:** 1492–1503.

Perutka, J., Wang, W., Goerlitz, D., and Lambowitz, A.M. (2004) Use of computer-designed group II introns to disrupt *Escherichia coli* DExH/D-box protein and DNA helicase genes. *J Mol Biol* **336:** 421–439.

Pizarro-Guajardo, M., Olguín-Araneda, V., Barra-Carrasco, J., Brito-Silva, C., Sarker, M.R., and Paredes-Sabja, D. (2014) Characterization of the collagen-like exosporium protein, BclA1, of *Clostridium difficile* spores. *Anaerobe* **25:** 18–30.

Razaq, N., Sambol, S., Nagaro, K., Zukowski, W., Cheknis, A., Johnson, S., and Gerding, D.N. (2007) Infection of hamsters with historical and epidemic BI types of *Clostridium difficile*. *J Infect Dis* **196:** 1813–1819.

Redmond, C., Baillie, L.W., Hibbs, S., Moir, A.J., and Moir, A. (2004) Identification of proteins in the exosporium of *Bacillus anthracis*. *Microbiology* **150:** 355–363.

Rupnik, M., Wilcox, M.H., and Gerding, D.N. (2009) *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. *Nat Rev Microbiol* **7:** 526–536.

Sambol, S.P., Tang, J.K., Merrigan, M.M., Johnson, S., and Gerding, D.N. (2001) Infection of hamsters with epidemiologically important strains of *Clostridium difficile*. *J Infect Dis* **183:** 1760–1766.

Sebaihia, M., Wren, B.W., Mullany, P., Fairweather, N.F., Minton, N., Stabler, R., *et al*. (2006) The multidrug-resistant human pathogen *Clostridium difficile* has a highly mobile, mosaic genome. *Nat Genet* **38:** 779–786.

Songer, J.G., and Anderson, M.A. (2006) *Clostridium difficile*: an important pathogen of food animals. *Anaerobe* **12:** 1–4.

Stabler, R.A., He, M., Dawson, L., Martin, M., Valiente, E., Corton, C., *et al*. (2009) Comparative genome and phenotypic analysis of *Clostridium difficile* 027 strains provides insight into the evolution of a hypervirulent bacterium. *Genome Biol* **10:** R102.

Steichen, C., Chen, P., Kearney, J.F., and Turnbough, C.L., Jr (2003) Identification of the immunodominant protein and other proteins of the *Bacillus anthracis* exosporium. *J Bacteriol* **185:** 1903–1910.

Sylvestre, P., Couture-Tosi, E., and Mock, M. (2002) A collagen-like surface glycoprotein is a structural component of the *Bacillus anthracis* exosporium. *Mol Microbiol* **45:** 169–178.

Sylvestre, P., Couture-Tosi, E., and Mock, M. (2003) Polymorphism in the collagen-like region of the *Bacillus anthracis* BclA protein leads to variation in exosporium filament length. *J Bacteriol* **185:** 1555–1563.

Tan, L., and Turnbough, C.L., Jr (2010) Sequence motifs and proteolytic cleavage of the collagen-like glycoprotein BclA required for its attachment to the exosporium of *Bacillus anthracis*. *J Bacteriol* **192:** 1259–1268.

Theriot, C.M., Koumpouras, C.C., Carlson, P.E., Bergin, I.I., Aronoff, D.M., and Young, V.B. (2011) Cefoperazone-treated mice as an experimental platform to assess differential virulence of *Clostridium difficile* strains. *Gut Microbes* **2:** 326–334.

Thompson, B.M., and Stewart, G.C. (2008) Targeting of the BclA and BclB proteins to the *Bacillus anthracis* spore surface. *Mol Microbiol* **70:** 421–434.

Thompson, B.M., Binkley, J.M., and Stewart, G.C. (2011a) Current physical and SDS extraction methods do not efficiently remove exosporium proteins from *Bacillus anthracis* spores. *J Microbiol Methods* **85:** 143–148.

Thompson, B.M., Hsieh, H.Y., Spreng, K.A., and Stewart, G.C. (2011b) The co-dependence of BxpB/ExsFA and BclA for proper incorporation into the exosporium of *Bacillus anthracis*. *Mol Microbiol* **79:** 799–813.

Todd, S.J., Moir, A.J., Johnson, M.J., and Moir, A. (2003)

Genes of *Bacillus cereus* and *Bacillus anthracis* encoding proteins of the exosporium. *J Bacteriol* **185:** 3373–3378.

Waller, L.N., Stump, M.J., Fox, K.F., Harley, W.M., Fox, A., Stewart, G.C., and Shahgholi, M. (2005) Identification of a second collagen-like glycoprotein produced by *Bacillus anthracis* and demonstration of associated spore-specific sugars. *J Bacteriol* **187:** 4592–4597.

Wust, J., Sullivan, N.M., Hardegger, U., and Wilkins, T.D. (1982) Investigation of an outbreak of antibiotic-associated colitis by various typing methods. *J Clin Microbiol* **16:** 1096–1101.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

RESEARCH LETTER

Use of *Bacillus subtilis* PXN21 spores for suppression of *Clostridium difficile* infection symptoms in a murine model

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Clostridium difficile; spores; probiotics; *Bacillus subtilis*.

Abstract

Clostridium difficile is the primary cause of nosocomial diarrhoea in healthcare centres of the developed world. Only a few antibiotics are available for treatment, and relapses are common in patients undergoing antibiotic therapy. New approaches are required to reduce reliance on antibiotics, the use of which represents a primary risk factor for development of *C. difficile* infections. Supplementation of the gut flora with probiotics represents a key area for producing more successful treatment options for *C. difficile* infection (CDI). In this study, spores of *B. subtilis* have been evaluated as a potential probiotic treatment against CDI. Using a murine model of infection, we demonstrate that oral administration of *B. subtilis* spores can attenuate the symptoms of infection. We further show that (1) suppression of symptoms was better if spores were administered post infection, and (2) germination of the spore to a vegetative cell may be an integral part of how CDI is suppressed. The results of this study highlight the potential of this bacterium as a probiotic treatment for CDI.

Introduction

Clostridium difficile is the primary aetiological agent of nosocomial diarrhoea in developed countries. Disease occurs when the normal colonic flora is disrupted, typically associated with antibiotic treatment. Dysbiosis of the microbiota of the gastro-intestinal (GI) tract allows ingested spores of *C. difficile* to germinate and proliferate. Clinical symptoms are mediated by production of two major exotoxins, toxin A and toxin B. Infection results in a variety of manifestations ranging from a nonsymptomatic carrier state to diarrhoea and inflammation of the gut. Severe infections can lead to development of pseudomembranous colitis and toxic megacolon which is potentially fatal (Rupnik *et al.*, 2009). Hospitalised elderly individuals receiving antibiotic treatment are the primary group at risk for developing this infection, although incidence in other groups such as pregnant woman (Rouphael *et al.*, 2008) and younger individuals is increasing (Wilcox *et al.*, 2008). Production of highly resistant spores during the infection cycle presents a major challenge to the control of infection, as spores play a significant role in the spread and persistence of disease (Dawson *et al.*, 2012; Deakin *et al.*, 2012).

The implementation of mandatory reporting across the UK in 2007 has resulted in a decrease in the number of cases as awareness of *C. difficile* has increased (HPA, 2009). However, the emergence of 'hypervirulent' strains and high relapse rates of infection have contributed to the persistence of this infection. A limited number of antibiotics are available for use in the treatment of *C. difficile* infection (CDI), and around 20% of treated cases will experience a recurrence of infection following treatment, representing a key challenge for treatment of CDI. Use of antibiotics is also a principle risk factor for development of infection. Due to these limitations in the use of antibiotics, alternative treatment strategies are being investigated. Most promising is faecal transplantation therapy consisting of an extract prepared from homogenised stool sample which is administered to the patient via a nasogastric tube (MacConnachie *et al.*, 2009). A second approach is that of the use of probiotics and/or prebiotics which have shown some effect in reducing symptoms of CDI. As an adjunct to antibiotic therapy, species of *Lactobacillus*, *Bifidobacteria* and *Saccharomyces boulardii* appear to reduce the incidence of CDI in human studies although none has shown full protection (Pochapin, 2000; Surawicz *et al.*, 2000; Wullt *et al.*, 2003). No

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consensus has been reached as to how these bacterial supplements exert their effect, but one likely mechanism is by influencing the gut microbiota such that growth of *C. difficile* is inhibited. A number of human trials are in progress.

In this work, we used spores of *Bacillus subtilis*, a bacterial species already widely associated with probiotic use, to suppress symptoms of *C. difficile* CDI. Our objective was to assess the capacity of this bacterium, as a probiotic, to protect against disease.

Methods

Strains

(1) *B. subtilis:* PXN21 is one of fourteen strains in the commercial product Bio-Kult (Probiotics International Ltd, Lopen Head, UK) and was obtained from NCIMB where it is registered as NCIMB strain 30223. PY79 is a laboratory strain and derived from the type strain 168 (Youngman *et al.*, 1984). SC2376 *gerD-cwlD* is congenic to strain PY79 and carries a severe germination defect (Mauriello *et al.*, 2007).
(2) *Clostridium difficile:* R20291 is an epidemic strain of ribotype 027 and was obtained from T. Lawley (Wellcome Trust Sanger Institute, UK). *Clostridium difficile* VPI 10463 (provided by B. Wren, London School of Hygiene and Tropical Medicine, UK) was originally isolated in the USA in 1980 and has since been used as a reference strain.

General methods

Spores of *B. subtilis* strains were prepared in liquid medium by the exhaustion method as described previously (Harwood & Cutting, 1990). Heat-killed spores were prepared by autoclaving suspensions of spores at 120 °C for 20 min. Spores of *C. difficile* were prepared by growth on SMC agar plates using an anaerobic incubator (Don Whitley, UK) as described previously (Permpoonpattana *et al.*, 2011).

Animal models of infection

Female C57BL/6 mice (Charles River, UK) aged 6–8 weeks were used in all experiments, with animals housed in IVC units (Tecniplast, Italy). Sterile bedding was used in cages, and animals had *ad libitum* access to sterile water and food.

Two animal models were used to evaluate CDI: (1) a nonfatal colonisation model modified from that of Lawley (Lawley *et al.*, 2009) and (2) the fatal, severe, disease model of Chen *et al.* (Chen *et al.*, 2008).

(1) Nonfatal colonisation model: a single oro-gastric (o.g.) dose of clindamycin (30 mg kg^{-1}) was used 1 day prior to o.g. delivery of 1×10^4 R20291 spores. Faecal samples were collected daily. Faecal samples were homogenised and then incubated with 70% ethanol for 20 min at RT. Samples were pelleted and ethanol removed before suspension in sterile water for serial dilution on brain heart infusion agar (BHI) supplemented with 0.1% cysteine and 0.1% sodium taurocholate for enumeration of ethanol-resistant spore counts.
(2) Fatal model: An antibiotic cocktail containing kanamycin (40 mg kg^{-1}), gentamicin (3.5 mg kg^{-1}), colistin (4.2 mg kg^{-1}), metronidazole (21.5 mg kg^{-1}) and vancomycin (4.5 mg kg^{-1}) was delivered via the o.g. route in a final volume of 200 μL per dose. Mice received three doses of the cocktail on consecutive days (D1, D2 and D3). On D5, mice were given a single o.g. dose of clindamycin (30 mg kg^{-1}). Animals were then infected with 1×10^4 of VPI 10463 spores on D6. Animals were monitored for appearance of symptoms, with faecal samples and weights recorded daily. Animals were culled at the clinical end point of disease, which was considered when animals lost 20% of original body weight.

Probiotic treatment

Doses of 1×10^9 spores in 200 μL of sterile H_2O were delivered by o.g. gavage. Animals received treatment daily: preinfection doses commenced on day 7 (where day 0 was the point of infection with *C. difficile*) with a total of seven doses received, and postinfection doses started on day 1, and administered until day 7 of study.

Haematoxylin and eosin (H & E) staining

C57Bl/6 mice (Charles River) were culled 3 days post infection with *C. difficile*. Colon and caecum were removed from animals that had received probiotic treatment either pre- or post infection, using nontreated and noninfected animals as controls. Tissue contents were carefully washed out and tissues fixed using 4% paraformaldehyde. Tissues were then sectioned, fixed on slides and stained with H&E (TUPI Manufacturing, Woodbridge, UK). Appearance of tissue was assessed microscopically once stained and images were taken using associated camera and software (GT Vision, Haverhill, UK).

In vitro innate immunity tests

RAW264.7 macrophage cells were seeded to six-well plates (3×10^5) in antibiotic-free growth medium (DMEM with 10% foetal bovine serum) and incubated for 2 days to develop confluency. Cells were washed two

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times with fresh growth medium then infected with media containing bacteria (spores or vegetative cells of *B. subtilis* PXN21 at a CFU of 1×10^7 mL^{-1}).

For the Toll-like receptor 2 (TLR2) expression assay, a protocol previously used to study immunostimulatory properties of *B. subtilis* (Huang *et al.*, 2008a, b) was adapted for the assay. Cells were incubated for 4 h before washing two further times with sterile PBS. Macrophages were then lysed *in situ* and total RNAs extracted using an RNeasy Kit (Qiagen, the Netherlands) as per manufacturer's instructions. cDNA was produced using a Precision qScript Reverse Transcriptase Kit (PrimerDesign, Southampton, UK). qPCR used primers targeted at the TLR2 gene (forward: AAGAGGAAGCCCAAGAAAGC reverse: CAATGGGAATCCTGCTCACT), with β-actin (forward: AGAGGCAAATCGTGCCTGAC reverse: CAATAGTGATCATCACCTGGCCCT) as a reference gene. A three-step PCR cycle was run 50 times with the following conditions: 95 °C 15 s, 55 °C 30 s and 72 °C 10 s. Data were analysed using ROTORGENE 3000 software (Qiagen, the Netherlands).

For detection of cytokines, macrophages were incubated with antibiotic-free growth medium (DMEM with 10% foetal bovine serum) containing spores or vegetative cells of *B. subtilis* PXN21 at a CFU of 1×10^7 mL^{-1} bacteria for 24 h. Lipopolysaccharide, (Sigma, MO) was used as a positive control for activation of macrophages *in vitro*. Supernatants were then removed, and detection of cytokines in the medium was carried out using commercial ELISA Kits (eBioscience, Hatfield, UK). Plates were read at 450 nm using a Spectramax microplate reader (Molecular Devices, CA).

Results

Colonisation resistance

Nonfatal colonisation of *C. difficile* was induced in a murine model of infection by antibiotic treatment followed by infection with *C. difficile* R20291 spores (Fig. 1). Levels of *C. difficile* spores present in faecal samples demonstrated that oral delivery of *B. subtilis* PXN21 spores both prior to and post infection had no significant effect on reducing *C. difficile* colonisation. In all groups, peak levels of spores were detected on day two post infection, and although *C. difficile* levels were highest in mice receiving no probiotic treatment, this was not significant ($P = 0.1$).

Attenuation of symptoms in a model of fatal disease

A study model utilising *C. difficile* strain VPI 10463 to induce a fatal infection in mice was used to assess the ability of *B. subtilis* PXN21 to influence the clinical outcome of infection. This model of infection utilised predosing with a cocktail of different antibiotics including vancomycin that was designed to mimic the clinical situation of antibiotic therapy in humans (Chen *et al.*, 2008). PXN21 spores were administered to mice before and after infection with VP1 10463, and both pre- and post infection with *C. difficile* increased survival compared with nontreated animals (Fig. 2a). In pilot studies, we showed that administration of PXN21 spores did not reduce the efficacy of vancomycin in treatment of CDI in mice (data not shown). Delivery of PXN21 spores prior to infection resulted in a survival rate of 41.6% while 66.6% survival was achieved in animals treated post-CDI (Fig. 2a); this compared to a survival rate of 16.6% in nontreated groups. Interestingly, weight profiles of infected animals added further complexity to our results (Fig. 2b). Animals that survived infection having received probiotic treatment prior to infection displayed less weight loss than those infected with PXN21 spores post infection. Post infection-treated animals displayed a similar weight profile to the nontreated group, despite having the highest rate of survival.

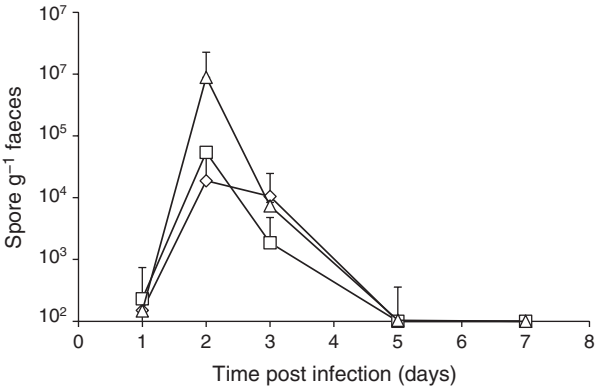

Fig. 1. Suppression of colonisation. Three groups of mice ($n = 6$) were treated as follows. (1) Preinfection, animals received seven daily o.g. doses of PXN21 spores (10^9) and were then infected with a single dose of *Clostridium difficile* R20291 spores (10^4) (◊); (2) postinfection, animals were infected with a single dose of R20291 spores (10^4) and then dosed o.g. for 7-days thereafter with PXN21 spores (10^9) (□); (3) no treatment, animals were infected with a single dose of R20291 spores (10^4) (Δ). In each treatment programme, the number of ethanol-resistant spores of *C. difficile* R20291 shed in the faeces was enumerated by plating serial dilutions of treated faecal samples on BHISS agar containing 0.1% sodium taurocholate.

Live *B. subtilis* spores are required for suppression of infection

Administering PXN21 spores post infection was more effective in preventing CDI than predosing. As killed

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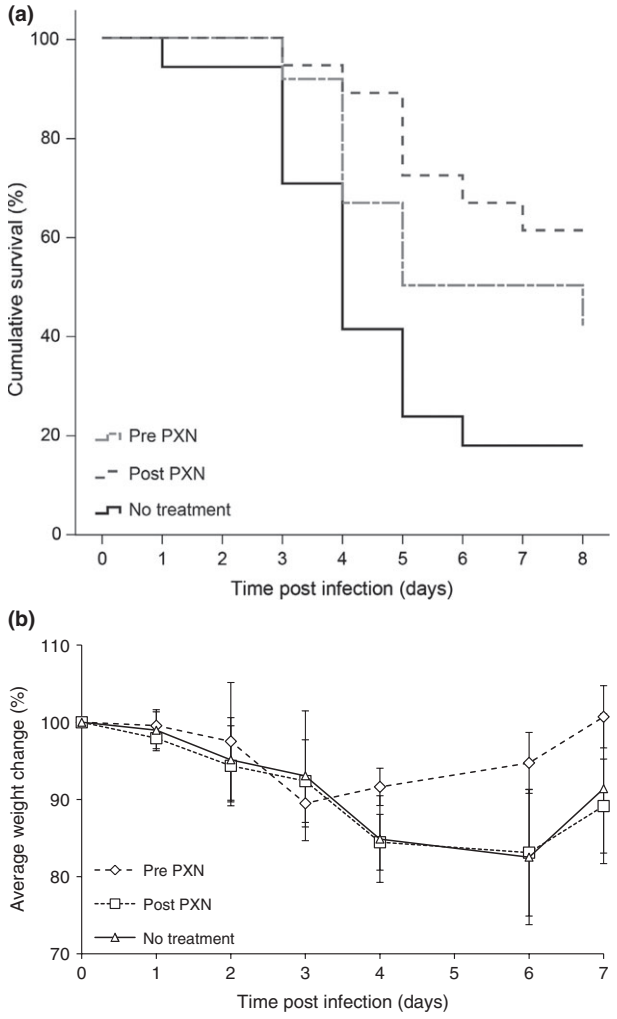

Fig. 2. Suppression of CDI using PXN21 spores. Groups ($n \geq 6$) of mice received daily o.g. doses of 10^9 *Bacillus subtilis* PXN21 spores either preinfection with *Clostridium difficile* VP1 10463 spores (10^4) or postinfection. The preinfection groups received 7 doses of PXN21 spores and postinfection seven doses of PXN21 after infection. (a) Kaplan–Meier survival plot for pre- and postinfection treatment groups. (b)% weight change for groups receiving either daily doses of PXN21 spores preinfection (◊), post infection (□) or no treatment (Δ).

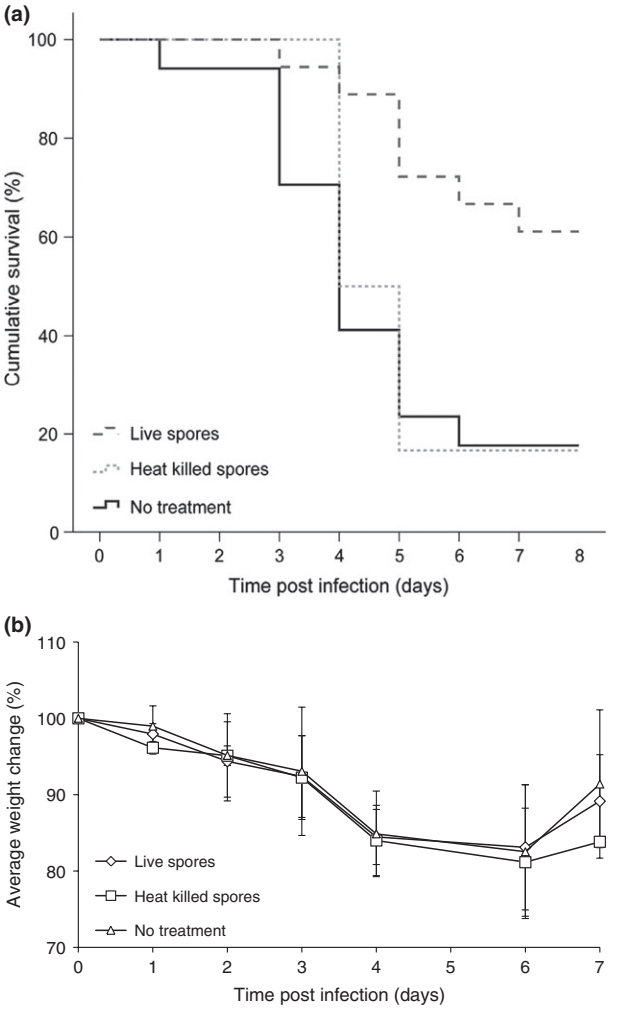

Fig. 3. Live PXN21 spores are required for suppression of CDI. Groups of mice were infected with *Clostridium difficile* VPI 10463 as described in Fig. 2 but in this case dosed post infection with live PXN21 spores (10^9) or heat-killed PXN21 spores. (a) Kaplan–Meier survival plot for pre- and postinfection treatment groups. (b) % weight change for groups receiving either daily doses of PXN21 spores preinfection (◊), postinfection (□) or no treatment (Δ).

spores have been shown to have adjuvant properties and can enhance the immunity of prototype vaccines (Barnes *et al.*, 2007; Huang *et al.*, 2010), we addressed whether spore viability might affect survival in mice administered PXN21 spores post infection. As shown in Fig. 3, a dosing of mice post infection with killed spores showed almost no improvement on survival. By contrast, dosing with live spores markedly improved survival. As shown earlier (Fig. 2a), treatment post infection with live PXN21 spores increases survival rate but did not induce an improvement in the weight profile of infected animals.

Histopathology

H&E-stained tissue sections were produced to assess pathology affecting gut tissues in groups receiving *B. subtilis* PXN21 spores. Images from tissue sections suggest that in animals given PXN21 spores, the gut tissues display reduced pathology, with less damage to cell structure than in untreated animals (Fig. 4). Tissues from healthy, noninfected mice exhibited well-defined tissue structure and cell integrity (Fig. 4a). Toxin-mediated damage is evident in the breakdown of tissue structure and disruption of epithelial cell membranes (Fig. 4d). The level of

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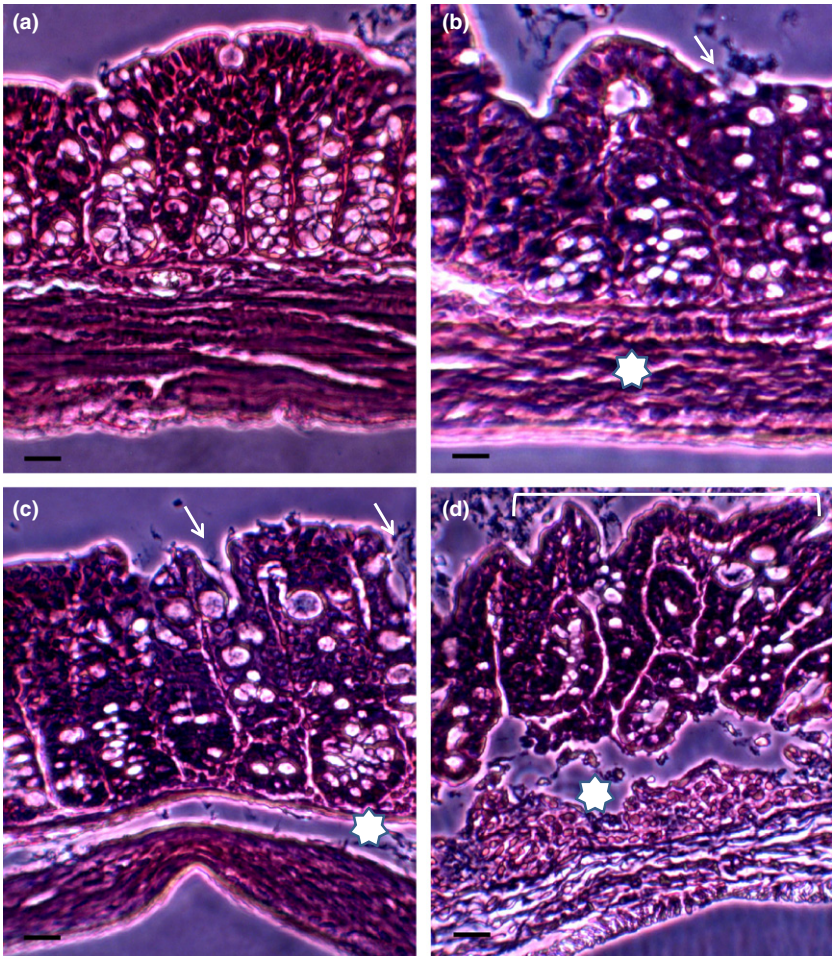

Fig. 4. H&E-stained sections of colon from *Clostridium difficile* VPI 10463 infected mice. (a) Uninfected mice, displaying healthy tissue and intact epithelial lining to colon; (b) mice treated with PXN21 spores preinfection with some damage to epithelial structure but with lining still intact although evidence of mild oedema in the submucosa; (c) mice treated with PXN21 spores post infection showed some damage to the integrity of the epithelial lining and some submucosal disruption; and (d) untreated mice displaying extensive damage to the colonic epithelial lining and erosion of the submucosa (shown by white arrows and stars). Scale bars 25 μm.

damage in terms of cell structure and tissue integrity was reduced in groups receiving PXN21 spores both before (Fig. 4b) and after infection (Fig. 4c). However, PXN21 treatment did not completely protect tissues, and some cellular damage was present in PXN21 treated animals, with minor disruption of cell membranes apparent.

Mechanisms of probiotic action

One mechanism for how PXN21 spores might suppress symptoms of CDI includes stimulation of the innate immune responses. To address this, first we examined stimulation of the Toll-like receptor 2 gene (TLR2) and, second, induction of two proinflammatory cytokines, IL-6 and TNF-α. Using an *in vitro* assay, expression of the TLR2 gene was measured using real-time PCR (RT-PCR) in RAW264.7 macrophages infected with PXN21 spores (live or killed) and PXN21 vegetative cells. This approach has been used previously to show potential TLR2 stimulants as TLR2 agonists can upregulate the TLR2 gene (Liu *et al.*, 2001). As shown in Fig. 5, vegetative cells and live spores of PXN21 efficiently stimulated expression of the TLR2 gene implying an interaction with the TLR2 receptor. Levels of expression were consistent with that of purified peptidoglycan which is a known TLR2 agonist (Liu *et al.*, 2001). By contrast heat-killed PXN21 spores showed a marked (four- to fivefold) reduction in TLR2 expression. Induction of the proinflammatory cytokines IL-6 and TNF-α was only apparent in macrophages infected with live PXN21 spores, and virtually no activity was present in cells infected with heat-killed spores (Fig. 6).

Discussion

This study shows that oral administration of *B. subtilis* PXN21 spores can reduce symptoms of CDI in a murine model of disease. PXN21 is a component of a commercial probiotic product and of course numerous products carry spores of *B. subtilis* as their active ingredient (Permpoonpattana *et al.*, 2012). In addition, some traditional foods such as natto contain live spores of *B. subtilis* and have

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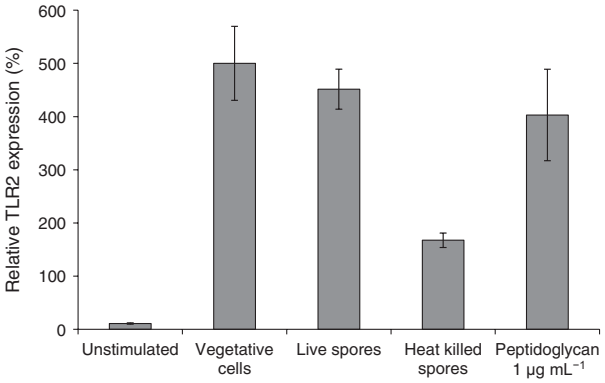

Fig. 5. *In vitro* analysis. TLR2 expression in murine macrophages (RAW264.7) after incubation with *Bacillus subtilis* PXN21. Vegetative cells, spores or heat-killed PXN21 spores were added to cells at a concentration of 10^7 mL^{-1} and incubated for 4 h. RNA was extracted from cells, and TLR2 gene expression was calculated relative to β-actin expression, using qPCR. Data are from three individual experiments.

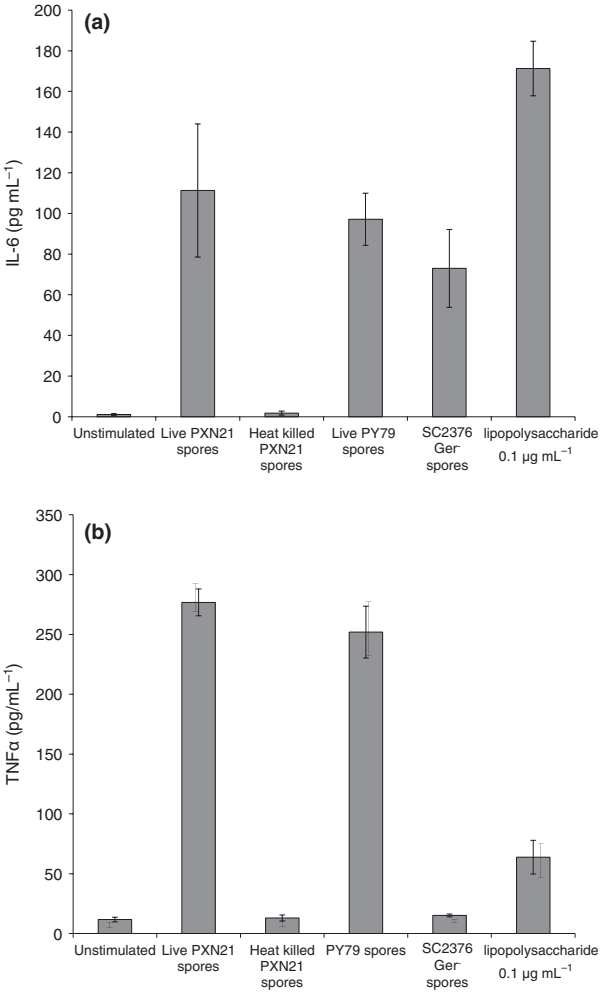

Fig. 6. Cytokine production. Production of the pro-inflammatory cytokines IL-6 (a) and TNFα (b) in murine macrophages (RAW264.7) after incubation with *Bacillus subtilis* PXN21. Live and heat-killed spores were added to cells at a concentration of 1×10^7 mL^{-1} and incubated for 24 h. Lipopolysaccharide was used as a control for induction of cytokines. Commercial ELISA kits (eBioscience) were used to quantify levels of IL-6 and TNFα in supernatants of cultured cells. Data are from three individual experiments.

been recognised as carrying health benefits (Hosoi & Kiuchi, 2004). There is also good data showing that oral consumption of probiotic bacteria or prebiotics can have some effect in reducing the symptoms or occurrence of CDI. As an adjunct to antibiotic therapy, species of *Lactobacillus*, *Bifidobacteria* and *S. boulardii* appear to reduce the incidence of CDI in human studies although no study has yet shown full protection (Pochapin, 2000; Surawicz *et al.*, 2000; Wullt *et al.*, 2003). Moreover, no consensus has been reached as to how these bacterial supplements exert their effect. A number of mechanisms can be considered. First, by influencing the gut microbiota such that growth of *C. difficile* is inhibited possibly by out-competing for available nutrients or possibly by secretion of antimicrobials. This is the basis of treatments that focuses on supplementation of the gut flora to prevent *C. difficile* from maintaining a niche within the GI tract. Faecal transplants, for example, while unpleasant, have been demonstrated to have a high success rate in resolving persistent infection (Gough *et al.*, 2011) although carry risk of further infections unless samples are heavily screened prior to use. More recently, 'synthetic stools' have been developed (Lawley *et al.*, 2012; Petrof *et al.*, 2013), selecting particular microorganisms that are able to resolve infections through rebuilding the microbial communities in the gut without the unpleasant and uncertain aspect of faecal transplants. Supplementation of the gut flora with a complex or even minimalist mix of microorganisms mimics the protection against colonisation by *C. difficile* that the normal healthy microbial community of the gut provides. Single strains of bacteria are less likely to be able to successfully act as a substitute for the complex microbial communities of the gut. However, used alongside conventional antibiotics, particular strains could provide enough influence in the gut environment to reduce the symptoms of infection and aid restoration of the gut flora. A second mechanism would be by stimulation of a robust innate immune response in the gut-associated lymphoid tissue (GALT). The importance of the innate immune response in CDI has been demonstrated previously, with mice carrying deficiencies in immune signalling (Lawley *et al.*, 2009) more vulnerable to infection. Direct stimulation of the innate immune receptor TLR5 has also been demonstrated to successfully protect against

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disease in infected mice (Jarchum *et al.*, 2011). Finally, for toxin producing pathogens, it is possible that probiotic bacteria might subtract toxins produced in the gut lumen. This is particularly true for probiotic spores as these entities have been shown able to efficiently adsorb toxins onto the surface of the spore (Huang *et al.*, 2010).

Our studies shown here support the role of innate immunity as we have shown that PXN21 spores can upregulate expression of TLR2. Peptidoglycan is one of many ligands associated with TLR2 induction and was used as a control in our *in vitro* experiments. Spores carry peptidoglycan in their cortex, a layer lying beneath the spore coat; however, when autoclaved spores were used, induction of TLR2 was substantially reduced. Vegetative cells of PXN21 showed strong induction of TLR2, so we assign this to peptidoglycan exposed on the cell envelope. This suggests that spore germination and release of the growing vegetative cell might be critical to induction of TLR2. We base this assumption on the fact that when the spore is inactivated by autoclaving, it is not broken but remains intact, so it is unlikely that peptidoglycan within the spore cortex is released in significant quantities. In other studies, TLR2 was shown to be activated by live *B. subtilis* PY79 spores but not by heat-killed PY79 spores or spores unable to germinate (Huang *et al.*, 2008a, b). Interestingly, in other work, we have shown that live spores of *B. subtilis* PY79 were shown to significantly delay symptoms of CDI in a hamster model of infection (Permpoonpattana *et al.*, 2011). Although hamsters did eventually die from CDI, PY79 spores were clearly shown to delay fatalities and most likely this is due to germination and release of live *B. subtilis* vegetative cells. A clear result of TLR2 activation would be induction of pro-inflammatory cytokines, and we have confirmed here that (1) both IL-6 and TNF-α were induced and (2) induction did not occur with heat-killed PXN21 spores. Again, proof that this must arise from germination of live spores to the vegetative cell comes from indirect evidence using germinating and nongerminating spores of PY79.

It is not clear why dosing of spores beginning at the onset of CDI (post dosing) was superior to pre dosing. Innate immunity is normally short-lived and would be induced pre- and post dosing and should not really affect the outcome. One possibility, spores might have some ability to adsorb and subtract toxins produced by *C. difficile*, and this process might be more effective if spores are dosed at the time infection begins. We have found that PXN21 spores can adsorb *C. difficile* toxin A *in vitro* but have not yet addressed whether this might occur *in vivo*. One problem with this explanation is practicalities of adsorption in the GI tract due to the interference of the gut microbiota. We are essentially sceptical but cannot completely exclude the possibility that with regular doses of spores, some level of adsorption could occur in the intestine.

Alternatively, prior dosing of probiotics could allow the probiotic strain to colonise the gut of the animal prior to infection allowing for some level of protection through supplementation of the gut flora. *Bacillus subtilis* can survive and reproduce in the murine gut (Hoa *et al.*, 2001; Tam *et al.*, 2006), and in the case of PXN21, this strain has been shown to persist in the GI tract of mice for 18 days following a single oral dose of spores (Permpoonpattana *et al.*, 2012). Although often considered a soil-based organism, it is likely that *B. subtilis* has adapted for at least transient colonisation of the animal GI tract (Hong *et al.*, 2009; Schyns *et al.*, 2014). Reduced success of predosing could therefore be due to individual variation in animals in terms of successful colonisation.

Clearly, suppression was not complete and animals succumbed to CDI. We are not necessarily alarmed by this, and it is quite possible that the dose of PXN21 spores used was not at an optimal level. It is also possible that the dosing regimen might need adjustment, and we cannot rule out that excessive dosing might induce some level of tolerance. PXN21 spores as a treatment for CDI might have some value, and we state only that symptoms are significantly suppressed. If translated to humans, spores might have some utility possibly as an adjunct therapy with no apparent side effects.

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References

Barnes AG, Cerovic V, Hobson PS & Klavinskis LS (2007) *Bacillus subtilis* spores: a novel microparticle adjuvant which can instruct a balanced Th1 and Th2 immune response to specific antigen. *Eur J Immunol* **37**: 1538–1547.

Chen X, Katchar K, Goldsmith JD, Nanthakumar N, Cheknis A, Gerding DN & Kelly CP (2008) A mouse model of *Clostridium difficile*-associated disease. *Gastroenterology* **135**: 1984–1992.

Dawson LF, Valiente E, Faulds-Pain A, Donahue EH & Wren BW (2012) Characterisation of *Clostridium difficile* biofilm formation, a role for Spo0A. *PLoS One* **7**: e50527.

Deakin LJ, Clare S, Fagan RP, Dawson LF, Pickard DJ, West MR, Wren BW, Fairweather NF, Dougan G & Lawley TD (2012) The *Clostridium difficile spo0A* gene is a persistence and transmission factor. *Infect Immun* **80**: 2704–2711.

Gough E, Shaikh H & Manges AR (2011) Systematic review of intestinal microbiota transplantation (fecal bacteriotherapy) for recurrent *Clostridium difficile* infection. *Clin Infect Dis* **53**: 994–1002.

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Harwood CR & Cutting SM (1990) *Molecular Biological Methods for Bacillus*. John Wiley & Sons Ltd, Chichester, UK.

Hoa TT, Duc LH, Isticato R, Baccigalupi L, Ricca E, Van PH & Cutting SM (2001) Fate and dissemination of *Bacillus subtilis* spores in a murine model. *Appl Environ Microbiol* **67**: 3819–3823.

Hong HA, To E, Fakhry S, Baccigalupi L, Ricca E & Cutting SM (2009) Defining the natural habitat of *Bacillus* spore-formers. *Res Microbiol* **160**: 375–379.

Hosoi T & Kiuchi K (2004) *Production and Probiotic Effects of Natto*. (Ricca E, Henriques AO & Cutting SM, eds), pp. 143–154. Horizon Bioscience, Wymondham, UK.

HPA (2009) *Clostridium difficile* Infection: How to Deal with the Problem. pp. 130. Health Protection Agency, London, UK.

Huang JM, La Ragione RM, Nunez A & Cutting SM (2008a) Immunostimulatory activity of *Bacillus* spores. *FEMS Immunol Med Microbiol* **53**: 195–203.

Huang JM, La Ragione RM, Cooley WA, Todryk S & Cutting SM (2008b) Cytoplasmic delivery of antigens, by *Bacillus subtilis* enhances Th1 responses. *Vaccine* **26**: 6043–6052.

Huang JM, Hong HA, Van Tong H, Hoang TH, Brisson A & Cutting SM (2010) Mucosal delivery of antigens using adsorption to bacterial spores. *Vaccine* **28**: 1021–1030.

Jarchum I, Liu M, Lipuma L & Pamer EG (2011) Toll-like receptor-5 stimulation protects mice from acute *Clostridium difficile* colitis. *Infect Immun* **79**: 1498–1503.

Lawley TD, Clare S, Walker AW *et al.* (2009) Antibiotic treatment of *Clostridium difficile* carrier mice triggers a supershedder state, spore-mediated transmission, and severe disease in immunocompromised hosts. *Infect Immun* **77**: 3661–3669.

Lawley TD, Clare S, Walker AW *et al.* (2012) Targeted restoration of the intestinal microbiota with a simple, defined bacteriotherapy resolves relapsing *Clostridium difficile* disease in mice. *PLoS Pathog* **8**: e1002995.

Liu Y, Wang Y, Yamakuchi M, Isowaki S, Nagata E, Kanmura Y, Kitajima I & Maruyama I (2001) Upregulation of toll-like receptor 2 gene expression in macrophage response to peptidoglycan and high concentration of lipopolysaccharide is involved in NF-kappa b activation. *Infect Immun* **69**: 2788–2796.

MacConnachie AA, Fox R, Kennedy DR & Seaton RA (2009) Faecal transplant for recurrent *Clostridium difficile*-associated diarrhoea: a UK case series. *QJM* **102**: 781–784.

Mauriello EM, Cangiano G, Maurano F, Saggese V, De Felice M, Rossi M & Ricca E (2007) Germination-independent induction of cellular immune response by *Bacillus subtilis* spores displaying the C fragment of the tetanus toxin. *Vaccine* **25**: 788–793.

Permpoonpattana P, Hong HA, Phetcharaburanin J, Huang JM, Cook J, Fairweather NF & Cutting SM (2011) Immunization with *Bacillus* spores expressing toxin A peptide repeats protects against infection with *Clostridium difficile* strains producing toxins A and B. *Infect Immun* **79**: 2295–2302.

Permpoonpattana P, Hong HA, Khaneja R & Cutting SM (2012) Evaluation of *Bacillus subtilis* strains as probiotics and their potential as a food ingredient. *Benef Microbes* **3**: 127–135.

Petrof EO, Gloor GB, Vanner SJ, Weese SJ, Carter D, Daigneault MC, Brown EM, Schroeter K & Allen-Vercoe E (2013) Stool substitute transplant therapy for the eradication of *Clostridium difficile* infection: 'RePOOPulating' the gut. *Microbiome* **1**: 3.

Pochapin M (2000) The effect of probiotics on *Clostridium difficile* diarrhea. *Am J Gastroenterol* **95**: S11–S13.

Rouphael NG, O'Donnell JA, Bhatnagar J *et al.* (2008) *Clostridium difficile*-associated diarrhea: an emerging threat to pregnant women. *Am J Obstet Gynecol* **198**: 635 e631–e636.

Rupnik M, Wilcox MH & Gerding DN (2009) *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. *Nat Rev Microbiol* **7**: 526–536.

Schyns G, Serra CR, Lapointe T, Pereira-Leal JB, Potot S, Fickers P, Perkins JB, Wyss M & Henriques AO (2014) Genome of a gut strain of *Bacillus subtilis*. *Genome Announc* **2**: in press.

Surawicz CM, McFarland LV, Greenberg RN *et al.* (2000) The search for a better treatment for recurrent *Clostridium difficile* disease: use of high-dose vancomycin combined with *Saccharomyces boulardii*. *Clin Infect Dis* **31**: 1012–1017.

Tam NK, Uyen NQ, Hong HA, le Duc H, Hoa TT, Serra CR, Henriques AO & Cutting SM (2006) The intestinal life cycle of *Bacillus subtilis* and close relatives. *J Bacteriol* **188**: 2692–2700.

Wilcox MH, Mooney L, Bendall R, Settle CD & Fawley WN (2008) A case-control study of community-associated *Clostridium difficile* infection. *J Antimicrob Chemother* **62**: 388–396.

Wullt M, Hagslatt ML & Odenholt I (2003) *Lactobacillus plantarum* 299v for the treatment of recurrent *Clostridium difficile*-associated diarrhoea: a double-blind, placebo-controlled trial. *Scand J Infect Dis* **35**: 365–367.

Youngman P, Perkins J & Losick R (1984) Construction of a cloning site near one end of Tn917 into which foreign DNA may be inserted without affecting transposition in *Bacillus subtilis* or expression of the transposon-borne *erm* gene. *Plasmid* **12**: 1–9.

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