Clostridium difficile:
Infection and Immunity

A thesis submitted for the degree of Doctor of Philosophy

by

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(September 2008 – January 2013)

January 2013

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DECLARATION OF AUTHORSHIP

I, Patima Permpoonpattana, 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: _____ (Patima Permpoonpattana)

Date: _____

ABSTRACT

Clostridium difficile is a Gram positive pathogen of significant importance in the UK, Europe and the USA. No vaccine has been developed and current treatments are focused on hospital management and the use of antibiotics. The disease is spread in hospitals in the spore form and the role of spores in *C. difficile* infecton is poorly understood. In this project spores of *C. difficile* have been characterised. The proteins from the outermost layers of the spore were identified and the genes cloned. Three of these surface proteins have unique enzymatic properties that maybe important for symptoms of disease. The ability of *C. difficile* spores to adhere to intestinal cells was found to be far greater than with live cells and through this we have identified that the spore may play an important role in colonisation. The regulation of spore coat gene expression during sporulation was also examined and temporal phases of genes expression identified. A major part of this project was to develop a mucosal vaccine to *C. difficile*. The approach used was to clone the C-terminus of toxin A onto the surface of *Bacillus subtilis* spores and use these recombinant spores to immunise mice and hamsters. We found that oral delivery of these spores conferred 75% protection to *C. difficile* infection in a hamster model of infection. Further, parenteral immunisation of the same antigens (toxin A and B) failed to generate mucosal responses and this showed that mucosal immunisation is critical for good protection. Finally, we found that antibodies to the C-terminus of toxin A were cross reactive to the C-terminus of toxin B. This showed that mucosal delivery of just the C-terminus of toxin A is sufficient to confer protection in an animal model of infection. The outcome of this work is that we have shown the parameters for successful immunisation and vaccination against *C. difficile*.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor, Prof. Simon Cutting for his excellent guidance, support, patience and providing me with an excellent atmosphere for doing research.

I appreciate Dr. Hong Huynh for expert advice on ELISA, animal model experiments and cell culture techniques. I would like to thank all Cutting lab members during 2008 – 2012 for their help.

I would never been able to finish my thesis without a full scholarship awarded by The Ministry of Science and Technology of Thailand for my complete four years of study. I also would like to thank my Thai Scholarship friends, especially Mr. Sukonmeth Jitmahantakul for his time and help on all professionally graphical drawings, Miss Penying Rochanakul and Mr. Jutarop Phetcharaburanin for their help.

Finally, I am indebted to my family in Thailand for their genuine love, all matters-support and for their great care. They were always there cheering me up and stood by me through good and bad times from afar.

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ABBREVIATIONS

AIM	anaerobic incubation medium
BHI	Brain Heart Infusion
BSA	bovine serum albumin
CD	*Clostridium difficile*
CDAD	*Clostridium difficile*-associated diarrhea
CDI	*Clostridium difficile*-associated infection
cDNA	complementary deoxyribonucleic acid
CDT	*Clostridium difficile* binary toxin
CDTA	C-terminus domain of Toxin A
CFU	colony-forming unit(s)
CR	core
CT	coats
CX	cortex
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
EM	electron microscopy
ERM	erythromycin
EX	exosporium
FBS	fetal bovine serum
FCS	fetal calf serum

GCW	germ-cell wall
GI	gastrointestinal
GM	genetically modified
HPA	Health Protection Agency
HV	hypervirulent strain of *Clostridium difficile*
IBD	inflammatory bowel disease
Ig	immunoglobulin
IgA	immunoglobulin isotype A
IgG	immunoglobulin isotype G (major systemic Ig)
IgG1	immunoglobulin isotype A subclass 1
IgG2a	immunoglobulin isotype A subclass 2a
IgY	immunoglobulin isotype Y
IL	interleukin
i.m.	intramuscular
i.p.	intraperitoneal
IPTG	isopropyl β-D-thiogalactopyranoside
kDa	kilodaltons
LB	Luria Bertani
LCTs	large clostridial toxins
MAbs	monoclonal antibodies
MALDI	Matrix-assisted laser desorption/ionisation
MVLA	multilocus variable-number tandem repeat analysis
mwt	molecular weight
TNF	tumour necrosis factor
o.g.	orogastric

OD	optical density
ORF	open reading frame
PaLoc	pathogenicity locus
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGL	pulsed-field gel electrophoresis
RHUL	Royal Holloway University of London
RNA	ribonucleic acid
RNAP	RNA polymerase
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcriptase PCR
σ	sporulation-specific sigma factor
SATH	spore adhesion to hydrocarbon
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sIgA	secretory immunoglobulin isotype A, major immunoglobulin found in mucosal secretions
Slp	S layer protein
SMC	sporulation medium supplemented with L-cysteine
TBS	Tris buffered saline
TEM	transmission electron microscopy
TGY	tryptic-glucose-yeast extract medium
Th-1	T helper lymphocyte type 1
Th-2	T helper lymphocyte type 2

TMB	tetramethyl benzidine
TRITC	tetramethyl rhodamin iso-thiocyanate
UC	ulcerative colitis
UT	untreated
UV	ultraviolet
VC	vegetative cells
v/v	volume per volume
w/v	weight per volume

GLOSSARY OF TERMS

Intraperitoneal	referring to injection into the abdominal cavity
Local Immunity	immune responses at the site of antigen delivery
Mucosal	the epithelial layers lining the gastrointestinal, the respiratory and the urogenital tracts
Orogastric	traversing or affecting the alimentary tract from the mouth to the stomach
Parenteral	delivery of antigen at a site other than mucosal (e.g. injection)
Recurrence	referring to the recurrence of symptoms and documentation of infection within two months of successful standard therapy

CHAPTER 1

INTRODUCTION

1.1 *Clostridium difficile* and its Impact as a Human Pathogen

C. difficile is an example of a pathogen that until recently was considered of minor importance to public health. As a nosocomial pathogen morbidity and mortality rates fell far below those of other hospital-acquired diseases. With the advent of vaccines and antibiotics other diseases have been controlled and managed which, in turn, has exposed this organism as a growing problem in the UK, Europe and USA.

C. difficile is a Gram-positive anaerobic bacterium that is able to form spores. It is the most common cause of nosocomial diarrhoea (*C. difficile*-associated diarrhoea, CDAD) and can cause a condition known as pseudomembranous colitis. Between 1990-2004 rates of *C. difficile* infection (CDI) increased significantly in the UK with the greatest changes in the elderly (age 60-64 years). Over 50,000 cases of CDI were reported in 2007, which included 20% in the younger age groups (<30 years) (see Figures 1.1 and 1.2). In 2007 alone 4,056 deaths were assigned to CDI (HPA, 2009).

The increase in CDI has become a national as well as international issue and attention is now focusing on the cause. Two principle factors appear to be important;

1. The appearance of hypervirulent strains of *C. difficile* that have increased toxin production referred to as hypervirulent strains (e.g. the 027 ribotype), and
2. The failure to diagnose and control the transmission of spores of *C. difficile* in hospitals.

The latter issue is particularly important and of a sensitive nature to national governments, especially the UK, where a potential link can be made between hospital cleanliness and transmission of disease.

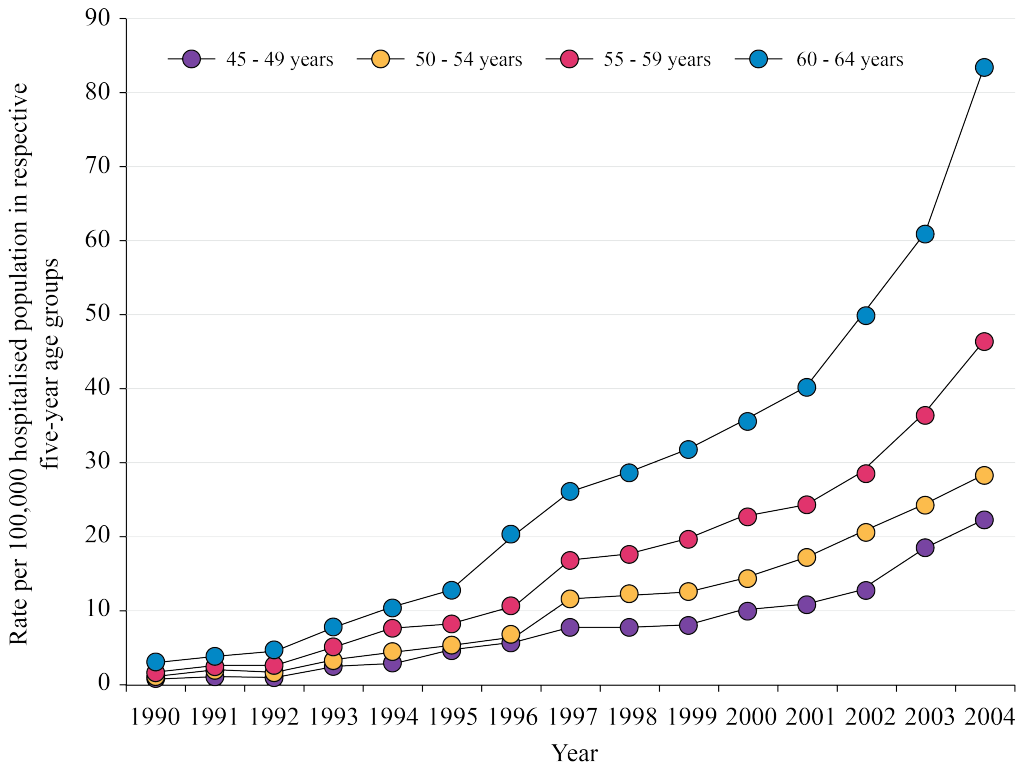


Figure 1.1 *C. difficile* infection rates in UK hospitals (modified from HPA, 2009).

The current situation with CDI in the UK is that the disease is controlled primarily by antibiotic therapy, which is universally considered unsuitable in the long-term

because of the associated risks of the development of antibiotic drug resistance. No vaccine is currently available and diagnosis of CDI remains ineffective. Improvements in the incidence of CDI have been achieved but mostly from strict management regimes in hospitals (HPA, 2009).

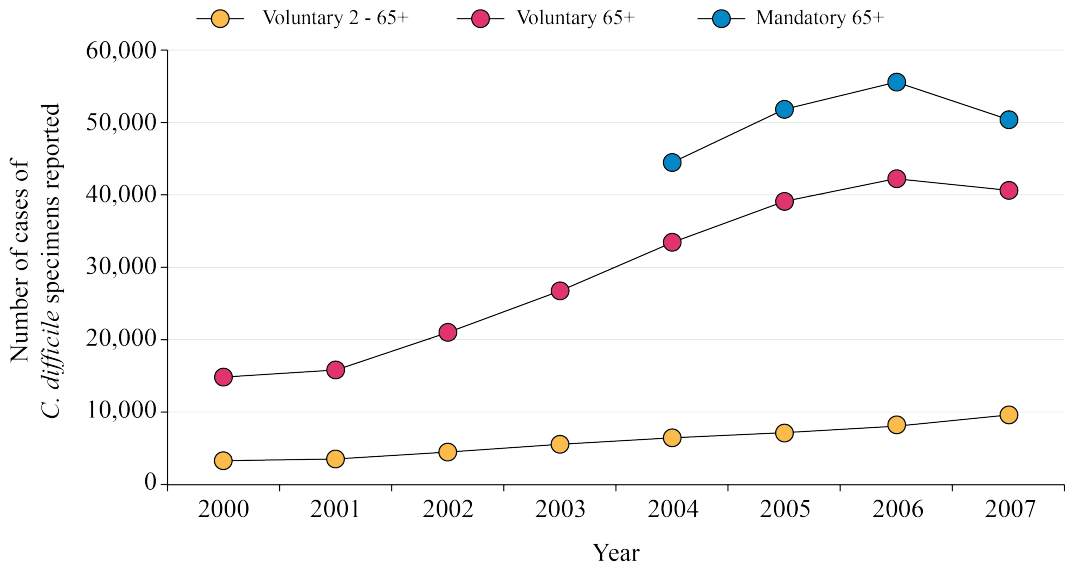


Figure 1.2 CDI cases in 2000-2007 by patient group (modified from HPA, 2009). CDI cases are reported in two age groups, 2-65 and 65+. Mandatory reporting of cases in over 65s was introduced in 2004, followed by mandatory reporting of all cases in 2007. Mandatory reporting of cases was introduced in 2004 as part of control measures to reduce *C. difficile* incidence.

1.2 *C. difficile* Infection

1.2.1 The disease

Spores of *C. difficile* exist in the environment but also in the human and animal GI-tract and most healthy adults are expected to carry *C. difficile* spores. The primary cause of CDI and its resulting illnesses (CDAD) result from antibiotic use and/or prolonged stays in hospitals (Kuipers & Surawicz, 2008). It is assumed that antibiotic

treatment disturbs the resident gut microbiota enabling spores of *C. difficile* to germinate, outgrow and proliferate. Illness nearly always results from the production of secreted toxins, toxin A and toxin B (Lyerly *et al.*, 1988, Rupnik *et al.*, 2009) (Figure 1.3). Patients will most probably become infected with *C. difficile* acquired from the hospital environment although resident strains may also account for primary infection. Whether a person then goes on to experience illness will depend on a number of factors, for example, if the host immune response is impaired illness is more likely. Another prerequisite is whether the *C. difficile* strain is a toxin producing strain since not all strains of *C. difficile* produce toxins. Transmission of *C. difficile* spores is also considered a growing problem and the emergence of hypervirulent strains of *C. difficile* that have increased levels of toxin production have been associated with recent outbreaks or epidemics in the USA, Canada, Europe and Japan (Kuijper *et al.*, 2007, Hubert *et al.*, 2007, Warny *et al.*, 2005).

CDAD can manifest itself in levels of severity ranging from mild diarrhoea to fulminant pseudomembranous colitis, sepsis, organ failure and potentially death. One of the major problems with CDAD is in diagnosis where early indicators such as fever and inflammation are confused with other illnesses. Ultimately, as the disease progresses severe colitis develops and at this point aggressive antibiotic therapy to prevent the most severe forms of the disease which can lead to toxic megacolon, perforation of the colon and progressive multi-organ failure leading to death. Mortality in CDAD patients is typically 3-30% mostly in the elderly (Kuijper *et al*., 2007, Loo *et al.*, 2005, HPA, 2009).

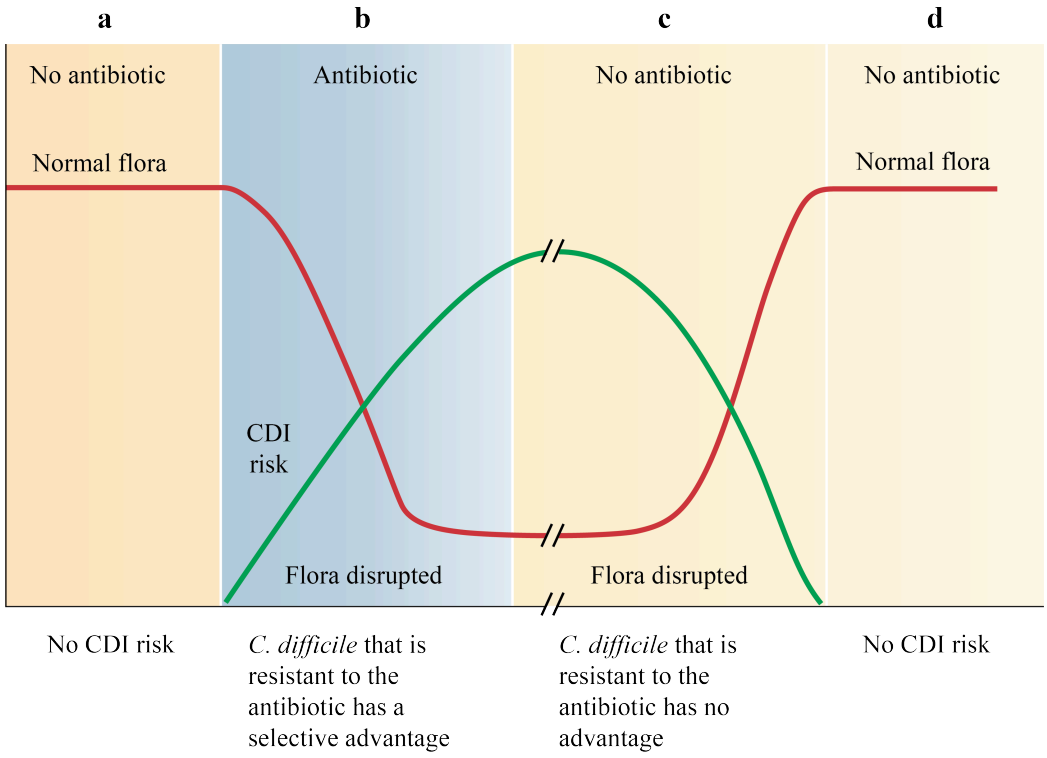


Figure 1.3 CDI in response to antibiotic. This figure shows the effects of antibiotics on the human microflora resident in the GI-tract. In the presence of antibiotics (b) the gut flora declines and *C. difficile* that is resistant to the antibiotic can proliferate. As soon as antibiotic treatment stops (c), the normal gut microflora recovers but there is a delay depending upon the antibiotic 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).

1.2.2 Recurrence

Possibly one of the most serious aspects of CDI is that the illness can reappear, a condition known as relapse where the original infecting strain causes a second bout of illness, or re-infection, where a different strain is responsible for the illness. Recurrence can affect up to 30% of patients experiencing CDAD (Lyerly *et al*., 1988, Williams & Spencer, 2009). Typically diarrhoea returns after 10-14 days but

this can be delayed by up to 1 month. What causes relapse is unclear but presumably the antibiotic treatment used to control CDAD may actually precipitate further rounds of germination and proliferation of *C. difficile* in the GI-tract, either of the original strain or alternatively, with a second, un-related strain of *C. difficile* acquired from the hospital environment.

1.2.3 Virulence Factors

Toxins

C. difficile strains produce three toxins, two glucosyltransferase toxins referred to as toxin A (TcdA) and toxin B (TcdB) and a binary toxin known as CDT (Figure 1.4). The toxin A and B genes are contained within a 19.6 kb chromosomal pathogenicity locus (PaLoc) (Lyerly *et al*., 1988, Rupnik *et al*., 2009). PaLoc carries the two toxin genes, *tcdA* and *tcdB*, a putative holin (TcdE) and two regulators, TcdC (a negative regulator) and TcdR (activator/sigma factor). Both of which regulate toxin gene expression. In non-toxigenic ($TcdA^-$ $TcdB^-$) strains of *C. difficile* the PaLoc sequence is replaced by an 115 bp non-coding sequence. Both toxins are produced during the late log and stationary phases of growth (Hundsberger *et al.*, 1997).

Substantial evidence shows that the toxins A and B are the primary determinants of virulence and pathogenicity and produce the classical symptoms of CDI (diarrhoea and inflammation) (Thelestam & Chaves-Olarte, 2000, Jank *et al.*, 2007). Most importantly, genetic inactivation of the *tcdA* and *tcdB* genes shows that in the absence of toxins A and B no symptoms of disease occur in a hamster model of infection (Kuehne *et al.*, 2010). TcdA and TcdB are both cytotoxic and will disrupt

the actin cytoskeleton and tight junctions which lead to decreased transepithelial resistance, fluid accumulation and destruction of the intestinal epithelium (Thelestam & Chaves-Olarte, 2000, Riegler *et al.*, 1995). A further consequence of the secretion of both toxins is that they cause the release of inflammatory cytokines from the intestinal epithelial cells, mast cells and macrophages. The consequence of these signalling molecules is an influx of inflammatory cells which further amplifies the inflammatory response (Figure 1.4).

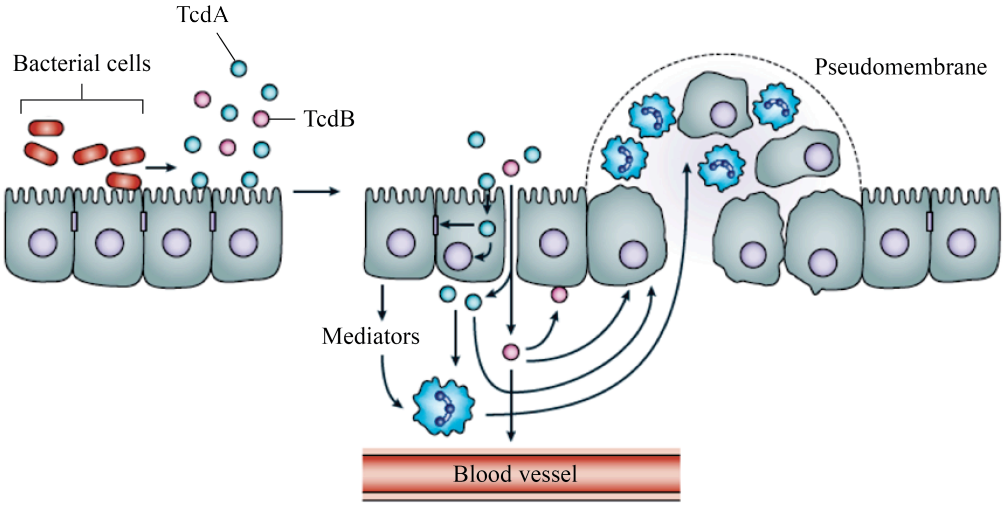


Figure 1.4 *C. difficile* pathogenesis. *C. difficile* colonises the intestine (colon) following disruption of the host gut microflora. Spores or vegetative cells of *C. difficile* adhere to the gut mucosa and toxigenic strains of *C. difficile* secrete toxins. The toxins initially insert into the membrane and then self-cleave releasing the catalytic (enzymatic) amino-terminal portion of each toxin into the cytoplasm. The toxins cause disruption of the tight junctions and loosening of the epithelial barrier ultimately leading to cell death or the production of inflammatory mediators that attract neutrophils. In animal models it has been shown that TcdB can enter the bloodstream (modified from Rupnik *et al*, 2009).

TcdA (308 kDa) and TcdB (269 kDa) are similar to a family of large clostridial toxins (LCTs) that all carry three functional domains (von Eichel-Streiber *et al.*, 1996, Amimoto *et al.*, 2007). An amino-terminal catalytic domain, a central translocation domain and a carboxy-terminal cell-binding domain. The catalytic domain is responsible for glycosylating small GTPases of the Rho and Ras families in host cells, this leads to their inactivation and causes cytoskeletal changes that initiate symptoms of CDI (Thelestam & Chaves-Olarte, 2000, Jank *et al*., 2007, Just *et al.*, 1995). The binding domain interacts with putative cell-surface carbohydrate receptors where seven potential carbohydrate binding sites have been identified for TcdA (Greco *et al.*, 2006, Ho *et al.*, 2005). In animals the TcdA receptor carries the trisaccharide Gal1(α1-3)Gal(β1-4)GlcNac (Krivan *et al.*, 1986) but it is thought that this receptor sequence is not found in humans where the glycoprotein gp26 is thought to act as a co-receptor (Na *et al.*, 2008). No receptor has yet been identified for TcdB.

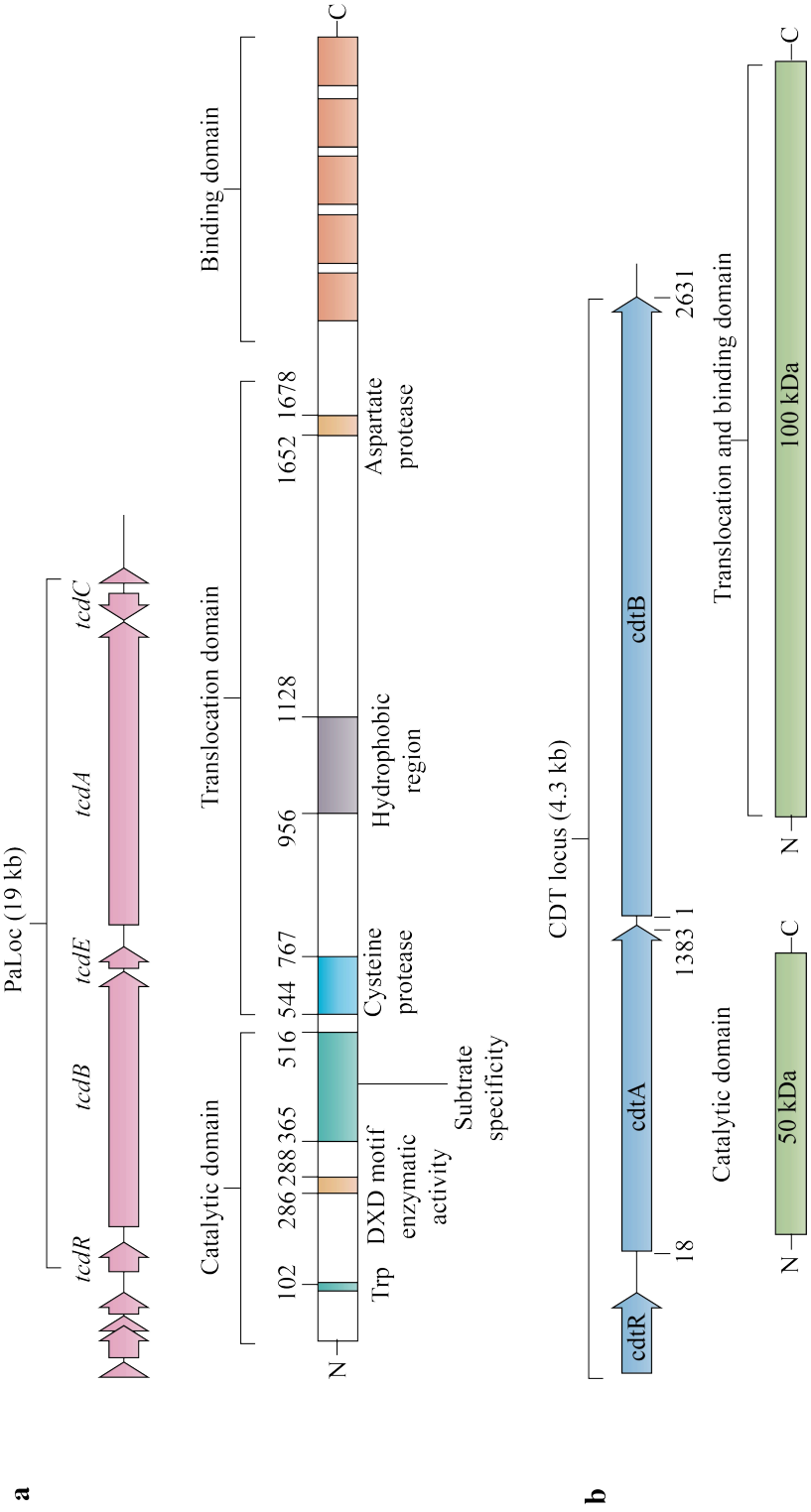


Figure 1.5 Toxins produced by *C. difficile* (see next page for legend).

Figure 1.5 (see previous page) **Toxins produced by *C. difficile.***

a) The PaLoc chromosomal locus encodes toxins A (308 kDa) and toxin B (269 kDa) in pathogenic ($ToxA^+$ $ToxB^+$) strains. In non-toxigenic strains this is replaced by a 115 bp segment of non-coding DNA. Both toxins are single chain polypeptides with distinct functional domains.

b) b) The CdtLoc pathogenicity locus encodes the binary toxin CDT comprised of two proteins, CdtA and CdtB. CdtB binds to cells while CdtA carries enzymatic activity. Figure taken from Rupnik *et al*., 2009.

A particularly interesting feature of the carboxy-terminal cell binding domains of TcdA and TcdB is that they carry multiple repetitive sequences referred to as CRD (C-terminal repetitive domain) (Figure 1.5). Different sequences have 21, 30 or 50 amino acids and repeated multiple times in the CRD of both toxins (von Eichel-Streiber *et al.*, 1992a, Dove *et al.*, 1990, von Eichel-Streiber *et al.*, 1992b). TcdA for example, carries 3- to 38 of these repeated sequences. Note that the number of repeated motifs depends upon how the motifs are defined. These sequences have some similarity with a number of bacterial proteins that bind to cell walls (Fernandez-Tornero *et al.*, 2001). It has been proposed that the multiple repeats may serve to amplify the affinity and avidity of receptor binding (von Eichel-Streiber *et al*., 1992b, Weis & Drickamer, 1996).

The crystal structure of TcdA has been resolved and shows that the CRD contains a β-solenoid fold containing five repeats. Each repeat is further defined as consisting of a β-hairpin followed by a loop of 7-10 residues in short repeats or long repeats of 18 residues (Ho *et al*., 2005). For TcdB only the amino-terminal catalytic domain

has been resolved demonstrating a classical glycosyltransferase with an interface for substrate binding (Wright *et al.*, 2008).

After cell binding and subsequent endocytosis the decrease in endosomal pH induces a conformational change in the toxin which results in exposure of the hydrophobic membrane insertion (MI) domain within the central translocation segment (Figure 1.4). This enables the amino-terminus to insert into the endosomal membrane via the formation of a pore (Jank & Aktories, 2008). The host cell is able to initiate autocatalytic cleavage of the amino-terminal region proximal to the cysteine protease (CP) site which releases the amino–terminal glucosyltransferase (GT) into the cytoplasm leaving the remainder of the toxin polypeptide attached to the membrane (Reineke *et al.*, 2007). GT is then, after a cascade of events, to inhibit Rho-GTPases that ultimately cause increased membrane permeability and loss of barrier function (Hecht *et al.*, 1992).

The binary toxin is least well understood although common with other clostridia is otherwise unrelated to TcdA and TcdB. In hamsters *C. difficile* strains that are *tcdA*$^-$ *tcdB*$^-$ *cdt*$^+$ produce no symptoms if CDI and it has been assumed that this toxin is not associated with disease (Perelle *et al.*, 1997). The binary toxin comprises two toxins, CdtA and CdtB. CdtB binds to host cells and translocates CdtA which is the catalytic component where it can ribosylate (ADP dependant) actin molecules. The *cdt* genes are located in the binary toxin locus known as CdtLoc which includes *cdtR*, encoding a regulator of toxin synthesis (Perelle *et al*., 1997).

S-layer proteins

Vegetative cells of *C. difficile* carry an exterior coat of proteins that are referred to as the S-layer (Calabi *et al.*, 2001). The S-layer lies above the peptidoglycan layer of the Gram-positive cell wall and is common in many Gram-positive and Gram-negative bacteria. The layer is comprised of two S-layer proteins (Slp) that arise from post-translational cleavage of a single precursor, SlpA (Eidhin *et al.*, 2006). The two forms exists as low and high molecular weights which self-assemble in an organised process and has been shown to mediate adhesion to enteric cells (Calabi *et al.*, 2002). Other functions have been proposed for the S-layer including protecting the cell from phagocytic attack or to avoid the immune system (Sara & Sleytr, 2000). SlpA is the predominant surface antigen and has been considered as a potential vaccine candidate (see 1.4.2). Interestingly, different variants of the *slpA* gene have been identified in *C. difficile* including the hypervirulent 027 strains (Fagan *et al.*, 2009, Eidhin *et al*., 2006).

Spores

The spore is the dormant form of *C. difficile* and like other Gram-positive spore formers is metabolically inactive and can remain in this state for long periods of time. Relatively little has been known about the spore until fairly recently. This has resulted from difficulties in preparing pure crops of spores. What is clear is that the spore is the most important agent of transmission since vegetative cells, being anaerobic, can not survive outside of the host. Person-to-person transmission of *C. difficile* is directly linked with the excretion of spores in the faeces of infected patients which creates a reservoir of *C. difficile* that has proven difficult to control

(Riggs *et al.*, 2007, Vonberg *et al.*, 2008). Germination studies on *C. difficile* spores has shown that they show increased levels of germination in response to cholate derivatives found in bile which demonstrates that they are adapted for life in the GI-tract (Sorg & Sonenshein, 2008, Sorg & Sonenshein, 2009). Hypervirulent strains of *C. difficile*, notably the 027 ribotype, show increased levels of sporulation which be associated with their increased virulence (Merrigan *et al.*, 2010). *C. difficile* spores resemble other spores from other *Bacilli* and *Clostridia* and carry a core containing the germ-line chromosome, a cortical layer of peptidoglycan and a proteinaceous spore coat. In addition, spores carry an exosporium which is a loose-fitting layer that encases the spore. The exosporium is found in some but not all spore formers and its role has yet to be defined, moreover, this layer is particular fragile and is easily lost during spore preparation (Panessa-Warren *et al.*, 1997, Henriques & Moran, 2000). Bioinformatic analysis based on the genome sequence of *C. difficile* has shown the presence of orthologues of many of the regulatory genes found in *Bacillus subtilis* (Paredes *et al*., 2005). For example, SpoOA, a regulatory protein that initiates spore formation and several sigma factors, σ^E, σ^F that control sporulation gene expression. This suggests that spore formation is likely to be similar in design to that of *B. subtilis* and other spore formers.

The most important study to date has been a proteomic analysis of *C. difficile* spores (Lawley *et al.*, 2009b). Here, pure spores of strain 630 (*tcdA*$^+$ *tcdB*$^+$) were prepared and total spore proteins solubilised and analysed using mass-spectrometry. Using the genome sequence of 630 (Sebaihia *et al.*, 2006) for comparison 336 spore-associated polypeptides were identified. Of particular interest as a potential vaccine candidate

was the BclA1 protein which has orthologues in *Bacillus anthracis* and *Bacillus cereus* (Sylvestre *et al.*, 2002, Henriques & Moran, 2000, Charlton *et al.*, 1999). BclA1 is found in the exosporium of other spore formers and is a collagen-like protein that is found in hair-like appendages that protrude from the exosporial layer (Henriques & Moran, 2000). *C. difficile* spores carry a fragile exosporium that encases the spore in a loose-fitting sac. The role of BclA1 in *B. anthracis* is that of increasing opsonophagocytic uptake by host cells and increasing spore germination (Brahmbhatt *et al.*, 2007, Hahn *et al.*, 2006). It may also be involved in adhesion or spore clumping at the mucosa (Bozue *et al.*, 2007).

1.2.4 Hypervirulent (HV) strains

Starting in the year 2000 highly virulent strains of *C. difficile* have emerged resulting in epidemics typically of a higher incidence, greater severity and increased mortalities (Loo *et al*., 2005, McDonald *et al.*, 2005, Eggertson & Sibbald, 2004). The strains responsible are referred to as hypervirulent and cluster into a distinct phylogenetic group (Killgore *et al.*, 2008). HV strains can be typed by a number of different methods include PCR ribotyping where the HV strains are referred to as 027 (Warny *et al*., 2005). Currently 20 sub-types of the ribotype 027 have been identified using an enhanced DNA fingerprinting method known as multilocus variable repeat analysis (MVLA) (Fawley *et al.*, 2008). What has become clear is that the 027 strains have spread widely and have been responsible for a large number of *C. difficile* epidemics worldwide. Interestingly, in the UK at least, MVLA analysis of *C. difficile* specimens isolated from faecal samples showed a gradual decline in 2010 compared to samples taken over the previous 24 months. The explanation for

this is not clear and it should be noted that there has been a compensatory increase in other ribotypes, for example, 078, 005, 023 and 002 (HPA, 2011). 027 strains have been characterised and shown to produce higher levels of sporulation (and therefore spores) and toxin production. The precise reason why toxin production and sporulation is more robust in 027 strains is unclear but one hypothesis involves TcdC, the negative regulator of toxin production, which is absent in 027 strains, and this may enable higher levels of sporulation and toxin production (Merrigan *et al*., 2010).

1.3 Control and Prevention of CDI

1.3.1 Antibiotic therapy

For patients showing symptoms of CDAD existing antibiotics being used are terminated (as these may have precipitated the disease) and oral treatment with vancomycin or metronidazole is initiated. Currently, resistance to these drugs is rare and metronidazole is preferred for initial treatment of CDAD and vancomycin reserved for the more severe cases (Nelson, 2007). For relapse prolonged treatments with vancomycin are prescribed although metronidazole is not used due to the risk of neuropathy. The use of live probiotic organisms as an adjunct to antibiotic treatment has been shown to have value based on human trials (see 1.4.2).

1.3.2 Diagnosis

The primary method for diagnosing *C. difficile* infection is by bacterial culture or by detection of the toxins in faeces (HPA, 2009, Kuipers & Surawicz, 2008, Williams & Spencer, 2009). In the case of the latter, methods are based either on tissue culture

where cytotoxicity is measured or immunodiagnostics where an ELISA-based method is used to detect the toxin. There are many problems with current strategies for diagnosis. First, culture-based methods do not discriminate between non-toxigenic/toxigenic strains. Second, methods for working with faecal samples are problematic and sensitivity can be low. Third, tissue culture based methods are expensive and there is an error of subjectiveness that can impede diagnosis. Fourth, some immune-based methods only detect toxin A and therefore can miss virulent strains that produce only toxin B. Fifth, the sensitivity of immune-based methods is low (60-85% compared to tissue culture based methods). Finally, the detection methods are all focused on detection of the live vegetative cell of *C. difficile* that produces the toxin and not the spore form. As mentioned below (1.3.3) during CDI the number of spores increases exponentially and a method to detect the spore rather than the live cell may prove to be a better indicator of the disease state.

1.3.3 Hospital management

Considerable effort has now been focused on improving the control of *C. difficile* in a hospital environment (Williams & Spencer, 2009). This ranges from standardised regimens of hand washing, use of gloves, disinfection and education (HPA, 2009). Studies in animals have shown some alarming insights to how *C. difficile* may spread in a hospital environment (Lawley *et al.*, 2009a). In mice virulent *C. difficile* can colonise the GI-tract and establish a carrier state that persists for many months. Spores of *C. difficile* are shed in the faeces but do not transmit to other mice. Upon antibiotic treatment though spores become able to transmit easily to other mice. *C. difficile* proliferates in the GI-tract and accompanies a drastic reduction in the normal

microflora of the GI tract. In this 'supershedder' state large numbers of *C. difficile* spores are shed in the faeces and infect other mice. If the other mice are immunocompromised (e.g., are Myd88-) they suffer severe intestinal disease that can be fatal. This study demonstrates that in a hospital environment the use of antibiotics may lead to a supershedder state of virulent *C. difficile* and under these circumstances transmission from person to person is highly probable and would require a rigorous program of containment to prevent an outbreak. Because of this methods need to be developed that facilitate destruction of the spore since this is the principal agent of transmission (Lawley *et al.*, 2010).

1.4 Prophylaxis

1.4.1 Correlates of protection

Compelling evidence suggests that the primary agent of pathogenesis and disease is the action of toxins A and B (Lyerly *et al.*, 1985, Kim *et al.*, 1987, Du & Alfa, 2004). The CDT binary toxin as mentioned earlier is not implicated in CDI. Toxigenic strains of *C. difficile* have been identified from patients that are $ToxA^+$ $ToxB^+$ and $ToxA^-$ $ToxB^+$ but to date no naturally occurring $ToxA^+$ $ToxB^-$ strains have been isolated (Drudy *et al.*, 2007a, Drudy *et al.*, 2007b). Despite this, insertional mutagenesis of the PaLoc locus in isogenic strains of strain 630 has shown that both $ToxA^+$ $ToxB^-$ and $ToxA^-$ $ToxB^+$ are virulent and can cause fulminant disease in a hamster model of infection (Kuehne *et al*., 2010).

In humans anti-toxin A and anti-toxin B antibodies have been shown to correlate with protection. For example, using a parenteral injection of toxoid A in 30

recipients, high level IgG responses specific to toxin A were reported (Aboudola *et al.*, 2003). In another study of 63 patients serum IgG specific to toxin A was associated with protection against recurrence (relapse) (Kyne *et al.*, 2001). Similarly, antibodies to both toxin A and toxin B correlated with protection to relapse in 29 subjects in a trial evaluating a mAb to toxin A (Leav *et al.*, 2010). Systemic antibody responses to toxin A or B develop in approx. 50-70% of patients who develop CDAD (Viscidi *et al.*, 1983, Johnson *et al.*, 1992, Aronsson *et al.*, 1985, Bacon & Fekety, 1994) with responses to toxin B being more common than to toxin A (Viscidi *et al*., 1983, Aronsson *et al*., 1985, Bacon & Fekety, 1994). Levels of serum IgA specific to the toxin have been shown to be higher than specific IgG (Johnson *et al*., 1992). For example, in one human study serum monomeric IgA has been shown to be important for neutralisation and is possibly more important than serum IgG (Johnson *et al.*, 1995). A later study based only on *in vitro* methods that suggests that polymeric IgA (i.e., secretory IgA) may play the most critical role in protection suggesting that to protect against CDI, mucosal, rather than systemic vaccination is a requirement (Stubbe *et al.*, 2000). Systemic IgG responses may reflect systemic infection brought about if the toxins have entered the bloodstream and arising during severe infections. Mucosal responses on the other hand may provide the primary response to CDI. Most studies in humans have shown mucosal IgA responses underlying the importance of mucosal immunity (Kelly *et al.*, 1992, Johnson, 1997). Interestingly, the role of antibodies in the prevention of relapse has not been resolved with limited studies and with different conclusions. (Johnson, 1997). In one study toxin A-specific IgG and IgA in serum were lower in patients recovering than those who went in to relapse (Johnson *et al*., 1992). In a second study, levels of serum IgG

and faecal IgA were lower in patients who relapsed (Warny *et al.*, 1994). There is currently no clear picture of what is required for protection and it is possible that protection requires other host responses, i.e., in addition to anti-toxin responses. An example might be decolonisation antibodies, i.e., antibodies directed against components of the live cell or spore that prevent *C. difficile* from colonising the GI-tract.

Antibodies specific to the cell-binding domain of toxin A can protect against CDI. Specifically, a defined region of the carboxy-terminal cell-binding domain of toxin A known as 14CDTA when expressed in a recombinant *Salmonella* vaccine (delivered nasally) generated mucosal and systemic antibodies against 14CDTA and toxin A neutralising activity (Ward *et al.*, 1999). This particular study is interesting because it shows that for vaccination just a small region of toxin A can be cloned and displayed and antibodies directed against it are sufficient to confer protection. This is supported by studies using 14CDTA-specific mAbs that abolish toxin A-mediated cytotoxicity (Sauerborn *et al.*, 1997). In hamsters immunized with IgY antibodies to toxin A or toxin B (i.e., passive immunisation) that had been raised in chickens protection against morbidity and mortality was observed (Kink & Williams, 1998). Interestingly, these studies showed that antibodies raised against the carboxy-termini of each toxin were most effective at generating toxin-neutralising antibodies suggesting these regions to be highly immunogenic. Formalin-inactivated toxoids have been evaluated as protective antigens by a variety of systemic and mucosal (oral, nasal and rectal in combination with cholera toxin as a mucosal adjuvant) routes using the hamster model of infection (Torres *et al.*, 1995). In all cases varying

levels of protection could be observed supporting the notion that antibodies to toxin A and toxin B are required for protection. What is particularly interesting is that delivery of either toxoid A or only toxoid B can generate protection, an observation best explained if the carboxy-terminal domains of each toxoid generate cross-reactive and cross-neutralising antibodies.

In patients with CDAD IgG responses to the S-layer proteins has been observed using immunoblotting of patient's sera (Pantosti *et al.*, 1989) or by mass-spectrometry (Wright *et al*., 2008). The conclusion of both studies was that the S-layer proteins were the immunodominant antigens present in vegetative cells of *C. difficile*. In addition, the S-layer proteins have been shown to induce inflammatory and regulatory cytokines in human monocytes and dendritic cells using *in vitro* studies (Ausiello *et al.*, 2006). Taken together, this suggests that these surface proteins are both immunogenic and inflammatory and antibodies directed against the S-layer proteins could be considered in a vaccine formulation. It is probable that other spore-associated proteins may be implicated in virulence and these together with the S-layer proteins might be important for protection against relapse. The S-layer proteins have been evaluated as vaccine candidates in a hamster model of infection. When administered in combination with cholera toxin (CT) as a mucosal adjuvant limited anti-Slp responses (IgG and IgA) were obtained suggesting that Slp should not be considered as a vaccine candidate unless better mucosal adjuvants can be developed (Ni Eidhin *et al.*, 2008).

1.4.2 Vaccines under commercial development

The principle approach considered for prevention of CDAD is by vaccination. In one documented phase I clinical study 30 healthy adults were injected (intra-muscular) with four spaced doses of formalin-inactivated toxoids A and B. 90% of subjects developed serum IgG responses and 50% faecal sIgA (Kotloff *et al.*, 2001). Current vaccination programmes under development all rely on the delivery of the toxoids by a parenteral route. Sanofi-Pasteur have entered phase II trials with a parenteral vaccine (3 doses) consisting of inactivated toxins A and toxin B. Although antibodies to toxin A and B are protective it is unclear whether this vaccine will be able to prevent CDI in the field. To be fully effective the vaccine must protect against hypervirulent strains of *C. difficile* as well as relapse and it is unclear that the phase II trials will demonstrate this. Other major vaccine manufacturers are all pursuing vaccine approaches using a parenteral approach based on the use of toxoids but these are all at the preclinical stage.

1.4.3 Probiotics

Probiotics are live bacteria that when ingested are able to confer health benefits to the host. Used extensively in humans these products often are comprised of just one bacterium, either as a dried formulation in a tablet or capsule, or as a fermented dairy product (e.g., Yakult, live yoghurt etc.). The rationale for using probiotics is confusing yet following antibiotic treatment there seems to be a compelling case for their use. The gut microflora consists of about 10^{12} bacteria per gram of intestinal content in a healthy adult and as many as 1000 different species. This population is stable yet antibiotic therapy can reduce the gut population by as much as 1-log and

this in turn can lead to classic symptoms associated with antibiotic treatment, for example, cramps, constipation and diarrhoea. Since CDAD is a result of antibiotic treatment that precipitates the growth and proliferation of *C. difficile* it might be possible that repeated doses of a probiotic bacterium could replenish the gut microflora. For CDAD there appears to be data to show that probiotic treatment can reduce the symptoms or occurrence of CDAD (reviewed in (Gougoulias *et al.*, 2007, Sleator & Hill, 2008)). Numerous animal studies have shown efficacy in reducing the incidence and severity of CDI in mice and hamsters. Various mechanisms have been put forward, for example, the production of certain antimicrobials (e.g., Abp118 and Lactican 3147 produced by *Lactobacillus salivarius* and *Lactococcus lactis* respectively (Corr *et al.*, 2007, Rea *et al.*, 2007)) that inhibit proliferation of *C. difficile*. While attractive this type of 'probiotic model' is not convincing since it is species and strain specific and a more universal solution is preferable (perhaps by prevention of colonisation or interference with toxin targeting). Despite this, human trials have been conducted, including studies with *Lactobacillus rhamnosus* GG (Pochapin, 2000), *Lactobacillus plantarum* 299v (Wullt *et al.*, 2003), *Lactobacillus acidophilus* + *Bifidobacterium bifidum* (Plummer *et al.*, 2004) and the yeast *Saccharomyces boulardii* (Surawicz *et al.*, 1989, Surawicz *et al.*, 2000a). The conclusions from these human studies as well as others reviewed by Gougoulias *et al* (Gougoulias *et al*., 2007) show a significant effect on the incidence of CDAD as well as relapse when used as an adjunct to antibiotic therapy (Surawicz *et al.*, 2000b). *S. boulardii* has also been shown to significantly enhance sIgA responses to toxin A in mice pre-fed with *S. boulardii* and then orally administered toxoid A (Qamar *et al.*, 2001). These studies suggest that this yeast may prime the immune mucosal immune

response and supports the use of probiotics as dietary supplements and/or mucosal adjuvants. As with many studies involving probiotics there are some issues that relate to study design and administration yet there seems to be a consensus, that in the absence of an alternative treatment, the use of probiotic bacteria could have a useful role as an adjunct or alternative treatment to the more conventional antibiotic therapies used to control the disease.

1.4.4 Faecal therapy

One of the most unusual methods shown to work against CDAD is referred to as a 'faecal transplant' or 'faecal therapy'. The treatment has been performed with 42 patients to date at Gartnavel General Hospital in Glasgow and in 92% of cases patients go on to show a full recovery (MacConnachie *et al.*, 2009). The method requires a stool sample (approx. 30 g) from a close relative which is then homogenized and solid matter removed by filtration. The faecal extract is then administered to the patient using a tube through the patients nose (nasogastric). To explain how this treatment works it is assumed that faecal therapy repopulates the GI-tract with gut commensals and thus this strategy shares some similarities with the use of probiotics. Clearly, there are some concerns over this treatment mostly relating to the use of faecal material and this is considered only a suitable and realistic option for patients who do not respond to vancomycin therapy.

1.4.5 Pre-colonisation with non-toxigenic *C. difficile*

A second strategy relates to reducing colonisation of *C. difficile* in the GI-tract. Studies in neonatal pigs (Songer *et al.*, 2007) and hamsters (Sambol *et al.*, 2002) has shown that when animals were administered oral doses of a strain of *C. difficile* unable to produce either toxin A or toxin B that, following infection with a toxigenic strain of *C. difficile* significant levels of protection were observed. In the hamster model three different non-toxigenic strains of *C. difficile* were used and 87-97% was observed following challenge doses of 10^6 spores (following clindamycin treatment) of each of the respective *C. difficile* strains (Sambol *et al*., 2002). These studies are interesting since they show that pre-colonisation of animals (and presumably humans) with non-toxigenic *C. difficile* can provide protection to CDI. The basis for how this works is not understood but several mechanisms can be proposed including, preventing the colonisation of toxin producing strains or possibly by a probiotic based mechanism. The strategy has been adopted by one pharmaceutical company, Viropharma (Exton, USA) who are completing phase II clinical trials in humans.

1.4.6 Passive immunisation:

Antibodies specific to toxin A and toxin B are required for protection against CDI. Patients with mild cases of CDAD carry high levels of anti-toxin IgG (Kyne *et al*., 2001, Warny *et al*., 1994, Viscidi *et al*., 1983) while patients who experience relapse have low anti-toxin Ig titres (Kyne *et al*., 2001, Katchar *et al.*, 2007). Patients who suffer CDAD develop anti-toxin A/B antibodies (IgG and IgA) (Giannasca & Warny, 2004, Viscidi *et al*., 1983). This then raises the possibility for using antibodies for passive immunisation, that is, administering anti-toxin A/B antibodies directly to an

affected person (Hussack & Tanha, 2010). In animals data has supported this therapeutic approach. For example, intra-peritoneal injection of toxin-specific antibodies prevented clindamycin-induced colitis in hamsters (Allo *et al.*, 1979). Monoclonal antibodies have also been used by the oral route and protected hamsters from a fatal injection of toxin A (Lyerly *et al.*, 1986). Using IgY antibodies (produced in poultry) specific to toxins A and B oral administration to hamsters prevented CDI and relapse but oral delivery of only anti-toxin A IgY prevented infection only but not relapse (Kink & Williams, 1998). The fact that an antibody can survive transit across the gastric barrier is surprising and may reflect the robustness of hen IgY antibodies. For human therapeutics humanised antibodies must be used to reduce immunogenicity and studies using anti-toxin monoclonal mAbs against toxin A and B and reduced hamster mortality following intraperitoneal injection (Babcock *et al.*, 2006).

A number of studies have now shown that passive immunisation is suitable for treatment of chronic relapsing CDI in humans. So far these studies have used systemic delivery. In the most successful study to date Lowy *et al* examined 200 patients who each received an intravenous dose of anti-toxin A/B mAbs. A significant reduction in CDAD recurrence (relapse) was observed with an incidence of 7% in treated individuals compared to a 25% relapse rate in patients receiving a placebo (Lowy *et al.*, 2010). This approach is now being pursued by Merck & Co. (USA).

Table 1.1 Summary of treatment options for CDI

Treatment options	Pro	Con
Antibiotic therapy	• Works in most cases	• Can induce relapse • Increases probability of antibiotic resistance • Side effects • Therapy only
Vaccine	• Will provide long term protection	• No vaccine yet • Not clear what are the required components for a vaccine
Probiotic	• Provides an adjunct to antibiotic therapy • Some efficacy shown	• Does not provide long term protection • Mechanism not understood • <100% protection
Faecal transplant	• Method works	• Therapy only • Complicated and unsuitable
Passive immunotherapy	• Method works	• Therapy only • Systemic delivery • Not 100% protection

1.5 Thesis Objectives

The long-term goals of this work are to develop a prophylactic treatment to CDI. This thesis project initiated with two questions.

1. Can a mucosal vaccine to CDI be developed?
2. What is the role, if any, of spores in CDI?

A mucosal vaccine

Current evidence suggests that parenteral vaccination relying on injection of toxoid antigens is insufficient for full protection to CDI. Since CDI is acquired by the oral route a mucosal vaccine may be most appropriate for full protection. To address mucosal vaccination a delivery system based on antigen expression using *Bacillus subtilis* spores will be used for delivery of toxin antigens. Heat-stable *B. subtilis* spores are convenient for delivery by the oral route and have been shown to provide mucosal immune responses to heterologous antigens displayed on the *B. subtilis* spore. The approach to be used was to express domains of toxin A and toxin B on *B. subtilis* spores and administer to animal models of CDI.

The role of the spore

Relatively little is known about the *C. difficile* endospore. Particularly its surface composition and the regulation of genes involved in differentiation. As a first step the spore coats will be examined and the principal spore coats proteins will; be identified. Secondly, the spore surface properties will be characterised and finally, the regulation of representative developmental genes defined. This will provide a framework for others to decipher the biosynthesis of *C. difficile* spores and their relationship to CDI.

CHAPTER 2

MATERIALS AND METHODS

2.1 General methods

General methods for working with *B. subtilis* are described elsewhere (Harwood & Cutting, 1990). Work with *E. coli* including cloning are as described by Sambrook *et al*. (Sambrook & Russell, 2001).

2.2 Bacterial Strains

630 is a toxigenic (*tcdA*$^+$ *tcdB*$^+$) strain of *C. difficile* isolated from a patient with pseudomembraneous colitis during an outbreak of CDI (Wust *et al.*, 1982) and obtained from N. Fairweather (Imperial College, UK). CD196 is a non-epidemic, hypervirulent, strain of *C. difficile* isolated in Paris in 1985 with the 027 ribotype, REA (restriction endonuclease analysis) type BI and PFGE (pulse-field gel electrophoresis) type NAP1 (Stabler *et al.*, 2009) and was obtained from T. Lawley (Wellcome Trust Genome Campus, UK). CD630 and M68 were routinely grown in vegetative culture by overnight growth (10ml) at 37°C in TGY-vegetative medium (3% tryptic soy broth, 2% glucose, 1% yeast extract, 0.1% L-cysteine) (Paredes-Sabja *et al.*, 2008). *S. mutans GB1* was obtained from Phan Nghia (Hanoi Univ., Vietnam); *B. subtilis* strain PY79 is a prototrophic (Spo$^+$) laboratory strain and a lab stock as was *B. clausii* O/C. *B. licheniformis* strain HU14 was obtained from the *Bacillus* Genetic Stock Center (Ohio, USA).

2.3 Sporulation of *C. difficile*

All manipulations were made in an anaerobic incubator (Don Whitley, UK). A single bacterial colony was grown on BHIS (brain heart infusion supplemented with L-cysteine, 0.1% and yeast extract, 5 mg/ml; (Smith *et al.*, 1981) agar overnight at 37^{o}C. One fresh single colony from the BHIS plate was inoculated in 10 ml of TGY medium (3% tryptic soy broth; 2% glucose; 1% yeast extract; 0.1% L-cysteine) (Paredes-Sabja *et al*., 2008) and incubated at 37^{o}C overnight. 1 ml of TGY culture was then sub-cultured into SMC broth (90 g, peptone; 5 g, proteose peptone; 1 g, $(NH_4)_2SO_4$; 1.5 g, Tris) containing 0.1% L-cysteine (modified from Wilson *et al*. (1982)) incubated overnight and then plated onto SMC agar. After 7 days incubation at 37^{o}C sporulation efficiency was confirmed by phase-contrast microscopy and measurement of heat-resistant CFU and spore crops harvested immediately or after overnight incubation at 4^{o}C.

2.4 Spore purification

The methods used were modified from Lawley *et al* (2009b). Spores were washed in water two times, then suspended in PBS containing 125 mM Tris, 200 mM EDTA, 0.3 mg/ml proteinase K (E00492; Fermentas) and 1% sarcosyl, and incubated with gentle shaking at 37^{o}C for 2 h. Spores were centrifuged (6,500 g, 10 min) and pellets resuspended in water and washed a further 10 times. After the final suspension in water, spores were heat-treated (60^{o}C, 20 min) to kill any residual cells; aliquots were stored at 4^{0}C until use. To calculate the spore CFU aliquots were serially diluted in PBS and plated onto BHIS agar supplemented with 0.1% sodium taurocholate (Sigma, UK). Plates were incubated for 24-48 h before CFU were enumerated.

2.5 Spore coat extractions (Chapter 3)

The spore coat extraction procedure has been described elsewhere (Tsuzuki & Ando, 1985) but in brief *C. difficile* spores (2 x 10^9) were suspended in freshly prepared 100 ml of sodium borate-SDS-DTT buffer consisting of sodium borate (0.1 M, pH 10), 0.5% sodium dodecyl sulfate (SDS) and 50 mM dithiothreitol (DTT), and then incubated at 68^{o}C for 75min with gentle agitation. After centrifugation (6500 g for 15 min) the supernatant was removed, mixed with 4X SDS-PAGE loading buffer and fractionated by SDS-PAGE. For *B. subtilis* and *B. clausii* spores, coat proteins were extracted using the SDS-DTT buffer described elsewhere (Nicholson & Setlow, 1990).

2.6 Peptide fingerprinting (Chapter 3)

Spore coat proteins were fractionated on 12.5% SDS-PAGE mini-gels and bands excised and digested with trypsin before analysis by MALDI mass spectrometry. Digestions and analysis were conducted by the University of Cambridge Protein & Nucleic Acid Chemistry Facility (PNAC) (http://www.bioc.cam.ac.uk/pnac).

2.7 Antibody production (Chapter 3)

pET28b expression vectors that express the complete *C. difficile cotA, cotB, cotCB* and *cotD* ORFs were constructed by amplifying the respective DNA by PCR from CD630 chromosomal DNA and ligating to cleaved pET28b. For *cotE* it proved impossible to clone the entire ORF so a fragment encoding the N-terminal, peroxiredoxin domain was cloned instead. Primers used for construction of pET28b clones are shown in (Table 2.1) High levels of expression were obtained upon IPTG

induction and purification of proteins by passage of the cell lysate through a HiTrap chelating HP column on a Pharmacia AKTA liquid chromatography system. Polyclonal antibodies were raised in mice immunized by the intra-peritoneal route with 2µg of purified recombinant proteins on days 1, 14 and 28. Anti-spore antibodies were made by treating spores in 2% formalin (2% v/v formaldehyde in PBS) overnight at 4^{o}C. Spores were washed 5-times with PBS and used to dose mice (2 X 10^{8} spores/dose) mice on days 1 and 14.

Table 2.1 Primers used for construction of pET clones for antibody production

Primer	Direction	Sequence[1]	Res. site
cotA			
CotA-NcoI-F	forward	GAT*CCATGG*CTGTGGAAAATAATAAATG	*Nco*I
CotA-XhoI- R	reverse	ATC*CTCGAG*TGCAATATAATCTATAGAATCTA CACATAC	*Xho*I
cotB			
CotB-NcoI-F	forward	GAT*CCATGG*CTATAGATAATCAAAAATATG	*Nco*I
CotB-XhoI-R	reverse	ATC*CTCGAG*CATGTTTTTATAACTCTC	*Xho*I
cotC			
CotC-NcoI-F	forward	GAT*CCATGG*CTTGGATTTATCAAAAAAC	*Nco*I
CotC-XhoI-R	reverse	ATC*CTCGAG*AAACTGATGCTTGCACTC	*Xho*I
cotD			
CotD-NcoI-F	forward	GAT*CCATGG*CTTGGATATATCAGAAAAC	*Nco*I
CotD-XhoI-R	reverse	ATC*CTCGAGG*AACATTTTTTGAGATTC	*Xho*I
cotEN			
CotEN-NcoI-F	forward	GGC*CCATGG*GCGTGATTTACATGCCAAATTT	*Nco*I
CotEN-XhoI-R	reverse	CCG*CTCGAG*TGGTACAAAACATAAGTACCAG	*Xho*I

[1] 5'-3', restriction site in italics

2.8 Confocal microscopy (Chapter 3 & 5)

Spores were labeled with mouse anti-Cot serum (1:1000 dilution) followed by an anti-mouse IgG-TRITC conjugate. Images were taken using a Nikon Eclipse fluorescence microscope equipped with a BioRad Radiance 2100 laser scanning system.

2.9 Transmission electron microscopy (TEM) (Chapter 3)

TEM methodology using suspensions of purified CD630 spores (7-days old) were as described previously for *B. subtilis* spores (Hong *et al.*, 2009). TEM studies were performed by Prof. Alain Brisson (Laboratoire de Microbiologie, UMR5248 CNRS-Université ENITA de Bordeaux, France).

2.10 Zeta potential measurements (Chapter 3)

Zeta potentials of spores were measured at 24°C with a 3000HS Malvern Zeta-sizer (Malvern Instruments Ltd, UK). Aliquots of 30 µl of spores suspended in Milli-Q water at a density of 5 x 10^9 spores ml^{-1} were added to 3 ml solutions of defined pH and ionic strength. The pH was adjusted using HCl or NaOH. The mean of two separate measurements from the same sample were determined. The zeta potential was calculated from the electrophoretic mobility using the Smoluchowski equation (Sze *et al.*, 2003).

2.11 Spore adhesion to hydrocarbon (SATH) assay (Chapter 3)

The surface hydrophobicity of spores was determined using the spore adhesion to hydrocarbon (SATH) assay using *n*-hexadecane as hydrocarbon (Seale *et al.*, 2008). Purified spores were washed in either Milli-Q water or 1 M NaCl in Milli-Q water by centrifugation at 16,000 g for 10 min and resuspended in 0.1 M NaCl at a density of 1 x 10^8 spores ml^{-1}. Spore suspensions (2 ml) were added to 1 ml *n*-hexadecane (Aldrich) and vortexed for 1 min, incubated at 37°C for 10 min, and vortexed again for 30 sec. The absorbance of the aqueous phase was measured at 600 nm. The mean of two measurements was determined. The percent hydrophobicity (*%H*) was determined from the absorbance of the original spore suspension (A_i) and the absorbance of the aqueous phase after incubation with hexadecane (A_f) according to the following equation : $\%H = [(A_i - A_f) / A_i] \times 100$.

2.12 *In vitro* cell adhesion studies (Chapter 3)

Adherence of spores to Caco-2 cells was carried out using a method modified from Rowan N.J. *et al* (Rowan *et al.*, 2001). Caco-2 cell monolayers of 70–80% confluence grown in a 5% CO_2 atmosphere at 37°C in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal calf serum (FCS; Gibco BRL) were seeded with approximately 10^5 cells per well on 24-well cell culture plates. Prior to assays, the monolayers were washed two times with DMEM, inoculated with 10^7 purified spores (in DMEM with 10% FCS) in duplicate, and incubated for 2 h at 37°C in a 5% CO_2 atmosphere. After incubation, the monolayers were washed five times with DMEM to remove any non-adherent cells, and then the tissue culture cells were lysed with 1 ml of 1% Triton X-100 (vol/vol in distilled water) for 5 min at

37°C. Samples (0.1 ml) of lysate from each tissue culture plate were serially diluted in 0.9 ml of sterile distilled water, with subsequent enumeration by plating of 100 µl of appropriate 10-fold dilutions on BHI supplemented with 1% sodium taurocholate agar plates. % adhesion =(number of adhered spores/initial number of spores) X 100.

2.13 Growth and sporulation (Chapter 4)

C. difficile strains were stored as glycerol stocks and routinely streaked out on BHIS (Brain heart infusion) agar supplemented with 0.1% (w/v) cysteine and 5 mg/ml yeast extract (Smith *et al*., 1981). All culturing of *C. difficile* was made in an anaerobic chamber (80% N_2, 10% H_2, 10% CO_2, Don Whitley, UK). Assays for growth and sporulation included optical density readings (OD_{600}, total cfu/ml by serial dilution and plating on BHIS agar (supplemented with 0.1% w/v sodium taurocholate), direct counting of phase bright spores using a haemocytometer (in duplicate), heat (60^{o}C, 1 h) and ethanol resistance (50% ethanol, 1 h, RT) and mRNA extraction.

2.14 Capture ELISA for detection of toxins A and B (Chapter 4)

Toxin A and toxin B were measured by capture ELISA. Briefly, ELISA plates (Greiner, high binding) were coated overnight, at RT, with rabbit polyclonal antibodies (1 mg/ml; Sigma) against toxin A or toxin B in PBS buffer. After blocking plates with 2% BSA (1 h at 37^{o}C), samples and reference toxin A or toxin B (AbD Serotec) were added to plates. Incubation was made at 30^{o}C for 3 h. For detection antibodies, monoclonal antibodies against toxin A (1/500; AbD Serotec) and toxin B (1/500; AbD Serotec) were used with incubation for 1 h at 30^{o}C. After

washing, the secondary antibody, HRP conjugated anti-mouse IgG was added, with incubation at RT for 1 h. Colour was developed with the TMB substrate and measurement at OD 450_{nm}. The titers of toxin in samples were calculated against the commercial reference toxins A or B (AbD Serotec). The sensitivity of the assay for toxin A was 5 ng/ml and 5 ng/ml for toxin B.

2.15. Cytotoxicity assays (Chapter 4)

The assays used to measure cytotoxicity of toxin A and toxin B in cell culture was that described by Kuehne *et al* (2010).

2.16 RT-PCR analysis (Chapter 4)

a) Synchronised sporulation in liquid broth

In each case a fresh, single, colony of the *C. difficile* strain was inoculated into BHIS broth (Brain heart infusion medium supplemented with 0.1% (w/v) cysteine and 5 mg/ml yeast extract (Smith *et al.*, 1981) and grown overnight (17-18 h) in an anaerobic incubator (Don Whitley, UK). The overnight culture was then diluted $1/10^4$ in BHIS in a volume sufficient for the planned sampling regimen. As discussed elsewhere, a $1/10^4$ dilution is essential to promote synchronized sporulation in liquid culture (a). Assays for growth and sporulation included optical density readings (OD_{600nm}), total CFU/ml by serial dilution and plating on BHISS agar (BHIS supplemented with 0.1% w/v sodium taurocholate to promote spore germination, (Wilson *et al.*, 1982), heat (60^oC, 1 h) and ethanol resistance (50% ethanol, 1 h, RT) and sampling for mRNA extraction.

b) RNA extraction

Culture samples (60 ml) were centrifuged at 6500 g for 10 min and the pellet dissolved in 1ml of denaturing solution and cells or spores disrupted (40 sec) using a Fastprep-24 (MP Biomedicals) cell disruptor. RNA was extracted using a single step method described elsewhere (Chomczynski & Sacchi, 2006). Extracted RNA was then dissolved in DEPC-treated water (Sigma) and stored at -80°C. The integrity of extracted RNA was verified using a NanoDrop spectrophotometer and by formaldehyde agarose gel electrophoresis. A purity ratio of between 1.9 and 2.1 (A_{260}/A_{280}) was acceptable for RNA work.

c) Real-time PCR (RT-PCR) analysis

cDNA was made using the SensiMix SYBR No-ROX kit (Bioline Reagents, London, UK) and stored at -20^{o}C. Primers for gene expression analysis are given in (Table 2.2) and were designed by PrimerDesign (PrimerDesign Ltd. Southampton, UK). Primers were first tested by conventional PCR with genomic DNA to confirm specificity and product sise. RT-PCRs were performed in triplicate in 20 µl volumes using 100 ng of cDNA, 100ng of primers and 10 µl of SYBR Green (Promega, UK). Gene expression of target genes was normalised using the housekeeping gene *rpoA* as a reference (Merrigan *et al.*, 2010, O'Connor *et al.*, 2006). To establish the efficiency of reaction a standard curve was generated for each reference or target gene by diluting primer concentration. This identified the optimal dilutions for use in subsequent analysis. Thermal profiles used were: initial denaturation (95^{o}C, 10 min) followed by 40 cycles of denaturation (95^{o}C, 15 sec), annealing (56^{o}C, 30 sec) and extension (72^{o}C, 10 sec). This was followed by a dissociation curve to check product specificity. The expression of target genes was normalised to that of *rpoA* and

calculated by the Pfaffl method (Pfaffl, 2001) using the amplification efficiencies (E) determined in each run according to the following equation: Ratio = $(E_{target})^{\Delta CT, target\ (calibrator - test)}$ / $(E_{ref})^{\Delta CT, ref\ (calibrator - test)}$, where ΔC_T, target (calibrator – test) is the C_T of target gene in the calibrator minus C_T of the target gene in the test sample and ΔC_T, ref (calibrator – test) is the C_T of reference gene in the calibrator minus C_T of the reference gene in the test sample. PCR was performed using a Rotor-Gene 6000 (Corbett Life Science).

2.17 Recombinant *B. subtilis* vaccine strains (Chapter 5)

B. subtilis merodiploid strains contained chimeric genes of *cotB* and/or *cotC* fused at their 3'-ends to the C-terminal domains of toxin A (A26-39; codons Ser_{2388}-Pro_{2706} (Dove *et al*., 1990)) or toxin B (B15-24; codons Glu_{2137}-Glu_{2366} (Barroso *et al.*, 1990)). The entire *cotC* gene was used for fusion to the toxin domains, while for *cotB* a 3'-deletion was used so that the encoded CotB segment was 33 kDa. Genes were integrated into the chromosome by a stable, double crossover, recombination and were placed in *trans* to the indigenous *cotB* or *cotC* genes. The general strategy for constructing strains carrying chimeric genes has been reported elsewhere (Isticato *et al.*, 2001, Duc le *et al.*, 2007, Hoang *et al.*, 2008) and is summarised as follows.

Step 1.

The *cotB* and *cotC* genes including their sporulation-specific promoters were cloned in either pDG364 or pDG1664 using primers CotBF and CotBR for *cotB* and CotCF and CotCR for *cotC* (Table 2.3). For *cotB* the 3'-codon (codon 275, serine) was used producing a truncated *cotB* gene (the entire *cotB* ORF is 380 codons) encoding a 33 kDa species while for *cotC* the terminal codon (codon 118, tyrosine). Both these plasmids enable cloned genes to be integrated into the chromosome of *B. subtilis* by a double crossover recombinational event (Guerout-Fleury *et al.*, 1996) occurring at either the *amyE* (pDG364) or *thrC* loci (pDG1664) by selection for either chloramphenicol (pDG364) or erythromycin (pDG1664) resistance.

Step 2.

Toxin domains A26-39 and B15-24 were amplified by PCR from chromosomal DNA of *C. difficile* strain 630. Primers (Table 2.4) were designed to enable restriction and ligation, in frame, to either the *cotB* or *cotC* 3'-termini contained in the pDG364 or pDG1664 clones described in step 1.

- Toxin A primers (TcdAF and TcdBR) were designed to amplify DNA between codons 2388 (serine) and 2706 (proline) of the *tcdA* gene (Dove *et al*., 1990) corresponding to toxin domain A26-39.
- Toxin B primers (TcdBF and TcdR) were designed to amplify DNA between codons 2137 (glutamic acid) and 2366 (glutamic acid) of the *tcdB* gene (Barroso *et al*., 1990) corresponding to toxin domain B15-24.

Recombinant plasmids were sequenced to confirm integrity of cloning.

Table 2.2 Primer pairs used to amplify genes studied by real time PCR

Gene	Direction	Primer sequence (5'-3')
tcdA	*F*	GTCGGATTGCAAGTAATTGACAATA
	R	TAACAGTCTGCCAACCTTTTGAGA
tcdB	*F*	ACCATATAGCTTTGTAGATAGTGAAGGAAA
	R	AAGAACTACATCAGGTAATTCAGATACAAA
tcdR	*F*	AACTCAGTAGATGATTTGCAAGAAA
	R	TCTGTTTCTCCCTCTTCATAATGT
cotA	*F*	CTTTCAAAAGTACCATCAAGTCCAG
	R	TCTCAGATTTCTTTATAGTAGGTGCTA
cotB	*F*	ATATCAAGAACATTACAGAAGAAACTC
	R	CATGGTCTGCTAAAAGTGGTAATAT
cotC	*F*	ATGTCTTTGGCGATTCGGTAA
	R	AATATATCTTTCAAATCAGGGTCATCA
cotD	*F*	CTTGTGACCCTAGAATGGCTAAA
	R	AGGCATTGTATATCTTTGGCTTAAAT
cotE	*F*	CCTAGATTTTTAAACCAATTACTTGCT
	R	TGTAGGTGTTAATGCTGCATCA
rpoA	*F*	GGATGATATGATGAAGGTTAGAAACCT
	R	CCCAATCCAAGTTCTTCTAGTTTTTG
spoOA	*F*	GCGCAATAAATCTAGGAGCA
	R	AGGTTTTGGCTCAACTTGTGT
sigH	*F*	GGGGGAGATTTACATGTTGG
	R	TTTTATCCCCTTCACTTGCTC
sigE	*F*	GTTGCTGGAATGCTTGGAAT
	R	TCATTTCTTTTTGCAACCTTGA
sigF	*F*	TGGAAGTAACTGTTGCCAGAGA
	R	TCTTTTGCCTCTTCATCTCCA
sigG	*F*	TGATGCCATACAAGACCCAAT
	R	TCTTGCAACCAATTTTCATCA
sigK	*F*	TCAACGGAAGATCAGGATGA
	R	GCATATGTTGCTAATCGAGTTCC

F=Forward, *R*= Reverse

Step 3

Plasmids were linearised by restriction digestion and then introduced into competent cells of *B. subtilis* PY79 (a laboratory strain of *B. subtilis* (Youngman *et al.*, 1984)). Chloramphenicol-resistant (pDG364-derived) or erythromycin-resistant (pDG1664-derived) transformants were then colony purified, checked by PCR and finally by immunoblotting for expression of the appropriate chimeric protein.

Table 2.3 PCR primers for vaccine constructions

Primer	Direction	Sequence[1]	Restriction site
TcdAF	forward	CCC*AAGCTT*GCC**TCAACTGGTTATAC**	*Hind*III
TcdAR	reverse	G*GAATTC*TTA**AGGGGCTTTTACTCCATC**	*Eco*R1
TcdBF	forward	CCG*AAGCTT***ATAGAATCTGGAGTACAAAAC**	*Hind*III
TcdBR	reverse	CCG*GAATTC*CTA**TTCACTAATCACTAATTG**	*Eco*R1
CotBF	forward	GC*GGATCC*ACG**GATTAGGCCGTTTGTCC**	*Bam*HI
CotBR	reverse	CCC*AAGCTT***GGATGATTGATCATCTGAAG**	*Hind*III
CotCF	forward	GC*GGATCC***TTCACAAAAATACTCG**	*Bam*HI
CotCR	reverse	CCC*AAGCTT***GTAGTGTTTTTTATGC**	*Hind*III

[1] restriction site is in italics; bold = *C. difficile* or *B. subtilis* sequence is in bold.
The following strains were made:

PP059 (*thrC::cotB-A26-39*): encodes a fusion of A26-39 to the C-terminus of CotB (predicted mwt. 69 kDa). The chimeric gene was carried at the *thrC* locus of *B. subtilis*.

PP052 (*amyE::cotC-A26-39*): encodes a fusion of A26-39 to the C-terminus of the CotC (predicted mwt. 49 kDa). The chimeric gene was carried at the *amyE* locus of *B. subtilis*.

PP108 (*thrC::cotB-A26-39 amyE::cotC-A26-39*): created by transforming competent cells of PP052 with chromosomal DNA of PP059 with selection for Erm^R conferred by the *thrC::cotB-A26-39* cassette.

PP132 (*thrC::cotB-B15-24*): encodes a fusion of B15-24 to the C-terminus of CotB (predicted mwt. 60 kDa).

PP142 (*thrC::cotB-B15-24 amyE::cotC-A26-39*): was created by transforming competent cells of PP052 with chromosomal DNA of PP132 (*thrC::cotB-B15-24*) with selection for Erm^R conferred by the *thrC::cotB-B15-24* cassette.

2.18 Recombinant proteins (Chapter 5)

Recombinant A26-39 (36.6 kDa) and B15-24 (29.4 kDa) were produced in *E. coli* BL21(DE3)(pLys) from a pET28b expression vector that separately carried the rA26-39 and rB15-24 ORFs fused to a C-terminal poly-histidine tag. Primers used for cloning are shown in (Table 2.4). High levels of expression were obtained upon IPTG induction and purification of rA26-39 and rB15-24 by passage of the cell lysate through a HiTrap chelating HP column on a Pharmacia AKTA liquid chromatography system.

Table 2.4 PCR primers for pET expression vectors

Primer	Direction	Sequence[1]	Restriction site
pETA26-39F	forward	CATG*CCATGG*CC**TCAACTGGTTATAC**	NcoI
pETA26-39R	reverse	CCG*CTCGAG***AGGGGCTTTTACTCCATC**	*Xho*I
pETB15-24F	forward	CATC*CATGG***ATATAGAATCTGGAGTCAAAA**	*Nco*I
pETB15-24R	reverse	CCG*CTCGAG***TTCACTAATCACTAATTCAG**	*Xho*I

[1] restriction site is in italics; bold = *C. difficile* or *B. subtilis* sequence is in bold.

2.19 Protein quantification (Chapter 5)

Coat proteins were extracted from purified spore suspensions of PP108 and PP142 using two extraction procedures, i) SDS-DTT and ii) alkali extraction as described elsewhere (Duc *et al.*, 2003). SDS-DTT solubilises both CotB and CotC while alkali extraction preferentially solubilises CotC. Quantification was made using a Bio-Rad Gel Doc imaging system.

2.20 Polyclonal antibodies (Chapter 5)

Polyclonal antibodies were raised in mice immunised by the intra-peritoneal (i.p.) route with 2 μg of purified protein on days 1, 14 and 28. Dilutions used were 1:3,500 for anti-A26-39 and 1:1,500 for anti-B15-24.

2.21 Immunisations in mice (Chapter 5)

Animals used in this work were pathogen-free, Balb/c mice (Charles River) for antibody production, and for analysis of immune responses. In all cases females, aged 6-8 weeks were used. All animal procedures were performed under the Home

Office project license PPL 70/6126. Mice were dosed o.g. (0.2 ml by gavage) on days 0, 14, 35 and 57 with a dose of $5x10^{10}$ spores (PY79, PP108 or PP142). A naïve group of un-immunised animals was included in all experiments as well a group receiving a mixture of the rA29-36 (10 μg) and rB15-24 (10 μg) proteins. i.p. immunisations consisted of doses on days 0, 7 and 28 with the rA26-29 + rB15-24 proteins (10 μg of each).

2.22 Determination of mouse antibody titers by indirect ELISA (Chapter 5)

For analysis of responses, serum was taken on days -1, 34 and 56 (o.g. groups) or day 42 (i.p groups) and feces collected on days 21, 42 and 67 and kept at -80°C. Sample extractions were made at 1/5 (weight/vol) dilution in extraction buffer (2% FCS DMEM medium 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 and feces were determined by indirect ELISA. Greiner 96 well plates (Maxisorp) were coated with purified 10ug/ml rA26-39 or rB15-24 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 in diluent buffer (0.01 M PBS [pH7.4], 0.5% (w/v) BSA, %5 (v/v) fetal bovine serum (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). 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 optical densities were read at 450nm. Dilution curves were created for each sample and end-point titers for each specific antibody were estimated at the maximum dilution of serum giving an absorbance reading of 0.1 units over the OD of naïve samples.

2.23 Neutralisation assays (Chapter 5)

The ability of the antibody samples to neutralise *C. difficile* toxins *in vitro* was determined as described recently (Lyras *et al.*, 2009) with some modifications. HT29 and VERO cells were grown in McCoys 5A and Dulbeccos Modified Eagle complete medium (10% v/v fetal calf serum and 1% v/v penicillin and streptomycin). Cells were cultured at 37°C, 5% (v/v) CO_2 in air (100% humidity). HT29 and VERO cells were seeded in 96-well plates ($0.5x10^4$/well $2.5x10^4$/well respectively). After 24 h cells were washed twice in sterile PBS. Before the assay, toxins were incubated with serially diluted pooled serum or fecal samples (1:1 v/v) in medium containing 2% FCS and incubated for 1 h at 37°C before addition to the pre-washed cell monolayer. Cells were evaluated at 24 h (HT29) and 48 h (VERO). Toxins used were a A+B toxin (toxin 630) obtained from the culture supernatant of strain 630 that had been partially purified by ammonium sulphate precipitation (60%). The presence of toxin A and B was confirmed in the supernatant of 630 cultures by Western blotting. Toxin A and toxin B had been purified from cell supernatants. Appropriate toxin

concentrations were determined by the highest dilutions causing 100% cytopathicity (i.e., cell rounding), Toxin 630 [120 ng/ml], Toxin A [4.5 ng/ml], Toxin B [6.2 pg/ml]. All assays were carried out in duplicate.

2.24 Hamster challenge (Chapter 5)

Groups of 6-10 female, Golden Syrian hamsters (70-80 g; Charles River, UK), were immunised o.g. (0.2 ml) with either, i) recombinant (PP108, PP142) and non-recombinant PY79 spores ($5x10^{10}$/ dose/ hamster), or ii) a mixture of the recombinant proteins rA26-39 (10 μg) and rB15-24 (10 μg). One further group was dosed with the protein mixture delivered by the i.p. route (0.2 ml). Hamsters were dosed o.g. on days 0, 14, 35 and 57 and i.p. on days 0, 7 and 28. The challenges were performed as described by Goulding *et al* (2009). with some minor modifications. In brief, 14 days after the last dose hamsters were transferred to individual sterile cages (including all food, bedding and water) and treated with clindamycin (30 mg/kg). 12 h later hamsters were o.g. infected with 100 *C. difficile* spores (strain 630). 24 h after challenge and every two days thereafter, hamsters were repeatedly transferred to new sterile cages. Hamsters were intensively monitored 38 to 60 h after challenge. Hamsters showing clear symptoms were killed and considered unprotected.

2.25 Statistics (Chapter 5)

The unpaired *t* test was used to compare between groups. A *P* value of > 0.05 was considered non-significant.

CHAPTER 3

CHARACTERISATION OF *C. difficile* SPORES

3.1 Introduction

Spores of *C. difficile* are dormant life forms that can remain metabolically inactive unless triggered to germinate. Spores are produced at the end of the stationary phase of growth after which a mature endospore is produced. Examined under microscopy (phase-contrast) the spore is approximately 1 μm in length and ellipsoidal in shape. Characteristically, the spores are phase bright and resemble other classical bacterial endospores of the *Bacilli* and *Clostridia*. Spores are only produced under anaerobic conditions and spores are resistant to high temperatures (~60^{o}C) and desiccation (Henriques & Moran, 2000). Spores of *C. difficile* carry a structure common to other bacterial endospores, a core containing the germ-line chromosome, a thick layer of loosely-linked peptidoglycan (the cortex) and a thick shell referred to as the spore coat which is thought to be proteinaceous in composition. Under electron-microscopy, the spore coat is electron dense and therefore grey-black in appearance and the cortex electron-transparent and therefore white. A thin layer of membrane, the inner forespore membrane lies between the core and cortex and will form the cell wall of the vegetative cell when the spore germinates. *C. difficile* spores carry an additional layer, the exosporium that encases the mature spore in a loose-fitting sac-

like layer and is thought, based on work conducted in other spore formers, to be rich in glycoproteins (Henriques & Moran, 2007). The function of the exosporium is unclear in all spore formers and is the least understood structure. Not all spore formers carry an exosporium, for example, *B. subtilis* lacks an exosporial layer, but a role in adhesion is a likely candidate role (Henriques & Moran, 2000). Studies examining the attachment of *C. difficile* spores to agar surfaces has shown that the exosporium of *C. difficile* may play an active role in anchoring the spore while the spore then proceeds to germinate (Panessa-Warren *et al.*, 1997). Some studies with other clostridia have shown hair like appendages that may aid attachment (Pope *et al.*, 1967, Yolton *et al.*, 1968). A likely candidate is the BclA protein, a collagen-like protein, that in spores of *B. anthracis* and *B. cereus* are linked to adhesion (Todd *et al.*, 2003, Sylvestre *et al.*, 2002). Interestingly, the BclA protein of *B. anthracis* has also been shown to be involved in enhancing uptake of spores by phagocytes (Bozue *et al.*, 2007) so a role in opsonophagocytosis can not be excluded for *C. difficile*. A BclA protein has been identified in *C. difficile* strain 630 based on a proteomic study (Lawley *et al.*, 2009b) and it seems highly probable that *C. difficile* spores carry hair like appendages on the spore surface.

The importance of the *C. difficile* spore should not be overlooked for a number of reasons. First, spores are the main agent of transmission and survival in the environment and the control of CDI can only be achieved after the role of the spore in infection is fully understood. Second, almost all studies on colonisation and adhesion of *C. difficile* have focused on the vegetative cell and not the spore. Third, spores are known to convert from a benign form in the GI-tract to a supershedder

state following antibiotic treatment and the onset of CDI (Lawley *et al.*, 2009a). This supershedder state leads to the production of large quantities of *C. difficile* spores being shed in the faeces and which are highly transmissible. It is not clear why or how this occurs. Finally, hypervirulent strains of *C. difficile* that are linked to numerous epidemics over the last decade are all strains that show increased levels of sporulation.

For these reasons, it is clear that the considerable work needs to be conducted on the *C. difficile* spore. This has not arisen because until recently because no suitable method for growing crops of *C. difficile* in liquid culture have been developed. This has been compounded by the lack of genetic methods that have prevented mutational analysis. In the last 5 years the genome of *C. difficile* has been completed (Sebaihia *et al.*, 2006), the spore proteome deciphered (Lawley *et al*., 2009b) and the CLOSTRON method for insertional gene developed (Heap *et al.*, 2010).

In this chapter the aim was to develop a reproducible method for producing *C. difficile* spores. Spores would then be examined for their surface properties (charge and hydrophobicity), adhesion and then the proteins found on the spore coat layers solubilised and examined by mass-spectrometry to identify the putative genes which could be retrieved from the genome sequence. This was work which could provide the first dissection of the surface composition of *C. difficile* spores.

3.2 Results

3.2.1 *C. difficile* spore formation

Using an empirical approach we adapted existing methods (Wilson *et al.*, 1982) to generate high levels of spore formation on a solid medium using CD630 (*tcdA*$^+$ *tcdB*$^+$). After seven days growth on agar we routinely obtained >75% sporulation (Figure 3.1) with crops consisting of mature, released, spores which were then purified further providing suspensions devoid of viable vegetative cells.

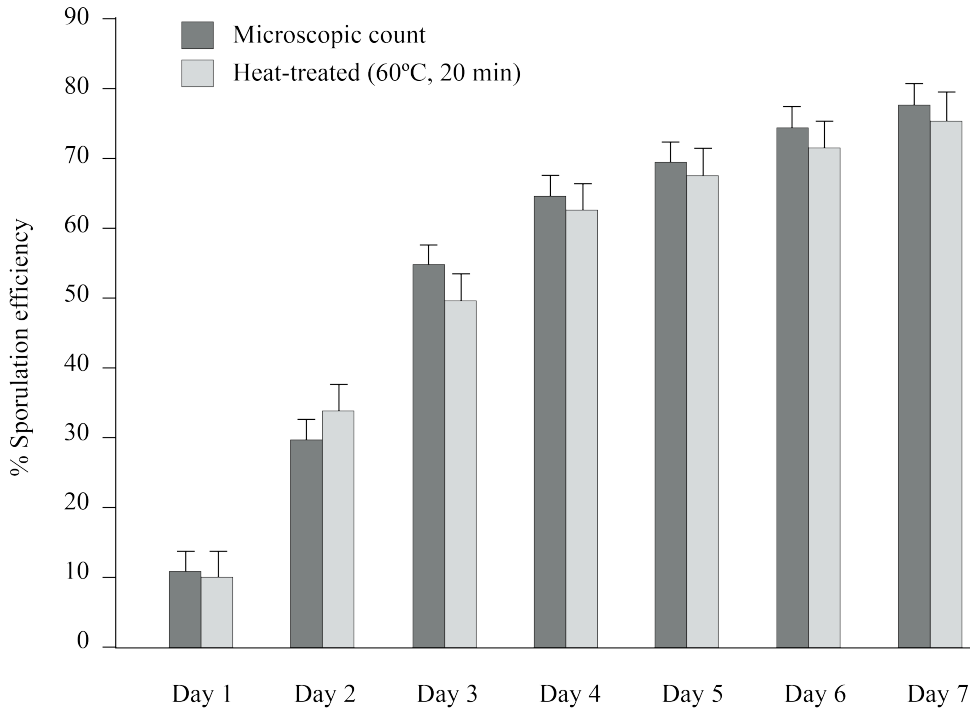


Figure 3.1 Sporutaion of *C. difficile* on solid medium.

C.difficile 630 was grown on agar at 37^{o}C. Each day samples were removed and examined for the number of spores by either phase contrast microscopy using a haemocytometer to count phase bright spores and vegetative cells, or, by heating the spore suspension for 60^{o}C for 20 min and plating for CFU/ml with comparison to un-treated CFU/ml.

TEM analysis of spores (Figures 3.2A and 3.2B) revealed a structure common to those produced by the majority of Gram-positive spore formers (Henriques & Moran, 2007), namely an inner core surrounded by a primordial germ cell wall (peptidoglycan derived from vegetative cell walls) and a thick cortical layer (loosely cross-linked peptidoglycan specific to the spore) (Figure 3.2B). Finally, above the cortex a thick, more electron dense, layer was present on all spores, this being the spore coat. Closer examination revealed further definition to this layer including laminations resembling the striated outer coats of *Bacillus* spores (Henriques & Moran, 2007). In other work spores of *C. difficile* have been reported to carry an exosporium (Lawley *et al*., 2009b, Panessa-Warren *et al*., 1997), a loose fitting sac-like structure enveloping the mature spore (Henriques & Moran, 2007). In our studies we have observed an exosporial layer resembling that of earlier studies and an example is shown in Figure 3.2C. However, we found observation of this structure to be inconsistent. The exosporium was only apparent in samples harvested and processed immediately (Figure 3.2C) and, for those spores where it was detected, the layer was only partially attached. In contrast, the images of Figures 3.2A and 3.2B were from CD630 spore preps that had been left overnight at 4^{o}C before spore purification.

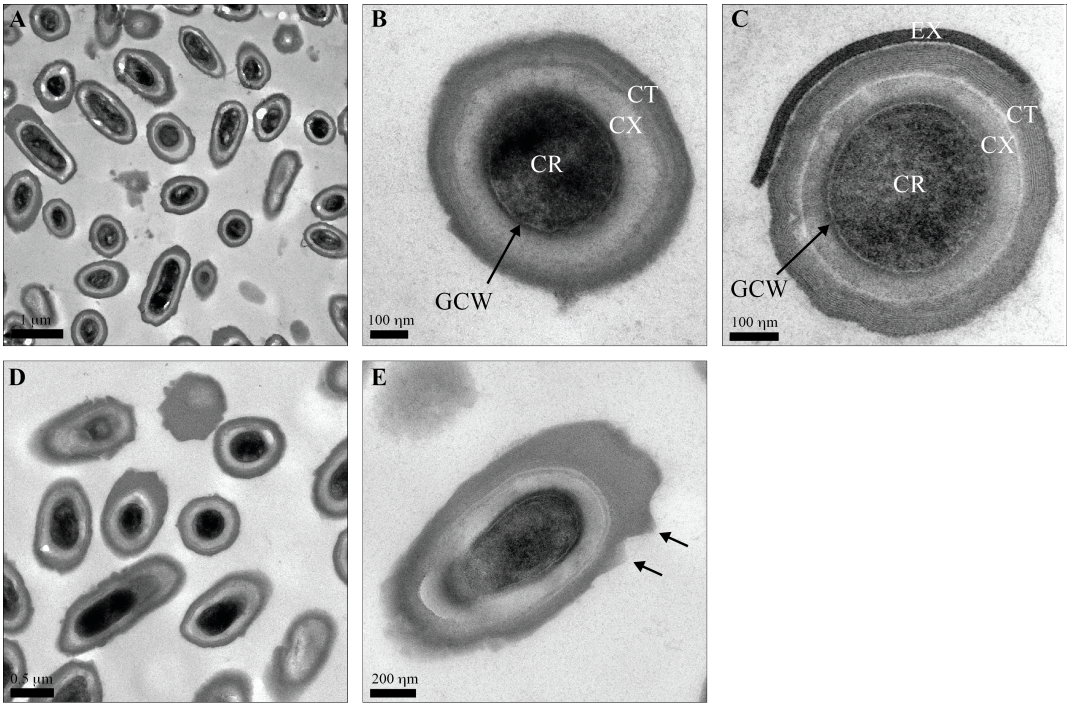


Figure 3.2 Ultrastructure of *C. difficile* 630 spores. Panels A to C show representative images of CD630 spores after seven days incubation on solid medium. **Panel B** shows the basic structural features found in a mature endospore; CR, core; GCW, germ-cell wall; CX, cortex and CT, coats. **Panel C** shows a spore containing a partially attached exosporium (EX). **Panels D** and **E** show seven-day-old spores subjected to ten cycles of sonication. **Panel E** shows angular projections found to be more abundant in sonicated samples (performed by Prof. A. Brisson, Univ. Bordeaux, France).

3.2.2 Surface properties of CD630 spores

The surface hydrophobicity of CD630 spores was determined using the SATH assay. The method measures the hydrophobicity of spores and has been used elsewhere as a reliable indicator of spore hydrophobicity (Huang *et al.*, 2010, Seale *et al.*, 2008). Spores of *B. subtilis* (hydrophobic) and *B. clausii* (hydrophilic) were used as

comparators. As shown in Figure 3.3A CD630 spores were markedly hydrophobic at low, medium and high pH (>90%).

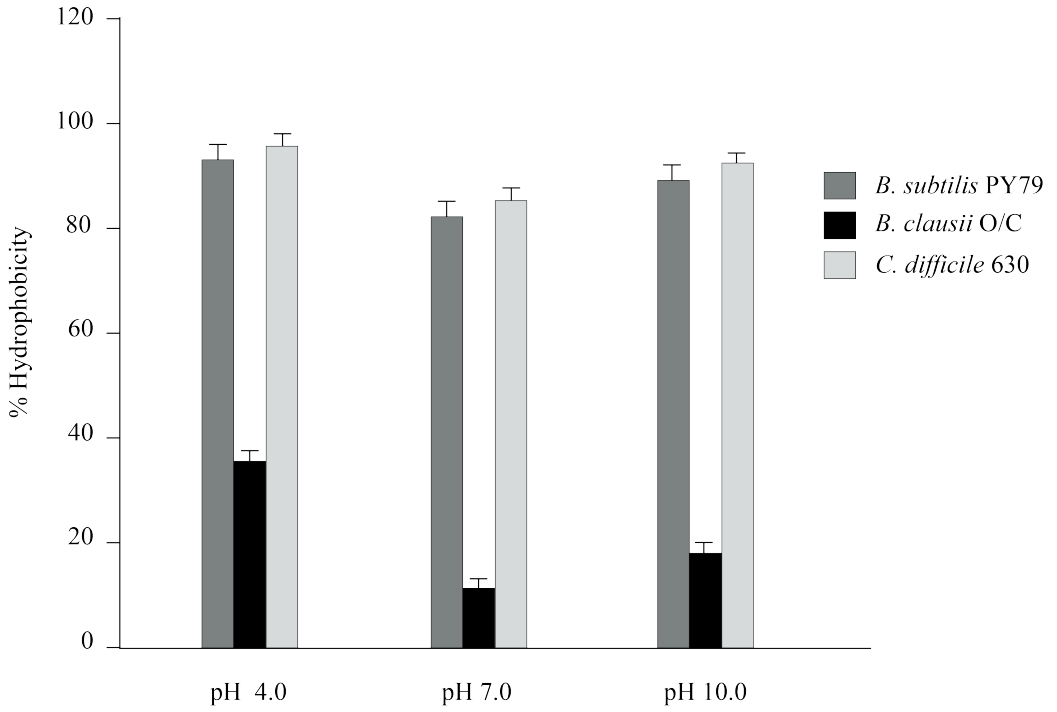


Figure 3.3A Surface hydrophobicity of CD630 spores. The SATH assay was used to calculate % hydrophobicity of purified CD630 spores. As controls, spores of *B. subtilis* PY79 and *B. clausii* O/C were used for comparison.

The hydrophobicity of vegetative cells was also measured and as shown in Figure 3.3B was substantially lower than spores. Moreover, sonicated CD630 spores (ten rounds of sonication) showed a reduced level of hydrophobicity. As will be shown later sonication efficiently removes not only the exosporium but also the other spore coat proteins from CD630 spores.

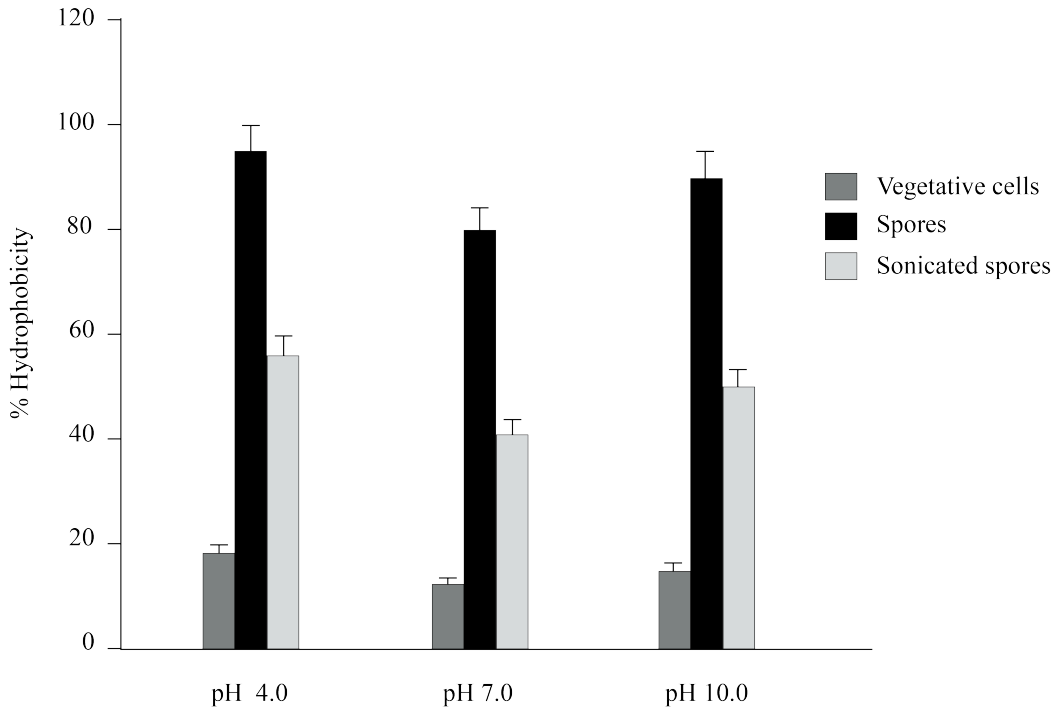


Figure 3.3B Surface hydrophobicity of CD630 spores and vegetative cells. The SATH assay was used to calculate % hydrophobicity of purified CD630 vegetative cells, spores and sonicated spores.

The electro-kinetic properties of CD630 spores were measured using a Zeta-sizer to determine zeta potential of CD630 spores at different pHs. The zeta potential is a measure of the net surface charge and has been used to show that spores of *B. subtilis* carry a net negative charge even at low pH (Huang *et al*., 2010). As shown in Figure 3.4 CD630 spores carried a net negative charge at all pH values. The charge diminished at lower pH but the spores still retained a negative charge (-2).

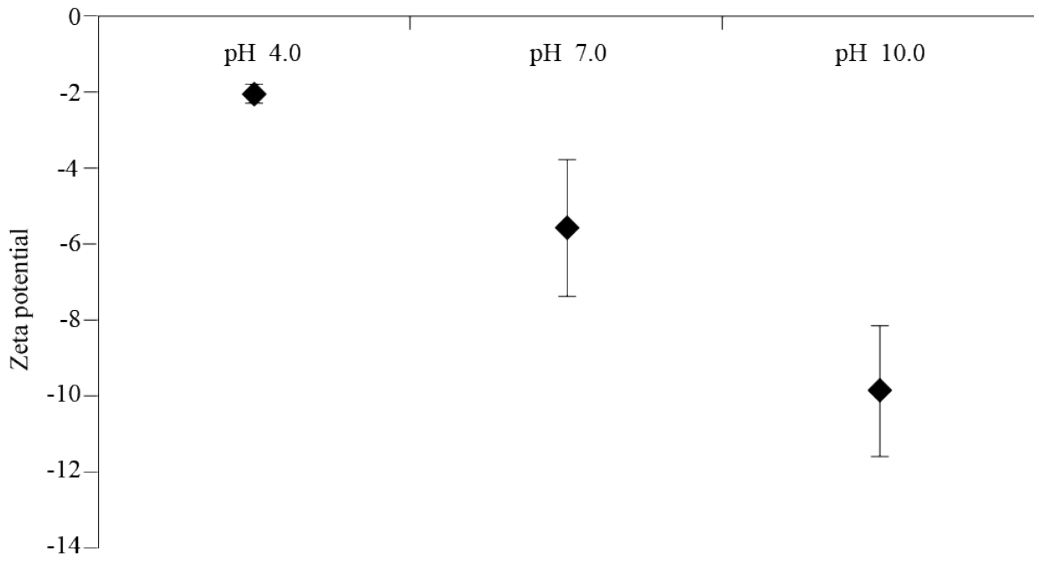


Figure 3.4 Surface charge. Zeta potential measurements of CD630 spores in water at low, medium or high pH.

3.2.3 Adhesion of CD630 spores to enteric cells

Next, we used purified suspensions of CD630 spores and measured the ability of spores to adhere to enteric cell lines using *in vitro* cell culture. Three human intestinal cell lines, Caco-2, HT29M and HT29 were used for this analysis and adhesion to 2-day old and 7-day old cultures was determined. HT29M is a mucus producing cell line and is isogenic to HT29 which does not produce mucus. As controls we also measured adhesion of vegetative cells of CD630 and of *B. subtilis* PY79 as controls. It was not possible to measure adhesion of spores of *B. subtilis* strain PY79 to cell lines since *B. subtilis* spores were shown to germinate and outgrow in the cell culture medium (data not shown).

Our results (Figure 3.5) which have been repeated more than two times in full and with similar results show that CD630 spores are very efficient in binding to the enteric cell lines we evaluated. Interestingly, we could not observe increased adhesion to the mucus-producing cell and indeed increased binding was observed to HT29 cells which do not produce mucin. This adhesion is in marked contrast to the binding of vegetative cells of CD630 where adhesion was almost 100-times less efficient when measured in parallel. This finding which will be discussed later and implies that the spore may play a role in colonisation.

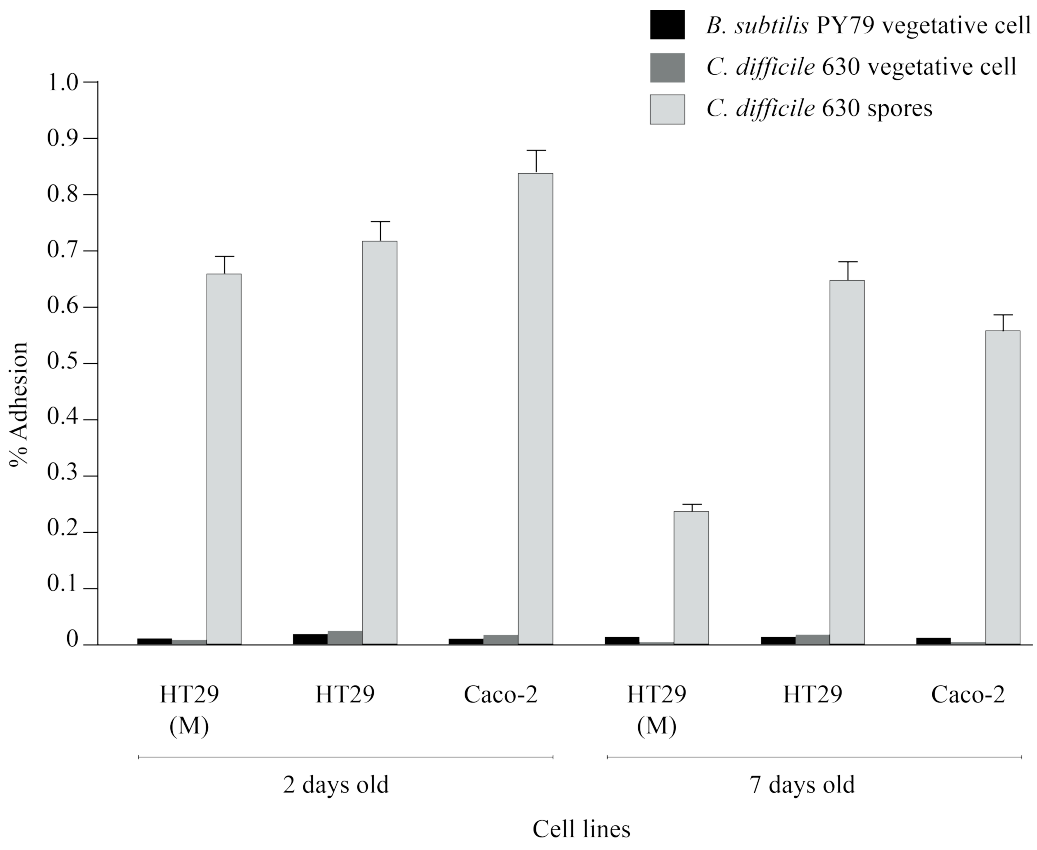


Figure 3.5 Adhesion of CD630 to enteric cell lines.

Caco-2 and HT29 (mucus and non-mucus producing) intestinal cell lines were cultured for 2 and 7 days and inoculated with spores of CD630 or vegetative cells of CD630 and *B. subtilis* PY79 as controls. The percentage of viable cells/spores adhering was determined as described in Materials and Methods (section 2.12).

3.2.4 Electron microscopic analysis of adhesion

Cultured Caco-2 cells (2 days) were co-incubated with spores or vegetative cells of CD630 and examined using TEM. Our results showed (Figures 3.6A and 3.6B) that *C. difficile* spores bound to the pseudopods of immobilised Caco-2 cells.

Caco-2 cells were immobilised on plastic supports and sections examined by TEM. Spores appeared were attached in clusters to protruding pseudopods. No binding of vegetative cells was observed. TEM studies were performed by Prof. A. Brisson, Univ. Bordeaux, France.

3.2.5 Identification of *C. difficile* spore coat proteins

In *B. subtilis* reverse genetics has been used to examine the spore coat; proteins are solubilised and subjected to Edman–amino-terminal sequencing and the resulting polypeptide sequences used to clone the genes from phage libraries (Donovan *et al.*, 1987, Zheng *et al.*, 1988, Cutting *et al.*, 1991). This approach was able to identify most of the principle spore coat proteins found in the spore coat of *B. subtilis* (Henriques & Moran, 2007). The approach adopted here was similar in principle but

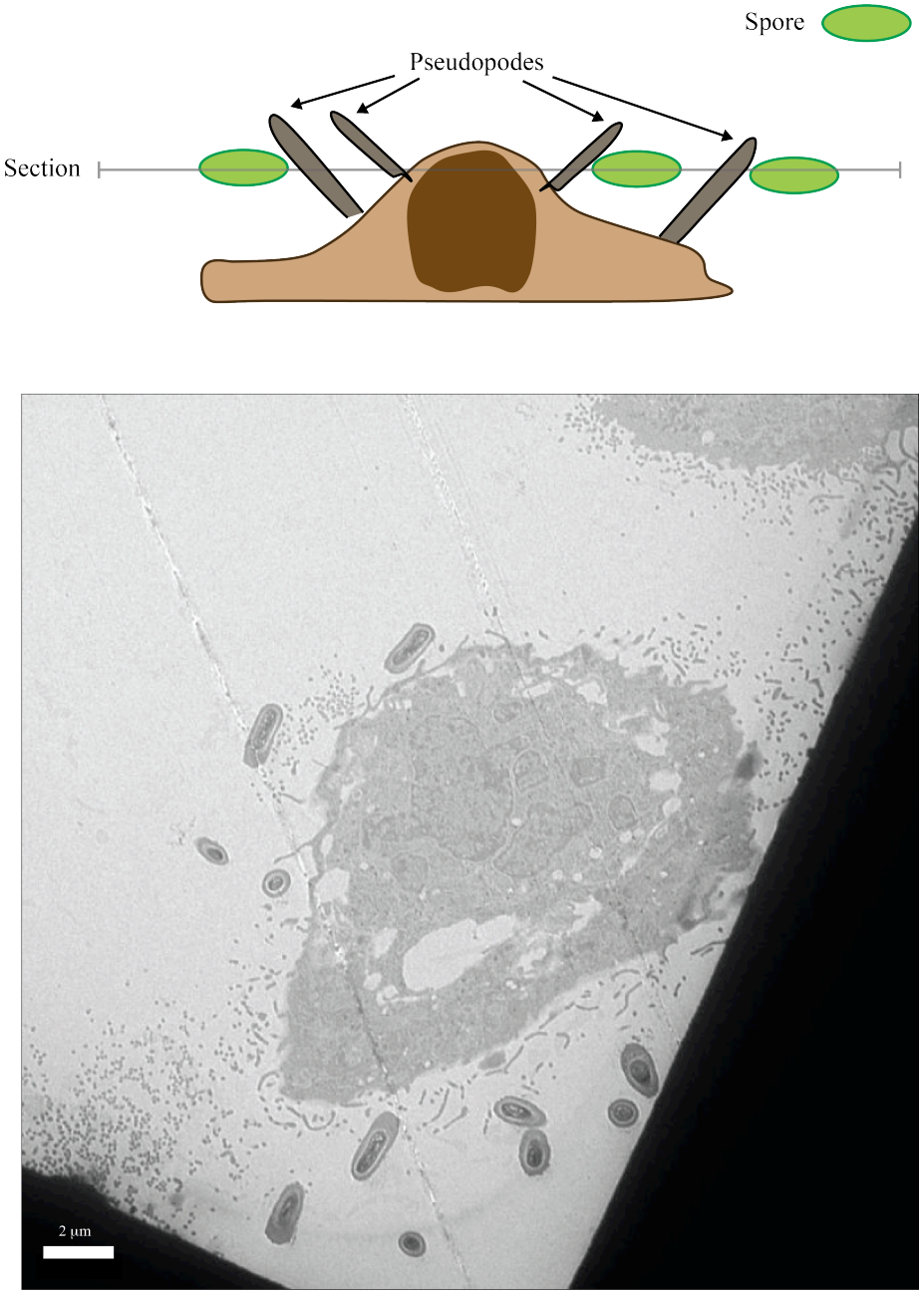
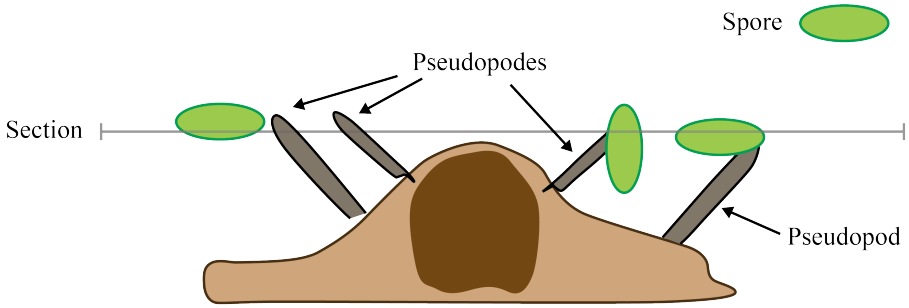


Figure 3.6A Adhesion of *C. difficile* spores to Caco-2 cells



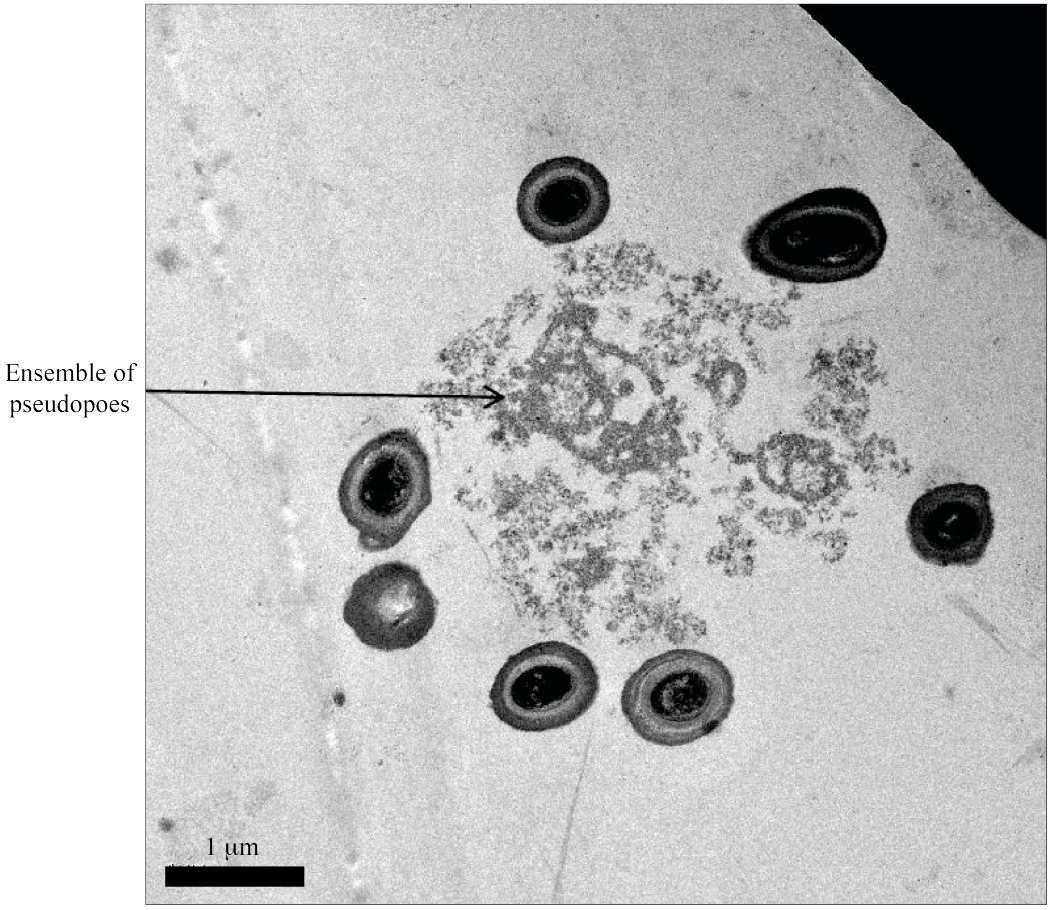


Figure 3.6B Adhesion of *C. difficile* spores to Caco-2 cells.

using mass-spectrometry to identify the amino acid sequences of fractionated spore coat proteins on SDS-PAGE gels. These sequences were then compared to the database of CD630 encoded proteins predicted from the CD630 genome sequence.

Coat proteins were extracted from freshly prepared spores of CD630 grown on solid medium using a sodium borate-SDS-DTT buffer and fractionated by SDS-PAGE (Figure 3.7). Since spores were processed immediately we reasoned that they may carry some residual exosporial material as shown in Figure 3.2C. Eleven protein bands were excised from Coomassie-stained gels and subject to peptide mass fingerprinting using trypsin digestion and MALDI mass spectrometry. This analysis revealed that a number of protein bands corresponded to truncated, breakdown, products (Table 3.1). One high molecular weight species of 118 kDa could not be identified and may be an aggregate. Two further bands, were chain E of proteinase K which was a contaminant derived from the spore purification process. The remaining eight protein species corresponded to five different proteins which we refer to as CotA, CotB, CotCB, CotD and CotE and their genes as *cotA-cotE* (Figure 3.8) based on nomenclature used in *B. subtilis* (Henriques & Moran, 2007); orthologues are shown in Figure 3.9. CotA shared no homology with other proteins in existing databases but CotB had orthologues in a number of *Bacilli* and *Clostridia*. CotCB and CotD were homologous with both each other (70% conserved residues) and to manganese catalases including the CotJC inner spore coat protein (and putative catalase) found in *B. subtilis* (Figure 3.10).

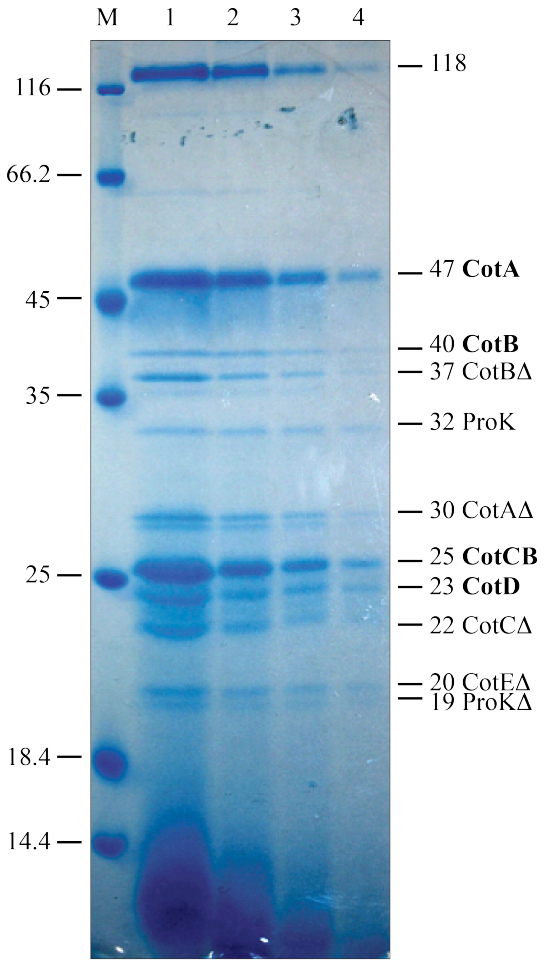


Figure 3.7 Proteins extracted from CD630 spores. Proteins extracted from CD630 spores (seven day old cultures grown on solid medium) using a sodium borate-SDS-DTT extraction buffer. Proteins were fractionated by SDS-PAGE (12.5%) and samples loaded as dilutions, lane 1, no dilution; lane 2, ½ dilution; lane 3, ¼ dilution; lane 4, 1/8 dilution. M, markers. Alongside the gel the identities of the bands excised and analysed by mass spectrometry are shown. Partially truncated proteins (D) are indicated.

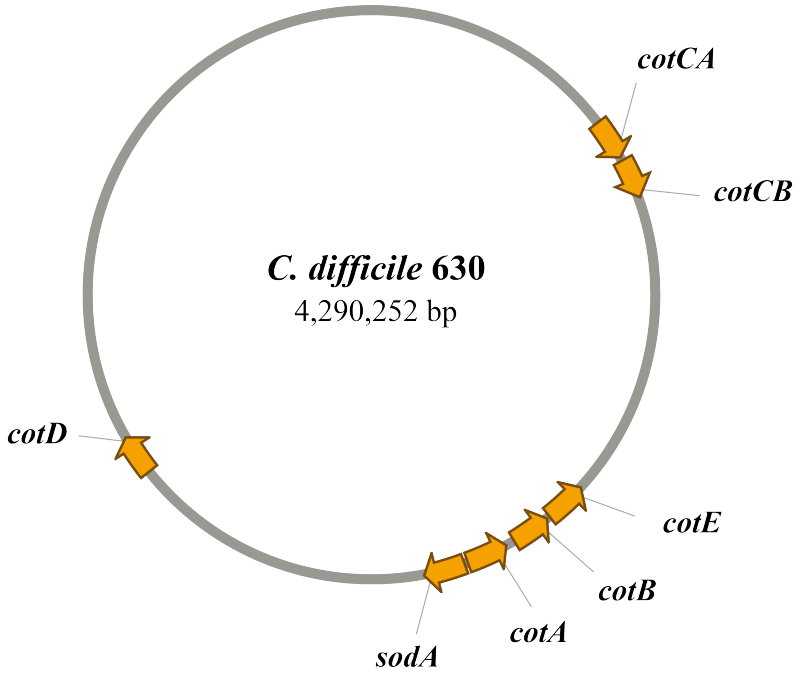


Figure 3.8 CD630 Spore coat genes. Chromosomal positions of genes referred to in this work.

As will be discussed later the 25kDa protein is most probably encoded by the second cistron of an operon so we refer to the gene and protein as *cotCB* and CotCB respectively. CotE based on its amino acid sequence, corresponded to a novel bifunctional protein with amino-terminal peroxiredoxin (1-cys peroxiredoxin) and carboxy-terminal manganese chitinase activity (Figure 3.11). The predicted mwt of this protein was 81 kDa although the full-length protein was not clearly discernable in our SDS-PAGE fractionations but rather a 20 kDa truncated species. CotE had orthologues in a number of spore formers (Figure 3.9). As a single bifunctional protein, no orthologues were found in other *Bacilli* or *Clostridia* but matches were found to either the peroxiredoxin or chitinase domains carried in CotE. This included a putative peroxiredoxin YkuU in *B. subtilis* (BS938810) and a number of putative

chitinases from exosporium-containing species including *Bacillus anthracis*, *B. cereus*, *Bacillus thuringiensis*, *Bacillus clausii* and *Bacillus halodurans* (Figure 3.9).

Table 3.1 SDS-PAGE and MALDI peptide fingerprint analysis of *C. difficile* 630 spore coat proteins.

Fragment mwt.	Protein ID[a]	Coding sequence No[b]	Predicted mwt	Assigned Gene
118	ND	-	-	-
47	Hypothetical protein	CD1613	34	*cotA*
40	Hypothetical protein	CD1511	35	*cotB*
37	Hypothetical protein	CD1511	35	*cotB*
32	Proteinase K (contaminant from purification steps)	-	-	-
30	Hypothetical protein	CD1613	34	*cotA*
25	Putative spore-coat protein; Manganese catalase; similar to CotJC of *B. subtilis*.	CD0598	21	*cotCB*
23	Putative spore-coat protein; Manganese catalase; similar to CotJC of *B. subtilis*.	CD2401	21	*cotD*
22	Putative spore-coat protein; Manganese catalase; similar to CotJC of *B. subtilis*.	CD0598	21	*cotCB*
20	Putative bifunctional protein: peroxiredoxin/chitinase	CD1433	81	*cotE*
19	Proteinase K (contaminant from purification steps)	-	-	-

[a]based on peptide mass fingerprinting of tryptic digestions ND, no determination.

[b]coding sequences as described in Sebaihia *et al* (2006).

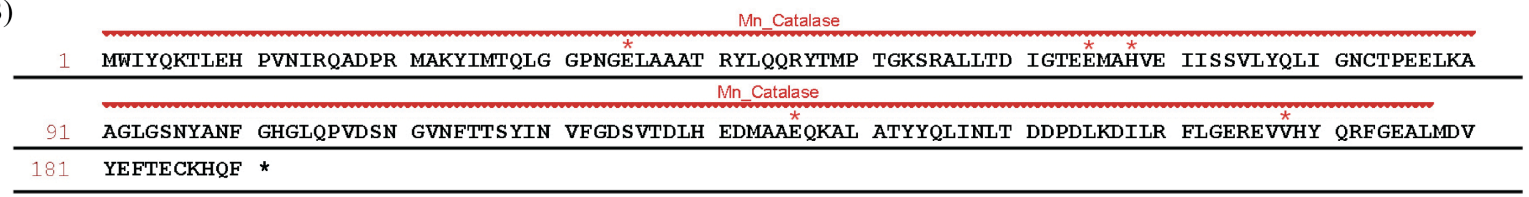
Proteins	Orthologues															
	C.d	***B.s***	***B.li.***	***B.a.***	***B.c.***	***B.t.***	***B.cl.***	***B.h.***	***G.k.***	***O.i.***	***C.p.***	***C.a.***	***C.t.***	***C.th.***	***C.n.***	***C.c.***
CotA	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CotB	+	-	+	+	+	+	-	+	-	-	+	+	+	+	-	+
CotCA	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	+
CotCB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
CotD	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
CotE-peroxchitinase	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CotE-peroxiredoxin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
CotE-chitinase	+	+	+	+	+	+	+	+	-	+	-	-	-	+	-	+

Orthologues were identified through BLASTP searches (www.ncbi.nih.gov) using proteins sequences against the following genomes:

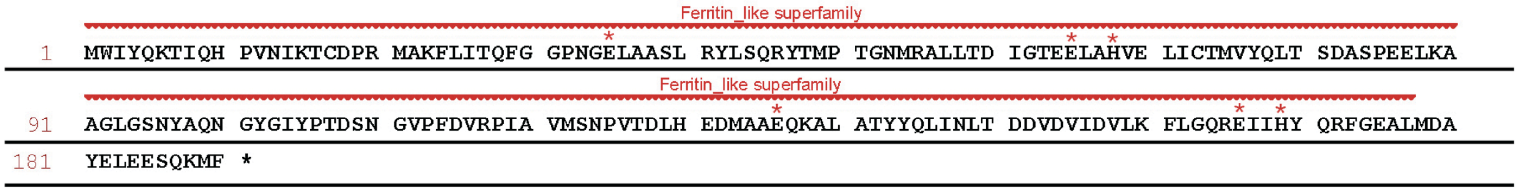
B.a., *Bacillus anthracis* Sterne
B.c., *B. cereus* ATCC 10987
B.cl., *B. clausii* KSM-K16
B.h., *B. halodurans* C-125
B.l., *B. licheniformis* ATCC 14580
B.s., *B. subtilis* 168
B.t., *B. thuringiensis* serovar konkukian str. 97-27
C.a., *Clostridium acetobutylicum* ATCC 824
C.c., *Clostridium cellulolyticum* H10
C.d., *Clostridium difficile* 630
C.n., *Clostridium novyi* NT
C.p., *Clostridium perfringens* ATCC 13124
C.t., *Clostridium tetani* E88 (asporogeneous)
C.th., *Clostridium thermocellum* ATCC 27405
G.k., *Geobacillus kaustophilus* HTA426
O.i., *Oceanobacillus iheyensis* HTE831

Figure 3.9 Orthologues of *C. difficile* 630 spore surface proteins CotA-E in other spore formers. The table shows possible orthologues of CotA-E found in *Geobacillus kaustophilus* and *Oceanobacillus iheyensis* as well as other common *Clostridium* and *Bacillus* spore formers. For CotE also shown are orthologues to the individual peroxiredoxin and chitinase domains. Hypothetical proteins are also included.

A (CotCB)



B (CotD)



C (CotCB vs CotD alignment)

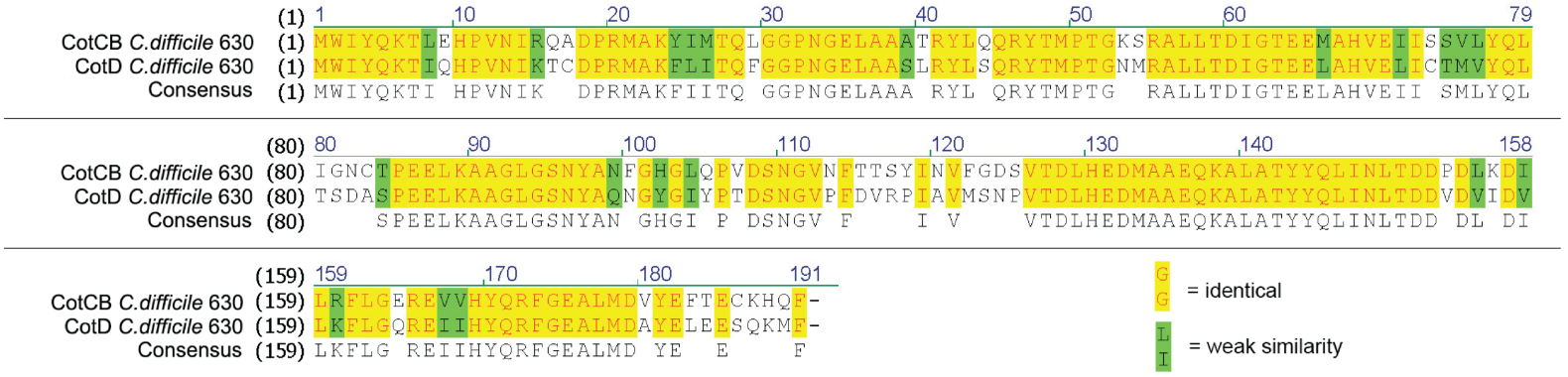


Figure 3.10 CotCB and CotD (see next page for legend)

Figure 3.10 (see previous page) **CotCB and CotD Panel A** shows the entire CotCB polypeptide and its similarity with the manganese catalases (a family of ferritin-like diron enzymes). Residues involved in forming the dimanganese centre are indicated (*). **Panel B** shows the homology of CotD with the ferritin-like family of catalases and amino acids involved in forming the dinuclear metal binding motif (*). **Panel C** shows the amino acid sequence homology between CotCB and CotD which share consensus and identity positions at 80.6% and 70.2% respectively.

3.2.6 Immuno-analysis of spore coat proteins

Polyclonal antibodies to recombinant Cot proteins were raised in mice. In the case of CotE we used the amino-terminal, peroxiredoxin domain, of CotE to generate antibodies. Using confocal imaging of antibody-labelled *C. difficile* spores (purified after overnight incubation at 4^{o}C) we observed uniform surface decoration using all antisera (Figure 3.12) while naive serum gave no labelling (not shown). Since, as mentioned earlier, these spores lacked an exosporium our data suggests that each of the five coat proteins must be exposed on the outermost layers of the spore and components of the spore coat, rather than the exosporium.

These antibodies were used in a Western blot to probe spore coat protein extractions (Figure 3.13A). CotA, CotB and CotD were present as single bands of 47, 40 and 23 kDa respectively corresponding to the predicted mwts of each of these proteins. CotE antisera identified two strongly reacting bands of 81 and 40 kDa. But the 20 kDa species (identified as a peptide fragment in Figure 3.13A) was not observed.

Figure 3.11 CotE. Figure shows the entire CotE polypeptide and its amino-terminal 1-cys-peroxiredoxin and carboxy-terminal chitinase domains. Active site residues are indicated.

Figure 3.12 Surface display of spore coat proteins. Surface display of CotA, CotB, CotC, CotD and CotE using confocal imaging of suspensions of CD630 spores (seven day old cultures grown on solid medium) labeled with mouse serum (1:1000 dilution) raised against each of the five Cot proteins. CD630 spores labeled with pre-immune serum served as a control and showed no labelling (not shown). Spores labelled with anti-spore serum is also shown ('Spores'). Anti-mouse IgG-TRITC conjugate was used for secondary labelling. Images were taken using a Nikon Eclipse fluorescence microscope equipped with a BioRad Radiance 2100 laser scanning system. (Image size = 37 X 37 mm). The upper row shows labelling of untreated spores and the lower panel shows labelling of spores that had been sonicated ten-times.

The most likely explanation is that this 20 kDa species is a C-terminal fragment that is not recognised by the polyclonal CotE antibodies that were raised against the N-terminus of CotE. For CotCB when probed with anti-CotCB we could sometimes discern two bands of 25 and 23kDa although this is not apparent in Figure 3.13A. Since CotCB and CotD were homologous we wondered whether these proteins shared related epitopes. Using recombinant proteins (rCotCB and rCotD) we probed each with anti-CotCB and anti-CotD sera. As shown in Figure 3.13B CotD was recognised by both anti-CotCB and anti-CotD sera. On the other hand, CotCB was detectable using anti-CotCB antibodies but only very weakly by anti-CotD.

Using antisera raised against formalin-inactivated CD630 spores we probed spore coat proteins extracted from CD630, *B. subtilis* and *B. clausii* (Figure 13C). *C. difficile* serum showed no cross-reaction against either *B. subtilis* or *B. clausii* spore coat proteins, the latter of which carries an exosporium (Henriques & Moran, 2007, Driks, 2007). Similarly, antiserum raised against formalin-inactivated *B. subtilis* spores showed no reaction against CD630 spores but some cross-reaction to *B. clausii* (Figure 13D). These results support bioinformatic analysis that has shown little conservation between *C. difficile* and *Bacillus* spores (Lawley *et al*., 2009b, Sebaihia *et al*., 2006).

3.2.7 Effect of sonication on spore coat proteins

An exosporium has been documented for *C. difficile* spores prepared in liquid medium (Panessa-Warren *et al*., 1997, Lawley *et al*., 2009b). The exosporium remains the least understood component of the bacterial endospore.

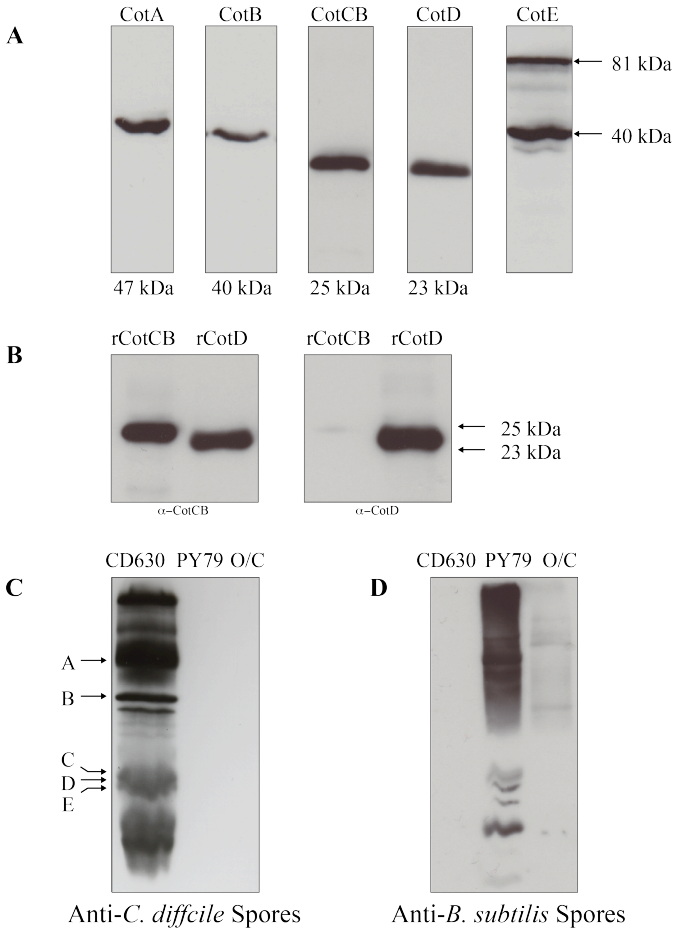


Figure 3.13 Immunoanalysis of spore coats. Panel A. Spore coats of CD630 were extracted and separate lanes probed with polyclonal (mouse) antibodies to CotA-E. Molecular weights of the relevant bands are shown. For CotE two principal bands of 81 and 40 kDa were found. Serum from unimmunized mice did not react with *C. difficile* spore coat proteins. **Panel B.** Purified recombinant CotC and CotD proteins (2 µg) were fractionated on SDS-PAGE gels and probed with either CotC or CotD antibodies at 1/1500 and 1/3000 dilutions respectively. Positions of CotC (25 kDa) and CotD (23 kDa) bands are indicated. **Panel C**. Coat proteins extracted from spores of CD630, *B. subtilis* PY79 and *B. clausii* O/C were fractionated and probed with antiserum to formalin-inactivated CD630 spores. Positions of CotA-E are shown. **Panel D**. As panel C but proteins were probed with antiserum to formalin inactivated *B. subtilis* PY79 spores.

remains the least understood component of the bacterial endospore (Henriques & Moran, 2007) and harsh physical methods such as sonication and shear stress are reported to remove the exosporial layer (Redmond *et al.*, 2004).

Although the majority of spores present in our preparations of CD630 did not appear to carry a recognisable exosporium we subjected CD630 spores to repeated cycles of sonication. Spore pellets and supernatants were then separated. Pellets were then extracted with Na borate-SDS-DTT buffer and extracts run on 12.5% SDS-PAGE gels together with supernatant fractions (Figure 3.14A). Our results showed that as few as two cycles of sonication were sufficient to remove almost the entire component of sodium borate solubilised proteins all of which were found in the supernatant fraction. Examination of the sonicated spores by phase-contrast microscopy revealed that phase-bright spores remained intact and analysis of CFU before and after sonication demonstrated no change in viability. Analysis by TEM revealed no clear-cut differences between sonicated spores (Figure 3.2C) and unsonicated spores (Figure 3.2D). Although, precise quantification was not possible we observed that in sonicated samples many spores carried angular projections on the surface layers (shown in Figure 3.2D). These crystalline-like structures might indicate an underlying layer of coat resulting from sonication and removal of one or more layers of coat material.

Following sonication the spore pellet and supernatant fractions were probed with antiserum to CotA-E (Figure 3.14). CotA, CotB and CotCB were not detectable in the spore pellets and found only in the supernatant fractions. CotD and CotE

although not visibly apparent in Coomassie-stained gels were present in both the spore pellet and supernatant fractions using immuno-analysis. If CotD was still present in the spore coat fraction then why was it not detected using anti-CotCB serum since CotCB and CotD share related epitopes? We reason that although the recombinant proteins, at high concentration, could be detected this does not reflect the composition and abundance of CotCB and CotD in the spore coat but rather the different binding strength and specificity of the respective antibodies. In the case of CotE only the 40 kDa CotE fragment was found in the spore pellet fraction. We used confocal imaging to examine antibody-labelled sonicated spores (Figure 3.12). This analysis revealed that in each case, following sonication, surface labelling was massively reduced. Labelling correlated well with the western blotting analysis (Figure 3.14B) with CotA, CotB and CotCB showing almost no labelling and weak labelling with CotD and CotE antibodies suggesting that some CotD and CotE remained in the sonicated spores. These results then show that all five Cot proteins are located on the spore surface and can be liberated either by sonication or using a sodium borate-SDS-DTT extraction buffer.

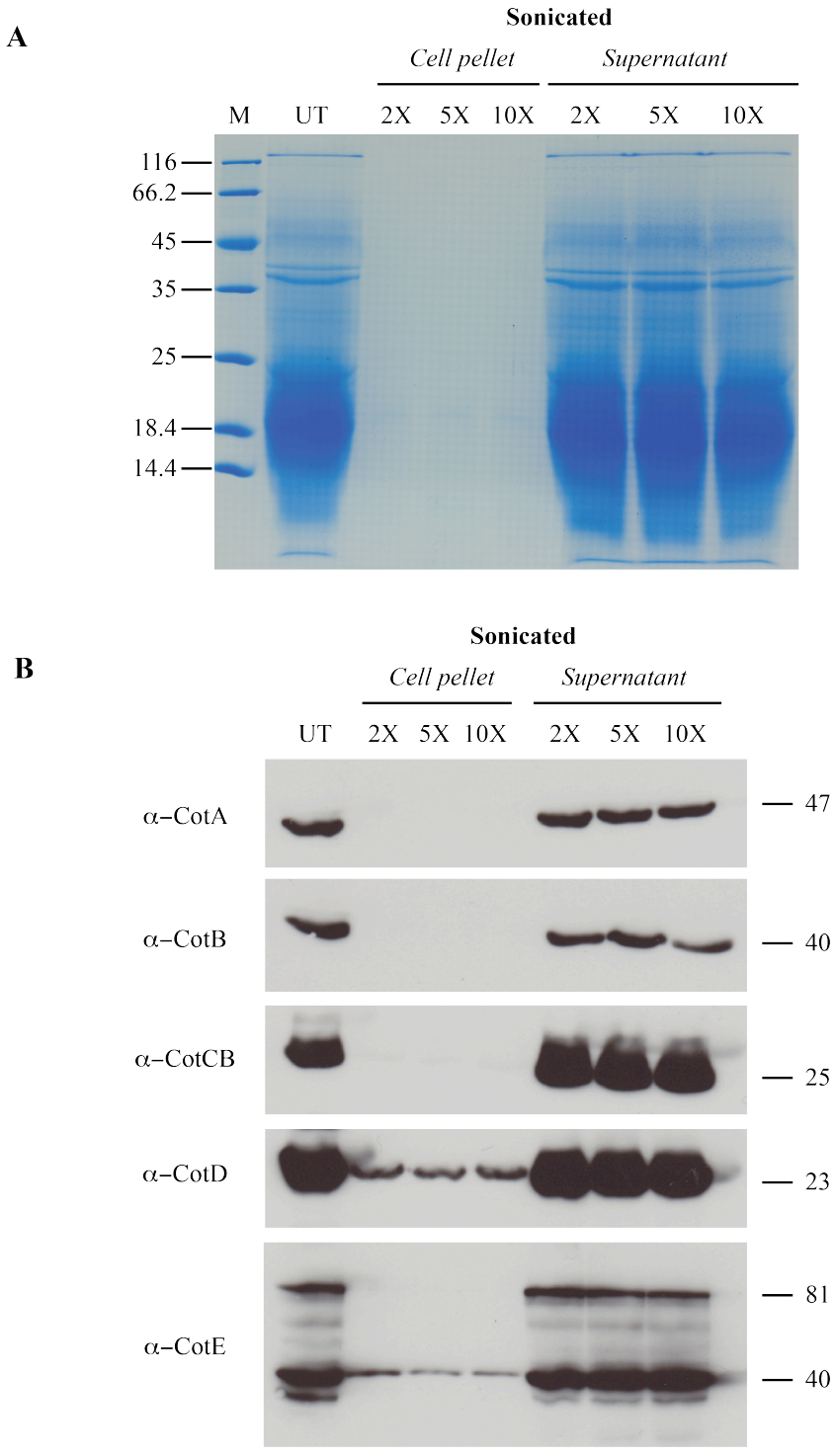


Figure 3.14 Removal of Cot proteins using sonication (see next page for legand)

Figure 3.14 (see previous page) **Removal of Cot proteins using sonication. Panel A.** *C. difficile* spores were sonicated (30 sec cycles), 2-times, 5-times and 10-times and pellets and supernatants separated. Pellets were treated with Na-borate-SDS-DTT extraction buffer and extracted proteins mixed with SDS-PAGE loading dye (4X) and fractionated by SDS-PAGE (12.5%). Supernatants were mixed with loading dye and run directly. **Panel B**. Spores were sonicated and pellet and supernatant fractions (**Panel A**) were probed with antiserum to each of the five Cot proteins. Molecular weights of Cot proteins are shown. For both panels UT, untreated spores. M, markers.

3.2.8 The enzymatic properties of spores

Based on the amino acid sequences of CotC, CotD and CotE and their surface location we would predict that spores carry enzymatic activity, either latent or active. Accordingly, we conducted a number of assays to measure catalase, peroxiredoxin and chitinase activity.

Using pure suspensions of spores the enzyme activities were evaluated by a PhD student working in the Cutting laboratory, Elisabeth Tolls. The summary of this data is presented in Table 3.2 and in Permpoonpattana *et al* (2011a).

Table 3.2 Enzymatic properties of CD630 spores.

Enzyme	Vegetative cells	Spores
catalase	-	+
peroxiredoxin	-	+
chitinase	-	+

3.3 Discussions

This study has provided an initial examination of the of *C. difficile* spores. This work has focused on two aspects, surface properties and the composition of the spore coat.

3.3.1 Surface properties

CD630 spores were found to be negatively charged and hydrophobic at all pH ranges evaluated. In one study of spores of 12 *Bacillus* species all were found to be hydrophobic yet this property has been proposed as being related to the exosporium, that is, species or strains carrying an exosporium were more hydrophobic than those that did not (Koshikawa *et al.*, 1989). In our study here we showed that *C. difficile* spores were as hydrophobic as *B. subtilis* which does not carry an exosporium. Moreover, we also found that vegetative cells were far less hydrophobic and that sonication reduced the hydrophobicity. Interestingly, spores of *B. clausii* were distinctly hydrophilic yet this species carries an exosporium. Thus, the role of exosporium in determining hydrophobicity is unclear. One potential issue is whether the *C. difficile* spores we produced do uniformly carry an exosporium and our belief is that using the methods described here the majority of spores probably lack an exosporium. Thus, the exosporium may actually increase the hydrophobicity further. In any event, we can state that CD630 spores are hydrophobic.

The most interesting finding we have made relates to adhesion. CD630 spores were found to bind efficiently to human enteric cells *in vitro* by as much as 100-times greater levels vegetative cells evaluated in parallel. This was unexpected since substantial data now supports the notion that live vegetative cells mediate adhesion

and colonisation to the gut mucosa. While this is true our data suggests that spores may themselves play a hitherto unnoticed role in gut colonisation and it possible that the hydrophoic nature of *C. difficile* spores may play a role in this process. Calabi *et al* (Calabi *et al.*, 2002) have demonstrated strong binding to the surface epithelium of gastrointestinal tissues including the epithelium lining of the digestive cavities and the lamina propria. This has been shown to be mediated by the high MW S-layer proteins (Calabi *et al*., 2002). Using Vero cells three surface proteins of *C. difficile* were found important for host cell attachment with mwts of 70, 50 and 40 kDa (Waligora *et al.*, 1999) one of which was identified as a 66 kDa cell wall protein, Cwp66 (Waligora *et al.*, 2001). Antibodies specific to Cwp66 were able to block adhesion, but only partially and this inhibition only occurred with heat-shocked bacteria. It has been proposed that there is likely to be multiple adhesins on *C. difficile* (Calabi *et al*., 2002).

3.3.2 Spore composition

We have identified five proteins that are exposed on the outermost layer of the spore coat. These have been named these as Cot proteins and their genes, *cot*, since this study clearly shows that they are located in the outermost layers of the spore coat. However, it should be noted that with two distinct structures, coat and exosporium, found on the outermost layers of the spore assigning names should be approached with caution. With the exception of an unidentified 118 kDa species these five proteins represented the major proteins extractable using procedures followed here. We believe however, that the coats of *C. difficile* are far more complex and that these five represent just a fraction of the total protein content of the spore coat. We base

this assumption on existing bioinformatic analysis and the extraction studies performed here. In *B. subtilis* more than 70 proteins are thought to be found in the coat layers (Henriques & Moran, 2007) and 18 orthologues have been identified in *C. difficile* (Henriques & Moran, 2007, Lawley *et al*., 2009b). Using anti-CD630 serum no cross-reaction was found with *B. subtilis* spore coat proteins suggesting that the functional composition and organisation of the coat may be very different in *C. difficile*. Interestingly, the recent spore proteome studies made on CD630 (Lawley *et al*., 2009b) identified five potential spore coat proteins which included CotCB and CotD but not CotA or CotB (n.b., CotE, as a chitinase, was identified but not as a spore coat bifunctional protein). Regarding extraction procedures, our method was based on the use of sodium borate, SDS and DTT and previously used for *Clostridium perfringens* (Tsuzuki & Ando, 1985), and shown here to efficiently extract five proteins. However, the presence of a number of truncated protein species suggests that this method may be overly harsh (it should be noted that inclusion of a proteinase K treatment step might contribute to this observed partial degradation of some proteins). It is also possible that these products arise from cleavage reactions occurring during spore maturation. SDS-PAGE analysis revealed that sonication of spores was able to remove all five Cot proteins recovered by extraction with buffer. Interestingly though, residual spores, which were still viable, released no additional protein when extracted with borate-SDS-DTT buffer. Sonicated spores when examined by TEM showed that the spore coat layers were still essentially intact suggesting that sonication removes one component (possibly one or more layers of coat) of the spore coat and that the remaining underlying coat is impervious to further extraction or sonication. We believe then that deciphering the inner layers of

the spore coat will require the development of new extraction procedures. One additional structure of the spore is the exosporium, although note that this should not be considered part of the spore coat *per se*. We have shown here that *C. difficile* spores do carry an exosporium but at best, this is loosely attached, to the spore, and it is possible that the stability of exosporium is linked to the conditions required to prepare (e.g., solid vs liquid medium) and/or store spores. By comparison the exosporium of *B. anthracis* spores is reasonably well characterised yet there are conflicting reports over how stable this structure is with recent studies suggesting that the exosporium is not easily removed by either sonication or shear stress (Thompson *et al.*, 2011). If the *C. difficile* exosporium is particularly fragile then what, if any, is the biological significance? Since our initial extraction and identification of proteins was made on spores carrying some exosporial material we believe one or more exosporial proteins may have been recovered and perhaps are present in low abundance in the our protein extractions shown in Figure 3.7. One candidate could be the collagen rich glycoprotein BclA1 (CD0332) which has orthologues in a number of other spore formers including *B. anthracis* where it forms filaments that are attached to the exosporium and facilitates interaction with host cells including enhancing spore uptake by macrophages (Bozue *et al*., 2007). Interestingly, BclA is extracted from exosporium-containing *Bacillus* spores as a high mwt. species and its identification using conventional proteomic tools is problematic (Sylvestre *et al*., 2002, Daubenspeck *et al.*, 2004). It will therefore be of interest to determine whether the 118 kDa species is in fact BclA.

One of the most interesting findings from this work are the enzymatic properties of the spores and the identification of three enzymatic coat proteins (CotCB, CotD and CotE) that most probably reside in the exosporium. Although absolute confirmation will require inactivation of the chromosomal genes and preferably, evidence of enzyme activity of the purified proteins this assumption is supported by several lines of evidence. First, vegetative cells were shown to exhibit no enzyme activity so this is unlikely to arise from any contaminating cells. Second, analysis of the spore proteome (Lawley *et al*., 2009b) has revealed no additional genes that could encode these enzyme activities although it must be emphasized that the spore proteome is incomplete.

What then are the functions of these putative spore-associated enzymes? The catalase (CotCB and CotD) and peroxiredoxin (CotE) activities are potential antioxidants and at first glance all three would reduce the cellular toxicity of H_2O_2 by conversion to oxygen and water. In the case of *C. difficile*, which is a strict anaerobe the presence of oxygen would, in turn, be harmful to the cell. Since the cell is irreversibly committed to dormancy it is conceivable that this is not actually harmful and *C. difficile* spores can be maintained in an oxic environment. Presumably though, there is a need to remove H_2O_2? Studies made on *B. subtilis* sporulation show that H_2O_2 may play a key role in spore coat synthesis and could serve as a substrate in the oxidative cross-linking of spore coat monomers (Henriques *et al.*, 1998). Here, the enzyme superoxide dismutase (SodA) is essential to the cross-linking of tyrosine-rich spore coat proteins and in CD630 a manganese-dependant SodA orthologue has been identified in the spore proteome (CD1631). CotE, as a 1-cys-peroxiredoxin would be

expected to have the same enzymatic activity as a peroxidase and could participate in the cross-linking of tyrosine-rich spore coat proteins. None of the other coat proteins identified in this work are tyrosine rich but examination of the *C. difficile* genome has revealed at least one gene (CD0597) that would encode a tyrosine rich protein (10.34% tyrosines). This protein is homologous to CotJB of *B. subtilis* and in *C. difficile* its ORF lies immediately upstream of *cotC* which, in turn, encodes an orthologue of *B. subtilis* CotJC. The ORFs are separated by 61bp and probably lie within the same operon so we propose to name CD0597 as *cotCA* and the downstream cistron as *cotCB* (Figure 3.8).

For chitinase activity the presence of this enzyme in the spore coat is intriguing since it would be expected to be involved in the breakdown of fungi and other biological matter whether in the soil or in the intestine. However, spores are dormant so we speculate that chitinase activity may be released (or activated) during spore germination enabling a potential source of nutrients as the *C. difficile* cell emerges from its coats. We have evidence to support this, firstly, chitinase activity decreased as spores matured but increased during both spore germination and secondly, following sonication; both of these being events that would rupture the spore coat layers. Interestingly, CotE (the putative chitinase) was detectable in the supernatant fraction as either a full-length species (81 kDa) or as a single 40 kDa species following sonication and we wonder whether the smaller species is actually the active chitninase enzyme. Another interesting aspect to CotE, based solely on its sequence prediction, is its bifunctionality and one of a growing number of 'moonlighting proteins' (Jeffery, 1999) which carry multiple functions including a

mammalian protein, 1-cys-peroxiredoxin, that carries peroxidase and phospholipase activities (Chen *et al.*, 2000). There is possibly, a more important consequence of a chitinase and peroxiredoxin displayed on the surface of *C. difficile* spores that should not be overlooked. This relates to the potential link between peroxiredoxins, chitinases and inflammation. Peroxiredoxin 1 (a 2-cys-peroxiredoxin), secreted from tumour cells (Neumann *et al.*, 2003), has been shown to induce proinflammatory cytokines in macrophages via interaction with Toll-like receptor 4 and to promote chronic inflammation which could support tumour growth (Riddell *et al.*, 2010). Regarding chitinases, it is now clear that some inflammatory conditions of the GI-tract (inflammatory bowel disease, IBD and ulcerative colitis, UC) lead to induction of host-cell chitinases by triggering the increased uptake of intracellular bacteria by colonic cells (Kawada *et al.*, 2008, Kawada *et al.*, 2007) and in potentiating the development of epithelial tumorigenesis (Eurich *et al.*, 2009). Considering that some symptoms of CDAD resemble both IBD and UC the *C. difficile* chitinase may play a direct role in infection and not simply in macromolecular degradation.

3.4 Conclusion

This work described the first detailed dissection of *C. difficile* spores. We have found that proteins can be solubilised from the spore coat but in reality better methods for solubilising the coat material will be required to reveal all. Despite this five proteins were resolved three of which had novel enzymatic properties. Two of these enzyme activities are undoubtedly required for maturation of the spore coat while the third, the chitinase is intriguing. It seems unlikely that a spore, which can in principal survive 100's of years, can maintain an enzyme on it spore surface for which it has no use; rather we predict that this enzyme is only activated when the spore germinates providing a useful mechanism to obtain digest macronutrients as the spore germinates. Our interest in this protein though is in its potential role as an agent of inflammation and as a potential vaccine target, i.e., as a antigen. This work has also illuminated that spores are better at adhering to enteric cells than vegetative cells and we wonder whether *in vivo* the is the spores key agent of colonisation.

The work described in this chapter has been published in Journal of Bacteriology (Permpoonpattana *et al.*, 2011a) and a second publication describing the in vitro adhesion of spores is in preparation. The following studies are now in progress or planned.

1. Inactivation of each of the cot genes using the ClosTron gene knockout system followed by characterisation of the *cot* mutants.

2. Inactivation of the *bclA* gene of CD630 using the Clostron system to evaluate the role of BclA in spore function.
3. Antibodies to BclA have been made with the aim to use this as a rapid tool to detect the exosporium.
4. A detailed mass-spectrometry study of spore coat proteins from *C. difficile* using soluble and insoluble fractions has been initiated with Prof. S. Brul and Prof. C. de Koster of the University of Amsterdam, Netherlands. This study will attempt to identify all components of the spore coat.
5. Antibodies to the Cot proteins are being used to examine inhibition of adhesion to enteric cells to determine if any of the spore coat proteins serve as specific ligands.

CHAPTER 4

TEMPORAL EXPRESSION OF SPORE COAT GENES

4.1 Introduction

Relatively few studies have been made on spore formation in *C. difficile*. Spores have been typically produced on solid medium and visualised by electron microscopy. This has revealed the spore structure and the presence of an exosporium. Spores are the primary agents of transmission of infective *C. difficile* and their control is of major importance to the prevention of epidemics especially with the emergence of hypervirulent strains in the last decade since these strains are known to form much higher levels of spore formation than the classical strains (Merrigan *et al.*, 2010, Kuijper *et al.*, 2007, Vohra & Poxton, 2011). Interestingly, the hypervirulent strains not only produce higher levels of spores but also of the toxins suggesting the possibility of co-regulation (Vohra & Poxton, 2011, Merrigan *et al*., 2010). Studies have shown that toxin production occurs at the same time as sporulation and when a sporulation inhibitor (e.g., acridine orange) is used to inhibit spore formation, toxin production is simultaneously reduced (Kamiya *et al.*, 1992), although not abolished. In these studies in liquid culture spores were first detected 12 h post inoculation and maximum levels >48 h. Toxins though were first produced 12 h post inoculation and maximum levels after 24 h. Expression of the *tcdA* and *tcdB* genes has been shown to increase as cells enter stationary phase (Dupuy &

Sonenshein, 1998). More recent studies have shown that in 027 hypervirulent strains maximal levels of toxin A and toxin B are produced after 12h in liquid culture (Vohra & Poxton, 2011). Interestingly, these studies also show a correlation between a developmental gene, *spoOA*, which encodes a regulatory protein that initiates spore formation and toxin production (Vohra & Poxton, 2011). In *C. difficile* strains that lack SpoOA reduced levels of spore formation and toxin production are found (Underwood *et al.*, 2009).

The control of toxin gene expression has recently been shown to be independent from sporulation gene expression (Saujet *et al.*, 2011). By examining gene expression in a *sigH* mutant that encodes sigma H (σ^H) a transcription factor that regulates developmental gene expression, it was found that although spore formation was essentially abolished, and *spoOA* expression blocked, expression of the *tcdA* and *tcdB* genes was upregulated (Saujet *et al*., 2011). This shows that toxin production is not associated with sporulation but it is subject to some level of developmental control mediated by σ^H.

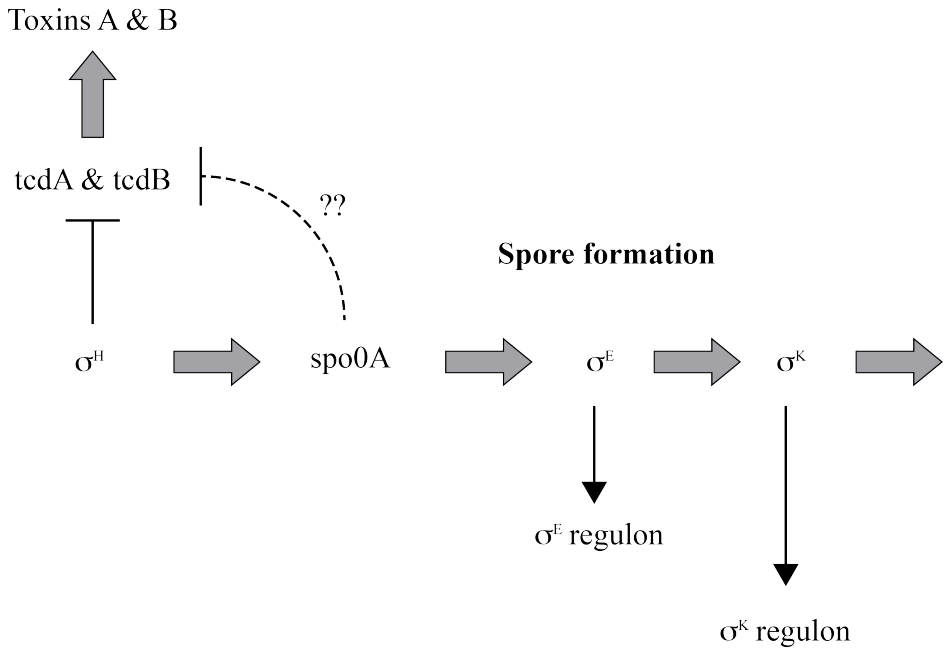


Figure 4.1 Hypothetical events during *C. difficile* spore formation. σ^H is the first developmental-specific sigma factor and controls the 1st phase of temporal gene expression. In a *sigH* mutant that cannot make σ^H, toxin gene expression is upregulated.

σ^H is the first transcription factor to initiate gene expression during spore formation in *C. difficile* and it carries a similar homolog in *B. subtilis*. Indeed, genomic analysis has revealed genes in *C. difficile* that are similar to many of the key sporulation genes and operons in *B. subtilis* that are required for spore formation (Paredes *et al.*, 2005). Although not all genes are currently identified the basic framework of spore formation is similar to that of *B. subtilis* with five principal sigma factors regulating development in an ordered cascade (Paredes *et al*., 2005). The major difference appears to be in the timing of sporulation. Whereas in *B. subtilis* heat resistant endospores are first detectable 5-6 h after the onset of sporulation in *C. difficile*

studies suggest 12 h (Merrigan *et al*., 2010, Kamiya *et al*., 1992, Vohra & Poxton, 2011).

The genetic control of sporulation in *B. subtilis* has been well characterised and serves as a useful model to compare with *C. difficile* (Stragier, 2002). In this work we have been focused on the expression of the spore coat (*cot*) genes identified in the *C. difficile* spore coat. In *B. subtilis* the expression of *cot* genes is controlled by two sigma factors, σ^E and σ^K (Henriques *et al.*, 2004) both of which have homologues in *C. difficile* (Paredes *et al*., 2005, Haraldsen & Sonenshein, 2003). σ^E directs the first phase of spore coat gene expression followed by σ^K acting about 1 - 1.5 h later. Both sigma factors control expression of *cot* genes in the mother cell chamber of the sporulating cells and the action of both of these sigma factors is further modulated by DNA-binding proteins (SpoIIID and GerE). Our aim in this chapter was to examine the profile of expression of the toxin genes, regulatory sigma factor genes and the spore *cot* genes using real time PCR, a technique successfully used in *C. difficile* (Vohra & Poxton, 2011, Saujet *et al*., 2011).

4.2 Results

4.2.1 Establishing synchronous sporulation in liquid medium

Although a number of publications report the production of spores and of sporulation in liquid culture (Merrigan *et al*., 2010, Vohra & Poxton, 2011, Saujet *et al*., 2011, Kamiya *et al*., 1992, Lawley *et al.*, 2009) we are not aware of any reports that have attempted to accurately define the initiation of sporulation. We established a protocol that could produce synchronous sporulation in liquid BHIS medium (Methods). First we evaluated a number of media for preparation of an overnight culture of CD630, specifically TGY (tryptic-glucose-yeast extract medium; (Paredes-Sabja *et al.*, 2008), BHIS and BHISS (BHIS supplemented with sodium taurocholate (0.1% w/v) to ensure spore germination in the starter inoculum (Wilson *et al.*, 1982). After 16 h standing growth at 37^{o}C all cultures were found to contain ethanol-resistant spores; TGY contained 485 spores/ml (total CFU/ml = 2 X 10^{8}); BHIS, 580 spores/ml (total CFU/ml = 3.4 X 10^{7}) and BHISS 386 spores/ml (total CFU/ml = 2.4 X 10^{7}). Since overnight cultures carried spores and therefore, by inference, an even greater number of cells 'committed' to sporulation, that is, cells that were irreversibly bound to form spores (Sterlini & Mandelstam, 1969) we reasoned that inoculation of fresh cultures with these overnight starter cultures would lead to non-synchronous sporulation unless diluted. Using BHIS as the medium of choice, since this has been used most commonly for sporulation in *C. difficile*, we made dilutions (1/10 to 1/10,000) of the overnight culture in fresh BHIS and evaluated growth and sporulation. We found that a 1/10,000 dilution of the overnight BHIS culture produced synchronous sporulation as judged by the production of ethanol resistant spores beginning at about hour 8-9 (Figure 4.2).

Next, using a 1/10,000 dilution we evaluated growth and sporulation of *C. difficile* strains, 630 (*tcdA*$^+$ *tcdB*$^+$) and the 027 hypervirulent strain CD196 (*tcdA*$^+$ *tcdB*$^+$) in finer detail

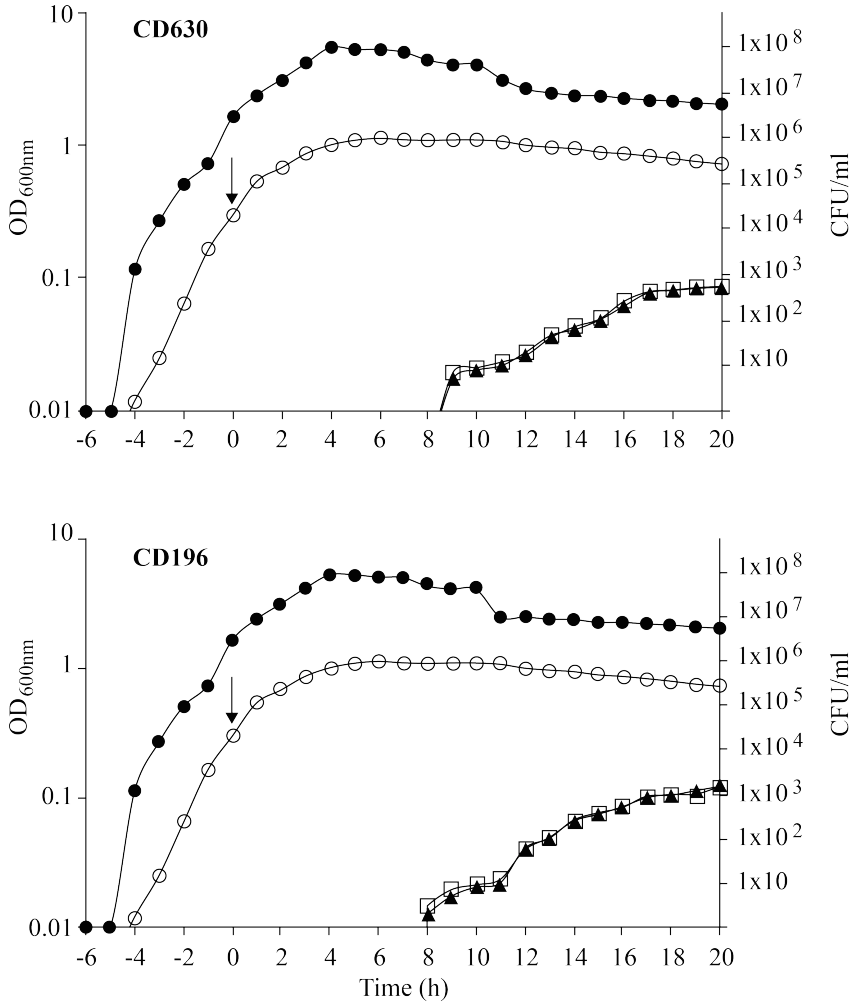


Figure 4.2 Growth and sporulation of *C. difficile* CD630 and CD196. *C. difficile* strains CD630 and CD196 were grown in BHIS medium at 37^{o}C. Growth was measured by OD_{600nm} (○) and determination of CFU/ml (●) over 20 h. Sporulation was assessed by the examination of spore formation by phase contrast microscopy and quantified by the determination of heat (▲) and ethanol (□) resistant spores at each time point. This experiment was repeated in its entirety three times with similar results. The arrows indicate the initiation of sporulation (T_0) determined as the point at which cells left the exponential phase of growth.

measuring optical density, viable counts (CFU/ml) and spores resistant to heat and to ethanol treatment (Figure 4.2). Cells reproducibly reached stationary phase of growth after approximately 6-8h with both strains containing equivalent numbers of cells. Growth characteristics and optical density readings were in good agreement with other liquid culture studies albeit it using different media (Vohra & Poxton, 2011, Merrigan *et al*., 2010, Kamiya *et al*., 1992). Based on the principles of defining the onset of sporulation by exhaustion in *B. subtilis* (Schaeffer *et al.*, 1965) we extrapolated the point at which cell growth was no longer logarithmic as the start point of sporulation; this point we refer to as T_0. The precise point at which cell growth left logarithmic phase was difficult to define and in Figure 4.2 T_0 could be later, at most by 1h. For simplicity we will use this point as a reference for all experiments discussed hereafter.

Based on our trial experiments the first spores resistant to either heat or ethanol (no differences between these treatments were observed) were detectable at about eight hours (T_8) after the onset of sporulation (note, as mentioned above that if T_0 is in fact later than out prediction then the earliest appearance of heat resistant spores would then be after seven hours). No substantial differences could be detected between CD630 and CD196 although with the latter resistant spores were detectable approximately 30 minutes earlier in CD196 and at slightly higher amounts. The levels of resistant spores steadily increased and after 20h post initiation of sporulation had reached maximum levels of ~10^3/ml.

4.2.2 Toxin production

Considerable evidence has shown that production of toxins A and B occurs in late stationary phase but also during sporulation *per se* (Kamiya *et al*., 1992, Merrigan *et al*., 2010, Vohra & Poxton, 2011). Moreover, expression of the genes encoding toxin A (*tcdA*) and toxin B (*tcdB*) are subject to regulation by both SpoOA and σ^H, both key regulators of the early stages of spore formation in *C. difficile* (Underwood *et al*., 2009, Saujet *et al*., 2011). We examined production of toxins A and toxins B during sporulation in BHIS media (Figure 4.3). Toxin A was expressed at higher levels than toxin B in both CD630 and CD196. This is the first report showing the levels of both toxin A and toxin B, as opposed to other studies which have measured toxins A + B together (Merrigan *et al*., 2010) or have used a modified cytotoxicity assay (Vohra & Poxton, 2011) and demonstrates that, at least in these strains, toxin A levels are at least five-times than that of toxin B. Toxin A was produced at much higher levels (five-times) in strain CD196 and is consistent with reports showing that toxin production is higher in hypervirulent strains of *C. difficile* (Merrigan *et al*., 2010, Vohra & Poxton, 2011). As a measure of functional toxin production a cell culture based cytotoxicity assay was used (Figure 4.3) demonstrating that peak cytotoxicity correlated with maximum levels of toxin accumulation.

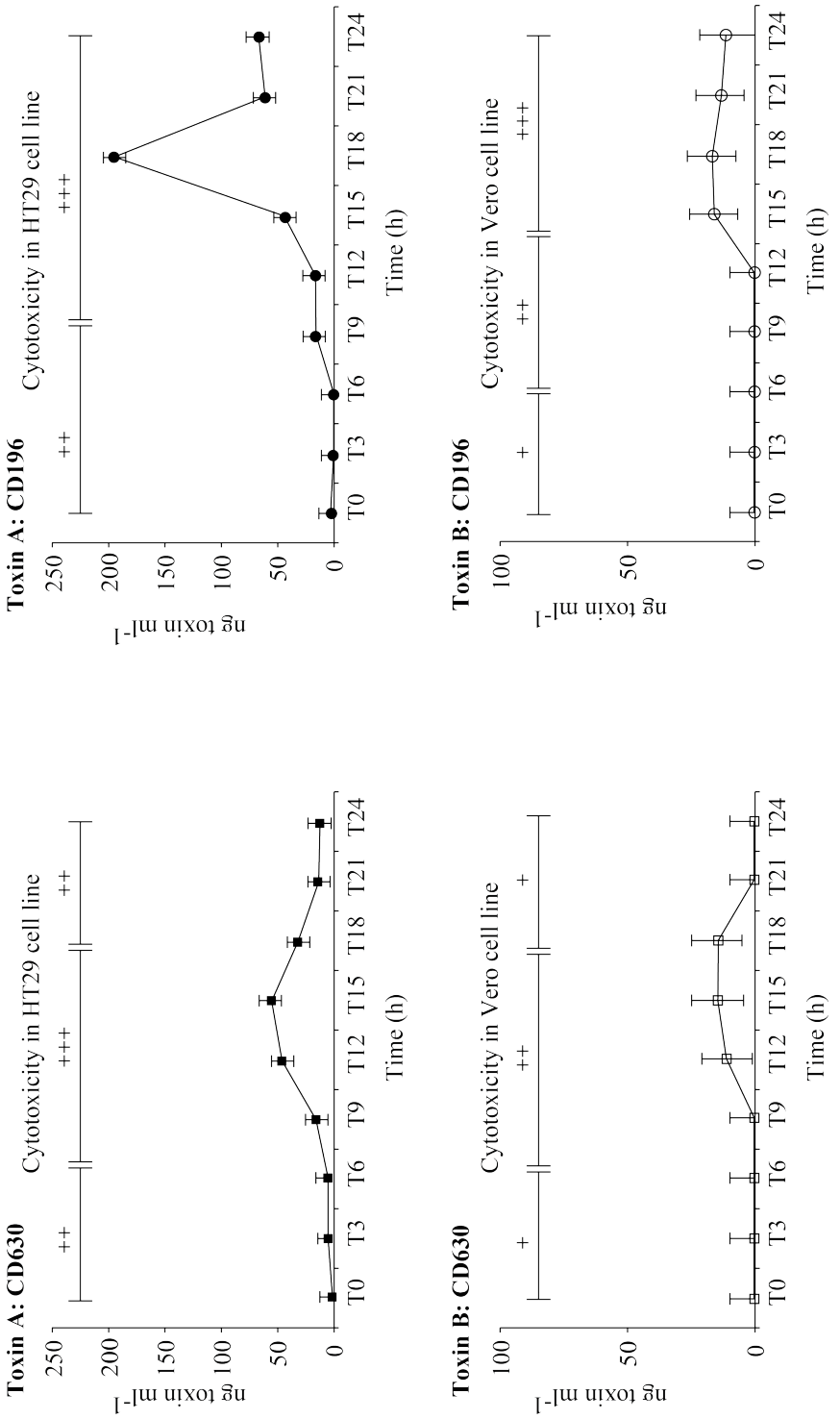


Figure 4.3 Toxin production (see next page for legend).

Figure 4.3 Toxin production (see previous page). Production of toxins A and toxins B in cell supernatants were measured from the growth and sporulation experiments shown in Figure 4.2 using ELISA-based methods described in Methods. Duplicate samples were examined and SD error bars shown. The sensitivity of the ELISA was 5 ng/ml for toxin A and 5 ng/ml for toxin B. Cytotoxicity assays were made using HT29 or Vero cells where HT29 cells show greatest sensitivity to toxin A and Vero cells to toxin B (Lyras *et al.*, 2009, Torres *et al.*, 1992).

4.2.3 Expression of the toxin genes, *tcdA* and *tcdB*

To evaluate expression of the *tcdA* and *tcdB* genes that encode toxin A and toxin B respectively, a quantitative RT-PCR assay was used (Figure 4.4). Our findings show some interesting differences between strains. CD630 showed that *tcdA* expression initiated in early stationary phase approximately 3 h before the onset of sporulation (T_0). By contrast *tcdB* expression initiated 3 h later corresponding to the commencement of sporulation. In CD196 *tcdB* expression commenced 6h prior to the start of spore formation and, comparing to the growth curve of Figure 4.2, this would equate to constitutive expression. On the other hand *tcdA* expression was 3h later. Comparison of both strains then, shows that *tcdA* expression initiates at the same time (3 h prior to T_0) while the timing of *tcdB* expression is delayed significantly in CD630.

4.2.4 Expression of the developmental transcription factors

Based on what is known from the regulation of spore formation in *B. subtilis* (Errington, 1993) six regulatory factors encoded by the *spoOA, sigH, sigF, sigE, sigG* and *sigK* are present in the *C. difficile* genome and, based on comparative

genomics most probably control sporulation-specific gene expression (Paredes *et al*., 2005). To date some studies have examined expression of two regulatory genes, *spoOA* and *sigH* (Underwood *et al*., 2009, Saujet *et al*., 2011, Vohra & Poxton, 2011) but expression of the other genes has not been examined. *spoOA* and *sigH* are present in all *Clostridia* and, based on their functions in *B. subtilis*, are essential to initiating the sporulation cascade. SpoOA when phosphorylated leads to expression of *sigH* which encodes the first sporulation-specific sigma (σ) factor, σ^H, in turn, can then transcribe both the *sigF* and *sigE* genes encoding the sigma factors, σ^F and σ^E. After these proteins are activated they are then able to transcribe the *sigG* (σ^F) and *sigK* (σ^E) genes leading to production of σ^G and σ^K. Using quantitative RT-PCR we examined expression of each of these six regulatory genes in sporulating cultures of CD630 and CD196 (Figure 4.5).

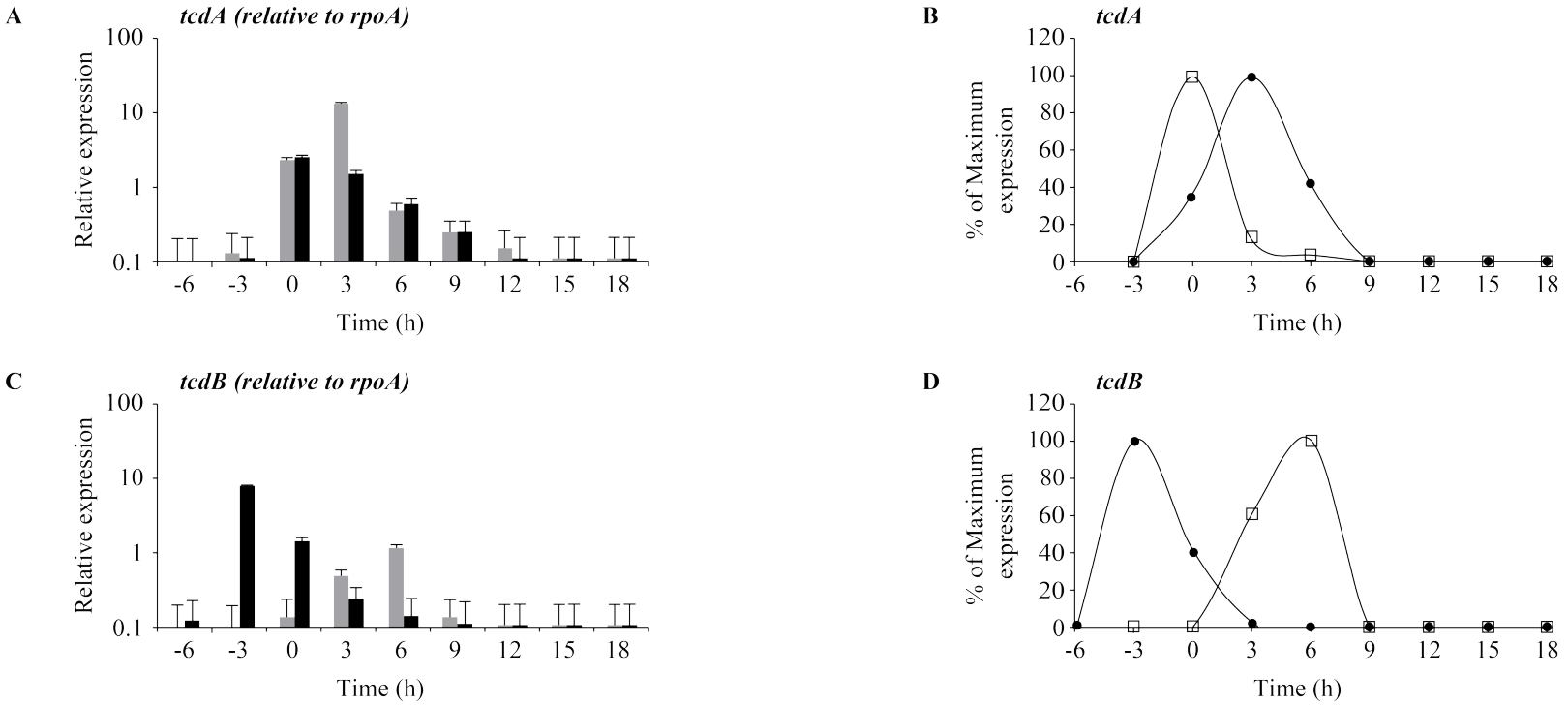


Figure 4.4 Expression of *tcdA* and *tcdB*. Transcription of *tcdA* and *tcdB* during growth and sporulation of CD630 and CD196 (as shown in Figure 2) was determined using quantitative RT-PCR. **Panel A** shows expression of *tcdA* relative to the housekeeping gene *rpoA*. **Panel B** shows the temporal profile *tcdA* expression in both strains (□, CD630; ●, CD196) as a percentage of maximum. **Panel C** shows expression of *tcdB* relative to *rpoA* and **panel D** the temporal expression of *tcdB* in these strains (□, CD630; ●, CD196) as a percentage of maximum. Means and standards errors of three replicates are shown in **panels A** and **B**. The experiment has been repeated two-times in its entirety with similar results.

Expression of *spoOA* and *sigH* initiated first with expression commencing at about T_0 and maximum levels reached at T_9 for SpoOA and T_6 for *sigH*. The timing of expression was essentially identical in both strains although more rapid in CD196. For *sigE, sigF* and *sigG* expression was noticeably different in CD630 and CD196. In CD196 expression of *sigF, sigE* and *sigG* initiated at about T_3 and reached maximal levels at T_9. In CD630 however, expression commenced between T_5 and T_6, at least 2h later than in CD630. Maximum levels were reached at the same time in both strains however. Finally, *sigK* expression commenced at T_6 in both strains and with similar kinetics with maximum levels being reached at T_{12}. In sum, expression of the key regulatory genes in *C. difficile* could be broken down into three phases. An early phase consisting of transcription of *spoOA* and *sigH*, a second, middle, phase consisting of *sigF*, *sigE* and *sigG*, and a final late phase consisting of *sigK*. For the early and middle phases, expression was more rapid in the hypervirulent strain CD196.

4.2.5 Expression of the spore coat genes

In a recent seminal study of the spore coats of *C. difficile* CD630 five spore coat proteins, CotA, CotB, CotCB, CotD and CotE have been identified of which three are putative enzymes, CotCB and CotD being manganese catalases and CotE a novel bifunctional protein carrying a periredoxin and a chitinase domain (Permpoonpattana *et al.*, 2011a). Each of these proteins is exposed on the surface of the spore coats and, based, on what is known of the spore coat.

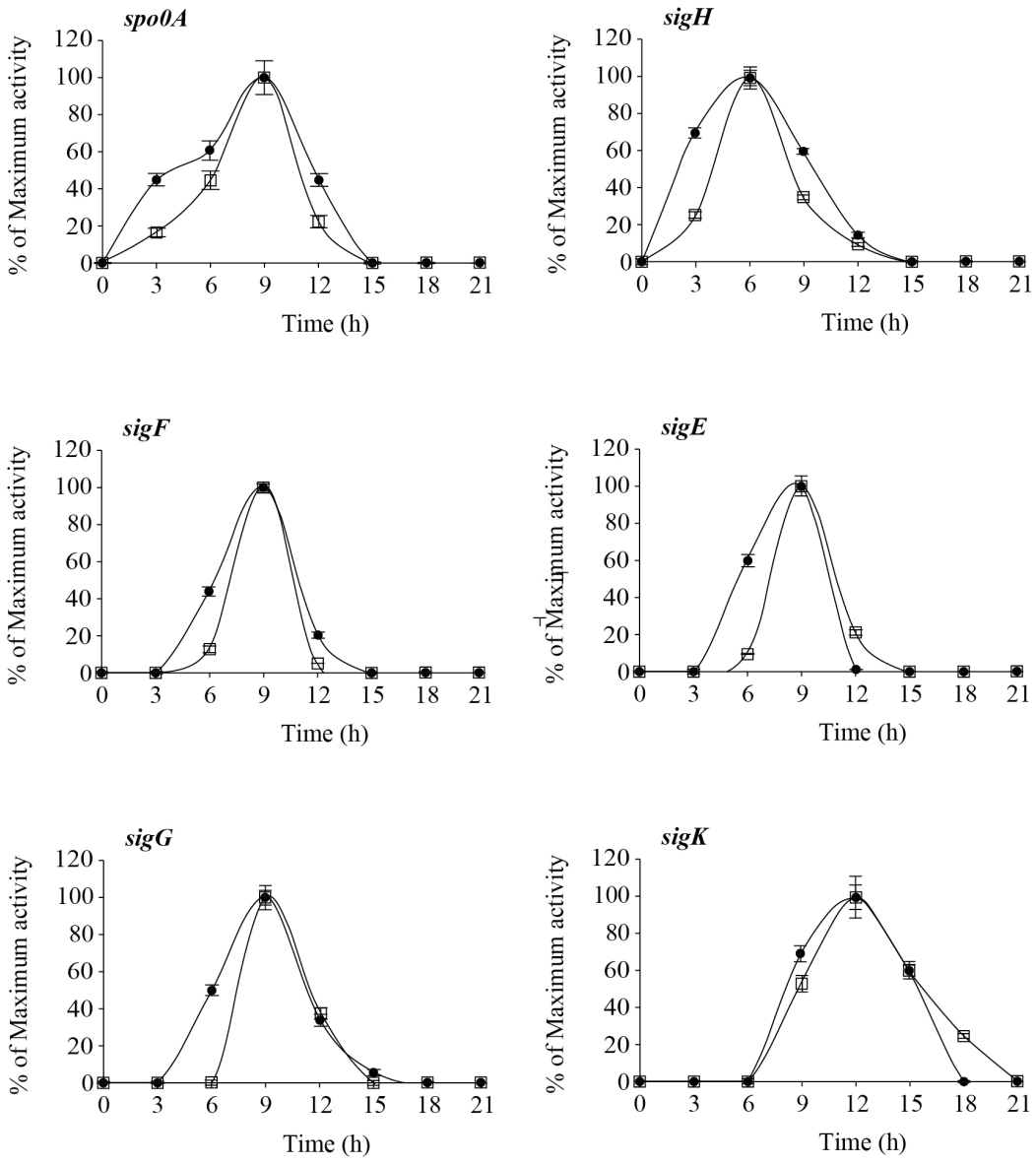


Figure 4.5 Temporal expression of regulatory genes. Transcription of *spoOA*, *sigH*, *sigF*, *sigE*, *sigG* and *sigK* in CD630 (□) and CD196 (●) sporulating cultures was determined using quantitative RT-PCR. The times indicate hours after the initiation of sporulation T_0. Expression is shown as percentage of maximum. The experiment has been repeated two-times in its entirety with similar results.

In *B. subtilis* spore coat biosynthesis the corresponding structural gene is likely to be transcribed in the mother cell compartment of the sporulating cell (Henriques & Moran, 2007). Using quantitative RT-PCR we examined expression of the *cotA, cotB, cotCB, cotD* and *cotE* genes in sporulating cultures (Figure 4.6). Based on this analysis for four genes, *cotA, cotB, cotCB* and *cotD*, we could identify two basic phases of temporal expression. Early expression beginning at T_6 (*cotA*, *cotB* and *cotD*) and late expression beginning at T_6 (*cotC*). Two genes, *cotA* and *cotB* showed distinctive biphasic patterns of gene expression with expression starting at T_3, dipping at T_{12} and then increasing again. This biphasic gene expression is seen in some genes of *B. subtilis* that are transcribed by two sigma factors, for example, *bofC*, a regulatory protein that is transcribed by σ^F followed by σ^G (Wakeley *et al.*, 2000). It is likely then that both *cotA* and *cotB* are subject to dual regulation in the mother cell chamber. Finally, for *cotE* in CD630 cells expression initiated at T_6 but in CD196 cells expression was 3h earlier beginning at T_3.

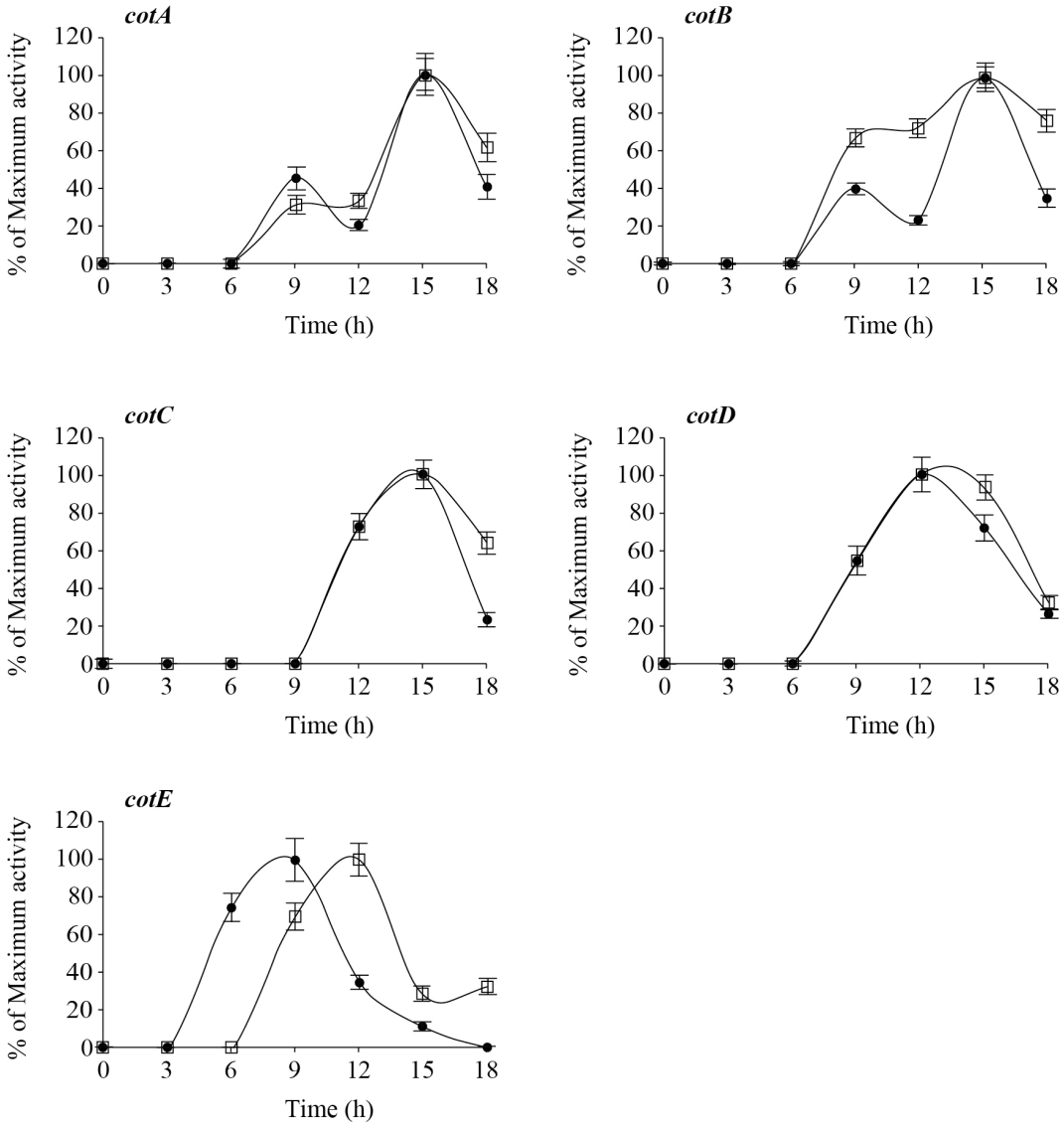


Figure 4.6 Expression of spore *cot* genes. Transcription of *cotA, cotB, cotCB, cotD* and *cotE* in CD630 (□) and CD196 (●) sporulating cultures was determined using quantitative RT-PCR. The times indicate hours after the initiation of sporulation T_0. Expression is shown as percentage of maximum. The experiment has been repeated two-times in its entirety with similar results.

4.3 Discussion

We have been able to demonstrate synchronous sporulation of *C. difficile* in liquid medium. It is not clear why this has not been approached previously but in part this may be due to the low levels of spore formation that occur in this organism. Our method has been synthesised from previous studies and the key attribute for achieving synchronous sporulation is generating a starting culture that contains cells in the vegetative phase of growth and devoid of cells committed to spore formation. Under these circumstances spore formation initiates at the end of the logarithmic phase of cell growth enabling the start point of sporulation, T_0, to be defined. Having defined T_0 we have shown that resistant spores are first detectable 7-8 hours post initiation. This is significantly faster than that reported for the solventogenic *Clostridia* (Paredes *et al*., 2005, Santangelo *et al.*, 1998, Jones *et al.*, 1982). The time required to form resistant spores in *C. difficile* is only 1-2h more than that required in *B. subtilis* (Schaeffer *et al*., 1965) and demonstrates that the basic program of sporulation is likely to be similar. In our studies we observed somewhat higher levels of sporulation in the hypervirulent CD196 strain but in all other respects spore formation as observed microscopically and at a physiological level that was no different between CD196 and CD630.

The number of cells that entered sporulation, while measurable, was very low (<0.1%). In other studies CD630 has been reported to produce much higher yields of spores, for example, 9.3% after 72 h growth in BHIS medium (Saujet *et al*., 2011) and 14% after 72 h growth in BHI medium (Underwood *et al*., 2009). In both of these studies incubation was considerably longer (2 days) and it is possible that in

our experiments counts of spores would have gradually risen. Indeed using BHI medium after 48 h spore yields of ~0.66% were found for CD630 (Merrigan *et al*., 2010) while using AIM (anaerobic incubation medium) ~2 X 10^2 spores/ml were found after 24 h (Vohra & Poxton, 2011) which more closely resembled the yields we achieved here. We have shown here that if the objective of the study is to produce synchronous sporulation then the quality of the overnight inoculum is critical and must be devoid of cells committed to sporulation. If the overnight inoculum carries spores or sporulating cells it is not possible to obtain synchronous sporulation.

Production of toxins A and B correlates with stationary phase and spore formation (Kamiya *et al*., 1992, Hundsberger *et al.*, 1997). Expression of the *tcdA* and *tcdB* genes is controlled negatively by TcdC and positively by the TcdR sigma factor both of which are encoded from the pathogenicity locus, PaLoc (Mani & Dupuy, 2001). Additional modulation of toxin production comes from CodY (Dineen *et al* 2007) and two regulatory proteins that also participate in the control of development, SpoOA and σ^H (Underwood *et al*., 2009, Saujet *et al*., 2011). We saw a marked contrast in transcriptional profiles of *tcdA* and *tcdB* expression in the CD630 and CD196 strains. In CD630 *tcdA* was transcribed during log phase while *tcdB* was expressed at late stationary phase and at the onset of sporulation. By contrast, in CD196 *tcdB* expression was constitutive and *tcdA* expression was expressed during late stationary phase/early sporulation. Analysis of toxins in cell free supernatants showed maximal levels of toxin A and toxin B at hours 15-18 after the onset of sporulation in agreement with other studies (Kamiya *et al*., 1992). Since toxins were being measured in supernatants the maximum levels would also correspond to the

presumed time at which the mother cell lyses releasing the mature endospore. Our results do show that toxin A levels were five-times higher in the hypervirulent strain CD196 than those found in CD630 which supports other studies suggesting that toxin A levels are higher in hypervirulent strains (Merrigan *et al*., 2010, Vohra & Poxton, 2011). Interestingly though, we did not see any difference in toxin B levels and we wonder whether toxin A, both its amounts and temporal appearance, plays the key role in defining the hypervirulent phenotype. Studies have shown that toxin A and B act cooperatively and, in the absence of toxin A, toxin B is unable to induce cell damage in the host. What is clear from our analysis of toxin gene expression is that the timing and order of toxin gene expression differs markedly between the two strains examined here. In CD630 *tcdA* expression is produced first while in the hypervirulent strain this is reversed with a basal level of *tcdB* expression being followed by a burst of *tcdA* expression. This temporal difference in toxin gene expression may play a defining role in the virulence of these strains possibly enabling the hypervirulent strain to release active toxins prematurely. In any event, two important steps now need to be addressed, first, to understand the physiological and/or genetic signals that modulate the temporal profiles of gene expression and second, to correlate this with toxin production in GI-tract. Other studies have shown considerable variation in the levels of toxin production in *C. difficile* strains although as mentioned above it is not possible to relate this to the present study since sporulation would not have been synchronous (Merrigan *et al*., 2010, Vohra & Poxton, 2011). Moreover, we believe it prudent not to pursue a direct link between toxin production '*in vitro'* to what occurs in the animal host. Studies in gnotobiotic mice have shown that while high levels of toxin A can be produced *in vitro* this is not

the case in the animal host ('*in vivo*') where levels of toxin A diminish substantially and in some *C. difficile* strains are no longer detectable (Vernet *et al.*, 1989). A similar lack of correlation between toxin production and virulence has been observed in hamsters (Borriello *et al.*, 1987).

Regarding sporulation-specific gene expression, we have shown that expression of the six key transcriptional regulators falls into three temporal phases. SpoOA and *sigH* are expressed at the onset of spore formation. This contrasts to *B. subtilis* where low levels of basal expression occur (Errington, 1993) although we recognise that our definition of T_0 could be ±1h so stationary phase expression cannot be wholly excluded. Our analysis of *sigF*, *sigE* and *sigG* show that all three genes appear to be expressed at the same time and about 3h after the onset of sporulation. Bioinformatic analysis of the *C. difficile* genome has shown that most of the genes required for activation of σ^F, σ^E and σ^G are present so it is likely that, as with *B. subtilis*, σ^F, σ^E and σ^G are activated post-translationally by intercompartmental signals or checkpoints (Losick & Stragier, 1992). Based on this we would expect expression of *sigG* to be later than that of *sigF* and *sigE* since both σ^F and σ^E are required for expression and activation of σ^G in the forespore. In *B. subtilis sigG* is transcribed approximately 1h after *sigF* and *sigE* (Errington 1993) and a more detailed dissection of *sigG* gene expression may reveal such a delay. *sigK* transcription was shown to differ in the two *C. difficile* strains. In CD196 expression was 3h after expression of *sigF-sigE-sigG* while for CD630 expression was concurrent. *sigK* in *C. difficile* carries a *sigK* gene that is interrupted with a 14.6 kb prophage-like insertion, known as $skin^{Cd}$ (Haraldsen & Sonenshein, 2003). *B. subtilis* also carries a *skin* element and

of all spore formers evaluated to date only *B. subtilis* and *C. difficile* carry this insertion (Haraldsen & Sonenshein, 2003). Excision of $skin^{Cd}$ occurs following expression of a σ^E-transcribed site-specific recombinase so *sigK* should be expressed soon after post-translational activation of σ^E which would account for the delay seen in CD196. In CD630 no delay was observed suggesting some other mode of regulation must account for this premature expression.

4.4 Conclusion

This work firstly demonstrated the establishment of synchronous sporulation in *C.difficile*. We have found that using overnight starter cultures could lead to the non-synchronous sporulation unless diluted. A 1/10,000 dilution of overnight BHIS culture was used to generate the synchronous sporulation as determined by the production of ethanol resistant spores at approximately hour 8-9 which differs from that of *Bacillus subtilis* at about hour 6. In addition to synchronous sporulation, we have also found that the expressional level of toxin A was higher than that of toxin B in both CD630 and CD196 in which toxin A was expressed five times higher than toxin B and the cytotoxicity was shown to correlate with the maximum levels of toxin accumulation. Moreover, the results showed that *tcdA* expression initiates three hours prior to the commencement of sporulation, at the same time in both strains while *tcdB* expression was significantly delayed in CD630. By examining the developmental transcription factors, our findings showed the initiation of *spoOA* and *sigH* expressions at T_0 reaching the plateau at T_9 and T_6, respectively with a more rapid expression in CD196 compared to CD630. Even though *sigF*, *sigE* and *sigG* expressions were different between both strains, *sigK* expression reached the maximum at the same time with similar kinetics. Finally, the expressions of five spore coat genes were analysed. *cotA*, *cotB* and *cotD* in both strains expressed at the same time, T_6 with the biphalic phase of *cotA* and *cotB* whereas *cotCB* expression initiates at T_9. Interestingly, *cotE* expression in CD196 commences three hours earlier than that of CD630.

The following study is now in progress or planned Inactivation of each developmental transcription factor (*sigH*, *sigE* and *sigK*) using ClosTron mutagenesis followed by the study of the developmental transcription factor dependency on spore coat genes

CHAPTER 5

MUCOSAL VACCINATION AGAINST CDI

5.1 Introduction

5.1.1 Vaccine strategies

Current strategies to vaccinate CDI are focused on generating humoral immunity, specifically anti-toxin A and anti-toxin B IgG responses. Although $toxA^-$ $toxB^+$ strains of *C. difficile* can produce disease most researchers are focused on both toxins A and B (Lyerly *et al.*, 1988). An effective vaccine to CDAD should target both toxin A and B since protection has been shown to correlate with high anti-toxin A and anti-toxin B antibody titres (Aboudola *et al.*, 2003, Kink & Williams, 1998, Leav *et al.*, 2010). Human MAbs directed against toxins A and B prevent *C. difficile*-induced mortality in hamsters (Babcock *et al.*, 2006) and reduced recurrence in humans (Lowy *et al.*, 2010). Although most studies to date have focused on parenteral delivery of the toxoids there is mounting evidence that mucosal immunity is the key determinant of protection and specifically secretory or polymeric IgA (Johnson *et al.*, 1992, Johnson *et al.*, 1995, Stubbe *et al.*, 2000, Kelly *et al.*, 1992, Johnson, 1997). It cannot be excluded though that mucosal IgG could also play a role in protection.

Both the *tcdA* and *tcdB* genes carry at their carboxy termini an elaborate array of repeated domains (Figure 5.1) (von Eichel-Streiber *et al.*, 1992a, von Eichel-Streiber *et al.*, 1992b). The carboxy-terminus of TcdA has been shown to be involved in host cell recognition and binding prior to insertion of the toxin in the endosomal membrane (Jank *et al.*, 2007). Substantial evidence suggests that this carboxy-terminal domain may be suitable as an antigen target for vaccination against CDAD. For example, a MAb directed against this domain prevents cytoxicity (Sauerborn *et al.*, 1997). A defined segment of the carboxy-terminal domain of TcdA known as 14CDTA expressed in a *Salmonella* vaccine elicited local (mucosal) and systemic immunity as well as toxin A neutralising activity when administered orally (Ward *et al.*, 1999a, Ward *et al.*, 1999b). The carboxy terminal cell-binding domain of TcdA has also been used in a DNA vaccine which when administered by injection conferred protection against challenge with a parenteral dose of toxin (Gardiner *et al.*, 2009).

Figure 5.1 The Carboxy-cell binding domains of TcdA and TcdB. Shown are the catalytic, glycosyltransferase domain (black), the cysteine protease domain (grey), the translocation domain (white) and the repetitive sequences involved in cell binding (stripes). The A26-39 and B15-24 segments expressed in *Bacillus* vaccine strains are labelled. A26-39 was formerly known as 14CDTA.

5.1.2 Animals models of CDI

Currently there exist a number of models with which to experimentally study CDI. Although a piglet model has been described recently (Steele *et al.*, 2010) only the following three, which are applicable to normal laboratory conditions will be described.

The hamster model of infection

This model uses Golden Syrian hamsters and is considered the gold-standard for assessing protective immunity (Goulding *et al.*, 2009, Sambol *et al.*, 2001, Sambol *et al.*, 2002). Hamsters are immunised and then, approximately 14 days after the last dose, given a single dose of clindamycin (30mg/kg body weight). Twelve hours later animals are given a dose of 100 spores of a pathogenic strain of *C. difficile*. Animals are monitored hourly for symptoms (diarrhoea) and unprotected animals will normally die after 42-45h. The challenge dose is either spores (100 CFU) or vegetative cells (10^5-10^6 CFU) of *C. difficile*. It is now considered more appropriate to use spores as the challenge dose since using live vegetative cells of *C. difficile* in an aerobic environment is technically impossible resulting in a reduction in the administered/challenge dose (Goulding *et al*., 2009). One of the problems with this model though is that the end point is death and it has proven difficult to examine the colonisation of animals during infection. In addition, obtaining bleeds from hamsters is technically demanding requiring withdrawal of blood either from the retro-orbital or saphenous veins. It has also been questioned as to whether an infection that kills animals in 2-3 days truly resembles the human disease.

The mouse models

In this model mice (C57 BL/6) are exposed to a mixture of antibiotics (kanamycin, gentamycin, colistin, metronidazole and vancomycin) for three days (Chen *et al.*, 2008). After two days they are injected with clindamycin and challenged 1 day later with *C. difficile* live cells. Disease manifests itself in mice varying from mild to fulminant depending on the challenge dose. Symptoms measured include colonisation, weight loss and histology and mice that survive CDI were protected against relapse.

A further model using mice has been reported recently (Sun *et al.*, 2011). Animals (C57 BL/6 mice) are treated for 3 days with the antibiotic cocktail described followed by 2 days of water and then clindamycin. Animals are then challenged with *C. difficile* (10^6 CFU of live cells). Surviving animals are then divided into two groups, one receives vancomycin only while the second group receives vancomycin + *C. difficile* to determine whether surviving animals suffer relapse. Colonisation, diarrhoea, humoral and mucosal antibodies and histology are all determinants of protection in this model.

5.1.3 Bacterial spores as a vaccine delivery system

The potential of bacterial spores as vaccines is based on two important attributes: first, their heat-stability and second, their use worldwide as dietary supplements (probiotics), in both humans and animals (Hong *et al.*, 2005). It is now also believed that bacterial spores possess immunomodulatory properties that can be utilised for adjuvant development. Using *B. subtilis,* the Cutting group at RHUL have

engineered a number of antigens for display on the surface of spores. Oral (or intra-nasal) dosing of these recombinant spores into mice has provided protection in the case of tetanus (Duc *et al.*, 2003), *Clostridium perfringens* (using alpha toxin; (Hoang *et al.*, 2008)) and *Bacillus anthracis* (using a parenteral approach; (Duc le *et al.*, 2007). More recently, a non-GM approach to using spores has been developed making use of a novel attribute of the spore surface, which is the capacity to adsorb heterologous antigens (Huang *et al.*, 2010). Remarkably, heat-killed spores are as efficient as live spores as an antigen carrier using adsorption. Studies on the nature of humoral and cell-mediated immune responses in mice have shown that spores co-administered with protein antigens augment antigen-specific sIgA (secretory IgA) and induce a balanced T_h1/T_h2 response (Barnes *et al.*, 2007, Huang *et al*., 2010).

5.1.4 A spore based vaccine to CDAD

The approach taken here is to focus on the carboxy-terminal cell-binding domains of toxin A and toxin B and to clone and express these antigens on the surface of *B. subtilis* spores. Next, to evaluate these spores in mice to evaluate immune responses and then to evaluate protection in a hamster model of infection.

5.2 Results

5.2.1 Expression of the C-terminal domains of *C. difficile* toxin A and toxin B on *B. subtilis* spores

B. subtilis was engineered to express the A26-39 domain of toxin A (TcdA) and the B15-24 domain of toxin B (TcdB) on the outermost layer of the spore coat. Both A26-39 and B15-24 lie within the C-terminal repeat domains of each toxin and carry repetitive sequences (von Eichel-Streiber *et al*., 1992a) (Figure 5.1). Expression was achieved by fusing A26-39 and B15-24 to the C-termini of the outer spore coat proteins CotB (43 kDa) and CotC (12 kDa) both of which have successfully been used for surface display and mucosal delivery of heterologous antigens (Isticato *et al.*, 2001, Mauriello *et al.*, 2004) (in the case of CotB, fusions were made to a C-terminally truncated, version of CotB). Two different spore constructions were made; PP108 (*cotB-A26-39 cotC-A26-39*) that expressed A26-39 attached to both CotB and CotC, and PP142 (*cotB-B15-24 cotC-A26-39*) that co-expressed two chimeras, CotB-B_{15-24} and CotC-A_{26-39}. Surface expression of A26-39 and B15-24 on PP142 spores and A26-39 on PP108 spores was confirmed by confocal imaging of spores (Figure 5.2A-C).

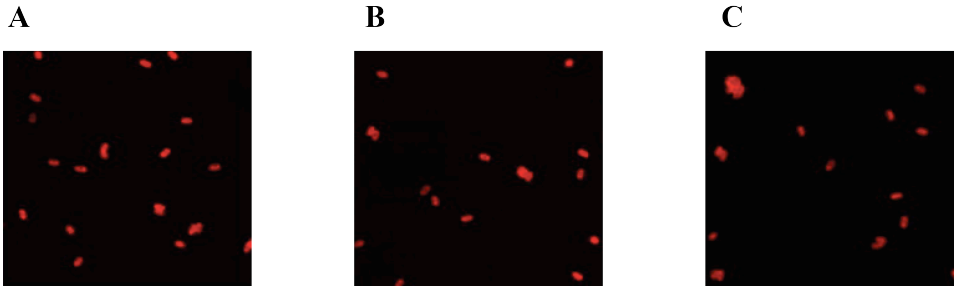


Figure 5.2 (A to C) Immonofluorescence microscopic images. Surface display of A26-29 and B15-24, confirmed using confocal imaging of samples labelled with mouse anti-A26-39 or B15-24 serum followed by an anti-mouse IgG-tetramethyl rhodamine iso-thiocyanate (TRITC) conjugate. Spores of PP108 were labeled with anti-A26-39 (A), and spores of PP142 were labelled with either anti-A26-39 (B) or anti-B15-24 (C).

5.2.2 Antibodies to the C-terminal domain of toxin A cross react with the corresponding domain of toxin B

The coat proteins of PP108 and PP142 were extracted and probed with antibodies to confirm expression of the chimeric CotB and CotC proteins (Figure 5.2D-E). PP108 spore coat protein extracts when probed with anti-A26-39 antibodies revealed bands of 69 kDa (CotB-A_{26-39}) and 49 kDa (CotC-A_{26-39}). Their molecular weights (mwt) were in agreement with the predicted size of the fusion proteins (Figure 5.2D) and corresponded in size to proteins in extracts taken from spores carrying each chimera alone. Quantification revealed that PP108 spores carried 2.2 X 10^{-4} pg/spore of A26-39. When PP142 extracts were probed with anti-B15-24, one principal band of 60 kDa was detectable (lane 10, Figure 5.2E) corresponding in size to CotB-B_{15-24}. Using anti-A26-39, CotC-A_{26-39} was observed as a 49 kDa species but also a second

band of 60 kDa (lane 5, Figure 5.2E). We predict that this higher mwt species is CotB-B_{15-24} and could be explained by the fact that the C-terminal cell-binding domains of TcdA and TcdB share some sequence identity (30% identical residues; Figure 5.3) and may, as shown elsewhere, carry related epitopes (von Eichel-Streiber *et al*., 1992a). In support of this we demonstrated that anti-A26-39 antibodies could cross-react with both purified rA26-39 (36.6 kDa) and rB15-24 (29.4 kDa) polypeptides yet anti-B15-24 antibodies reacted only with rB15-24 (Figure 5.2F). Protein expression was quantified and each spore of PP142 was shown to contain 2.45×10^{-5} pg of B15-24 and 1.9×10^{-4} pg of A26-39. In additional work we verified using anti-CotB and anti-CotC polyclonal antibodies that the expected change in the molecular masses of the chimeric CotB and CotC spore coat proteins in PP108 and PP142 spores (Figure 5.4).

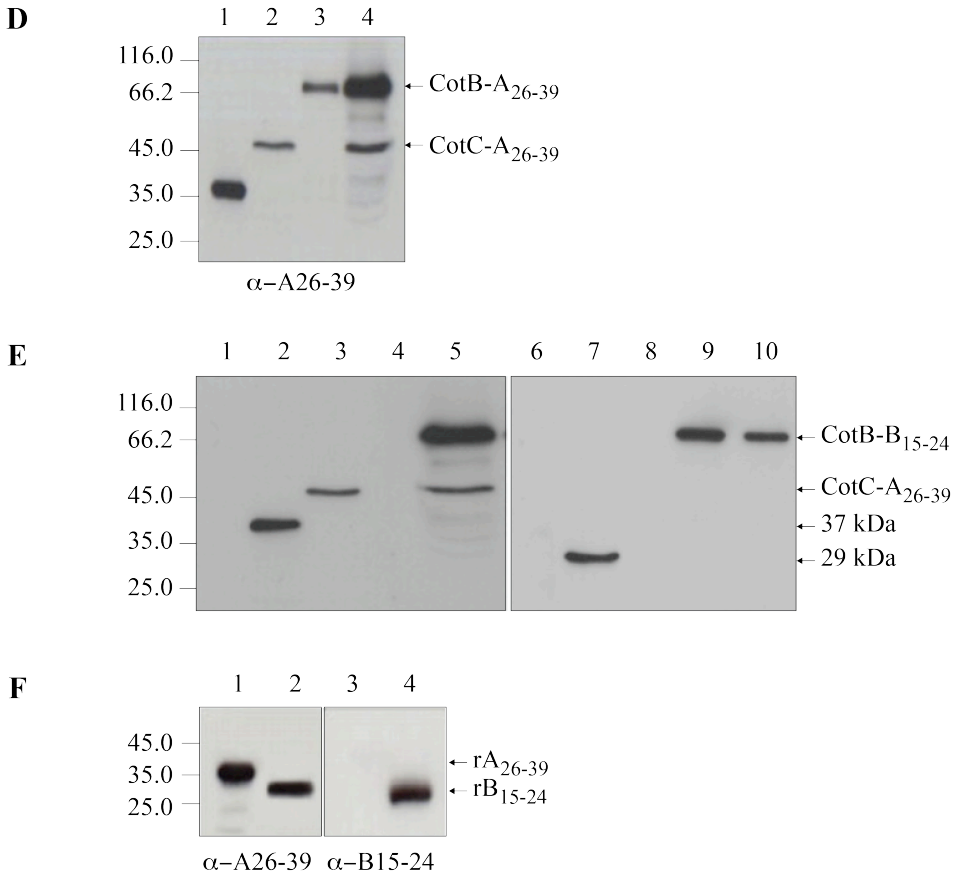


Figure 5.2 (D to F) Expression of A26-39 on spores of PP108 (CotB-A_{26-39} CotC-A_{26-39}) Lane 1 to 4 were probed with anti-A26-39. Lane 1 rA26-39 protein; lane 2, coat proteins from PP052 (CotC-A_{26-39}); lane 3, coat proteins from PP059 (CotB-A_{26-39}); and lane 4, PP108-extracted spore coat proteins. (E) Expression of A26-39 and B15-24 on spores of PP142 (CotB-B_{15-24} CotC-A_{26-39}) probed with anti-A26-39 (lane 1-5) and anti-B15-24 antibodies (lanes 6 to 10). Lanes 1 and 6, nonrecombinant PY79 spore coat proteins; lane 2, rA26-39 protein (36.6 kDa); lanes 3 and 8, PP052 extracts (CotC-A_{26-39}); lanes 4 and 9, PP132 extracts (CotB-B_{15-24}); lanes 5 and 10, PP142 extracts; lane 7, rB15-24 protein (29.4 kDa). (F) Purified rA26-39 (lanes 1 and 3) (36.6 kDa) and rB15-24 (lanes 2 and 4) (29.4 kDa) polypeptides were probed with anti-A26-39 or anti-B15-24. Numbers at the left of the blots are molecular masses (in kilodalton).

Figure 5.3: Homology between the cell binding domains of toxin A ($TcdA_{26-39}$) and toxin B ($TcdB_{15-24}$)

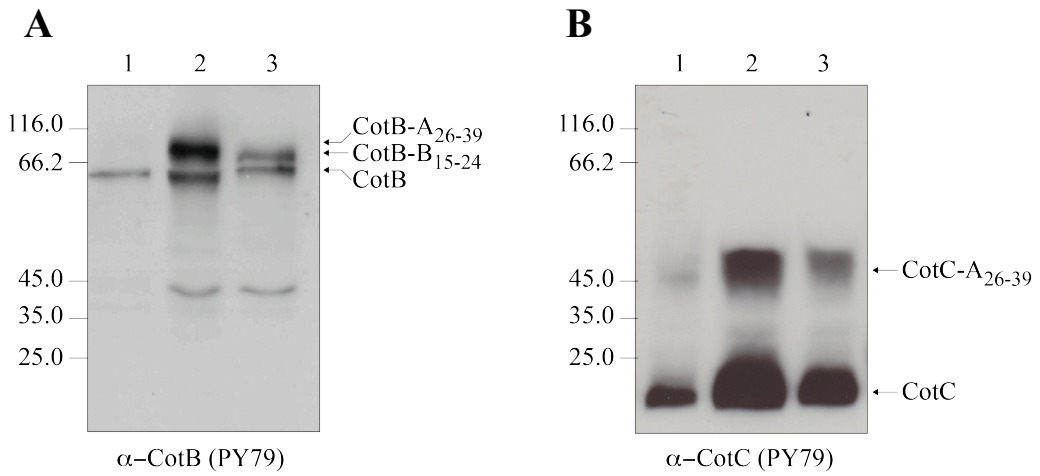


Figure 5.4 Analysis of CotC and CotB recombinant proteins in PP108 (CotB-A_{26-39} CotC-A_{26-39}) and PP142 (CotB-B_{15-24} CotC-A_{26-39}). Panel A shows blots of coat proteins extracted from PY79, PP108 and PP142 probed with anti-CotB (mouse polyclonal, 1/4000). CotB runs at 59 kDa, recombinant CotB-A_{26-39} at 69 kDa and CotB-B_{15-24} at 60 kDa. For PP108 and PP142 the 59 kDa species corresponds to CotB encoded by the endogenous *cotB* gene in these partial diploids where the recombinant genes are inserted *in trans* on the chromosome. **Panel B** shows western blots of spore coat proteins extracted from PY79, PP108 and PP142 spores probed with antiserum to CotC (mouse polyclonal, 1/3000 dilution). The 12 kDa CotC band and the 49 kDa CotC-A_{26-39} bands are indicated.

5.2.3 Oral delivery of the C-terminal domains of toxin A and toxin B displayed on spores induces systemic and mucosal antibodies

Immune responses were determined in mice dosed orogastrically (o.g.) with PP108 or PP142 spores. Control groups included, naïve and groups dosed (o.g.) with non-recombinant spores (PY79). In addition, we included one group dosed (o.g.) with a mixture of the recombinant rA26-39 (10 μg/dose) and rB15-24 (10 μg/dose) proteins using the same dosing regimen. 10 μg was chosen for each protein since this equaled

or exceeded the dose of rA26-36 or rB15-24 delivered in one o.g. dose of PP108 (11 µg of A26-39) or PP142 (9.5 µg of A26-39 and 1.25 µg of B15-24) spores.

Specific antibodies (serum IgG, and faecal IgA) against A26-39 and B15-24 were measured by indirect ELISA. Compared to control groups (naïve mice, mice dosed with PY79 spores or mice receiving proteins alone) significant ($p < 0.01$) levels of A26-39 and B15-24 (cross reacting) IgG were detected in the serum of animals dosed with PP108 (Figures 5.5A and 5.5B). In mice dosed with PP142 spores anti-A26-39 IgG responses were not significantly greater ($p > 0.05$) than control groups, while anti-B15-24 IgG responses were significantly greater ($p < 0.01$) (Figure 5.5B) and showed seroconversion. IgG isotypes were also determined (Figures 5.6 and 5.7) and significant ($p < 0.01$) levels of anti-A26-39 and anti-B15-24 (cross-reacting) IgG1 and IgG2a were found in the PP108 groups compared to the control groups. However, in the PP142 groups significant levels ($p < 0.01$) of the IgG1 and IgG2a isotypes were found only against B15-24. Analysis of the IgG1:IgG2a ratios over time (Figure 5.8) showed a clear increase (3-fold) after the third dose indicative of a Th2 biased immune response. Anti-spore IgG responses were also measured and found to be markedly low with no responses substantially greater than the control groups (Figure 5.9). IgG present in the serum of PP108 immunised mice was found to bind in a Western blot to rA26-39 and rB15-24 while from PP142-dosed mice IgG bound only to rB15-24 (Figure 5.5C).

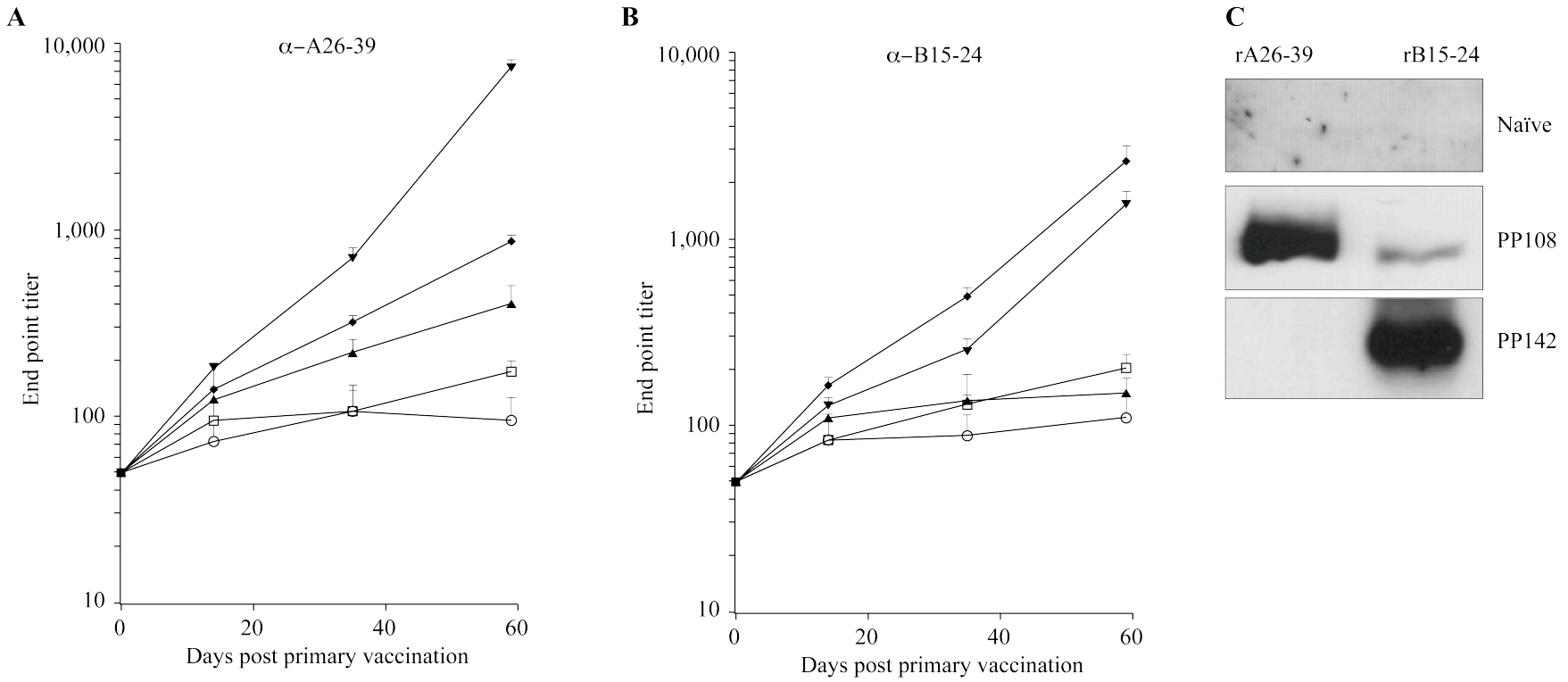


Figure 5.5 IgG responses after orogastric immunisation of mice with recombinant spores Anti-A26-39-specific IgG (A) and anti-B15-24 IgG (B) responses are shown. Mice were immunised with spores of strain PP108 (CotB-A26-39 CotC-A26-39) (▼), PP142 (CotB-B15-24 CotC-A26-39) (♦), nonrecombinant PY79 (□), a mixture of the rA26-39 and rB15-24 recombinant proteins (▲) (10 μg of each) and naïve mice (○). (C) Western blots containing pooled sera from naïve and PP108- and PP142-immunised groups were used to probe rA26-39 proteins and rB15-24.

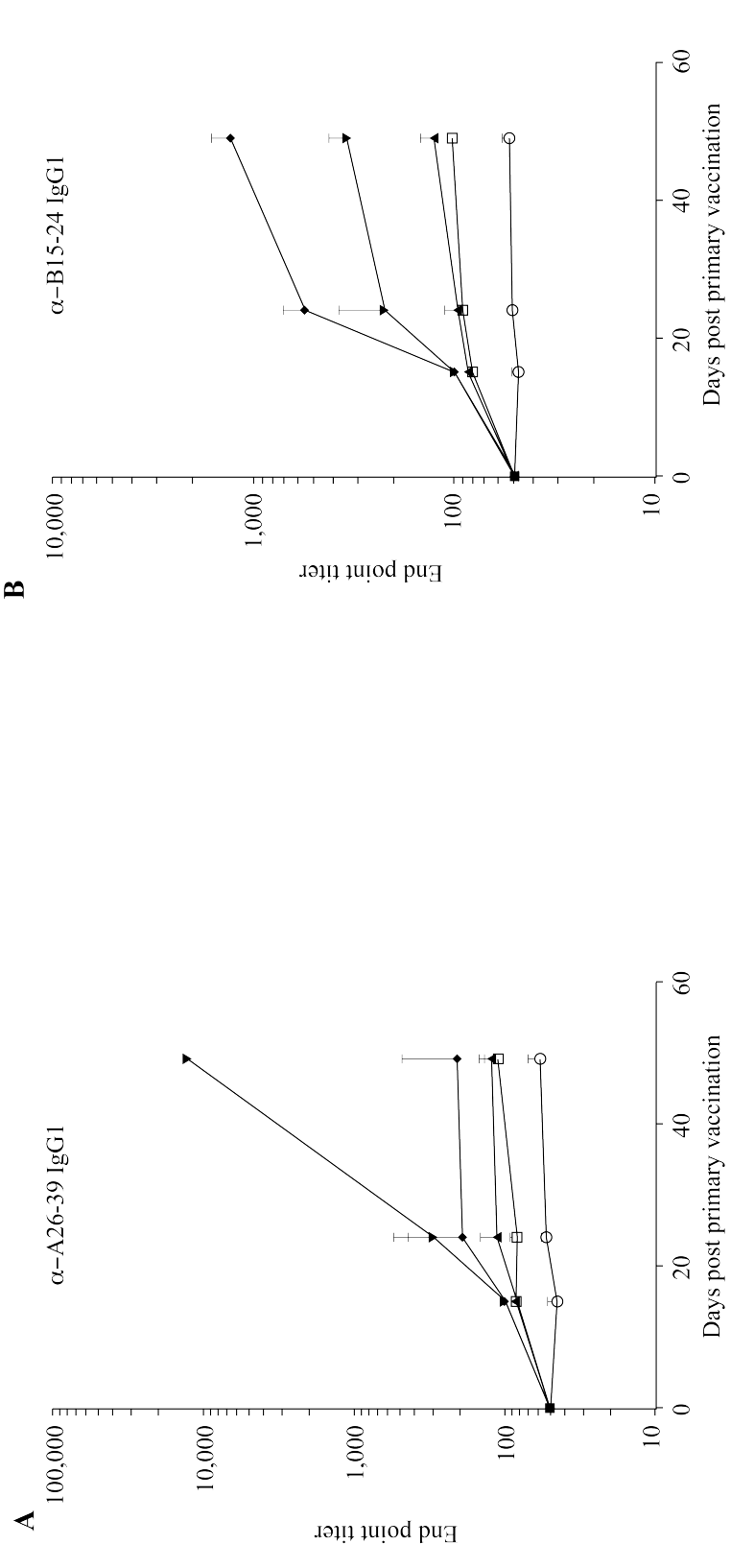


Figure 5.6 IgG1 responses in mice. IgG1 responses specific to A26-39 (A) and B15-24 (B) in mice dosed orally with PP108 (▼) and PP142 (♦) spores were determined by ELISA. Control groups were, naïve (○), mice dosed with non-recombinant PY79 spores (□) and mice dosed with a mixture of the A26-39 and B15-24 proteins (▲).

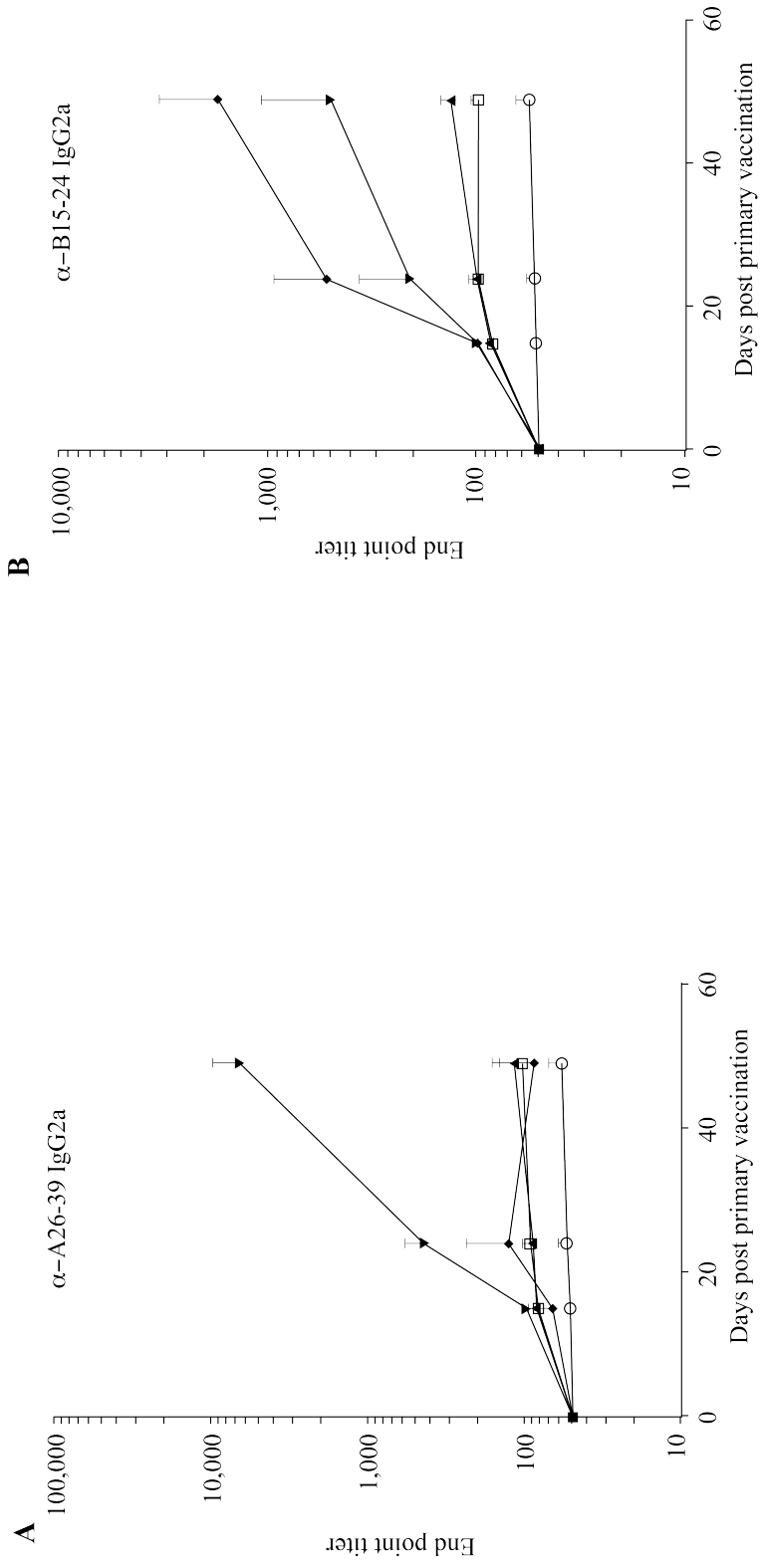


Figure 5.7 IgG2a responses in mice. IgG2a responses specific to A26-39 (A) and B15-24 (B) in mice dosed orally with PP108 (▾) and PP142 (♦) spores were determined by ELISA. Control groups were, naïve (○), mice dosed with non-recombinant PY79 spores (□) and mice dosed with a mixture of the A26-39 and B15-24 proteins (▲).

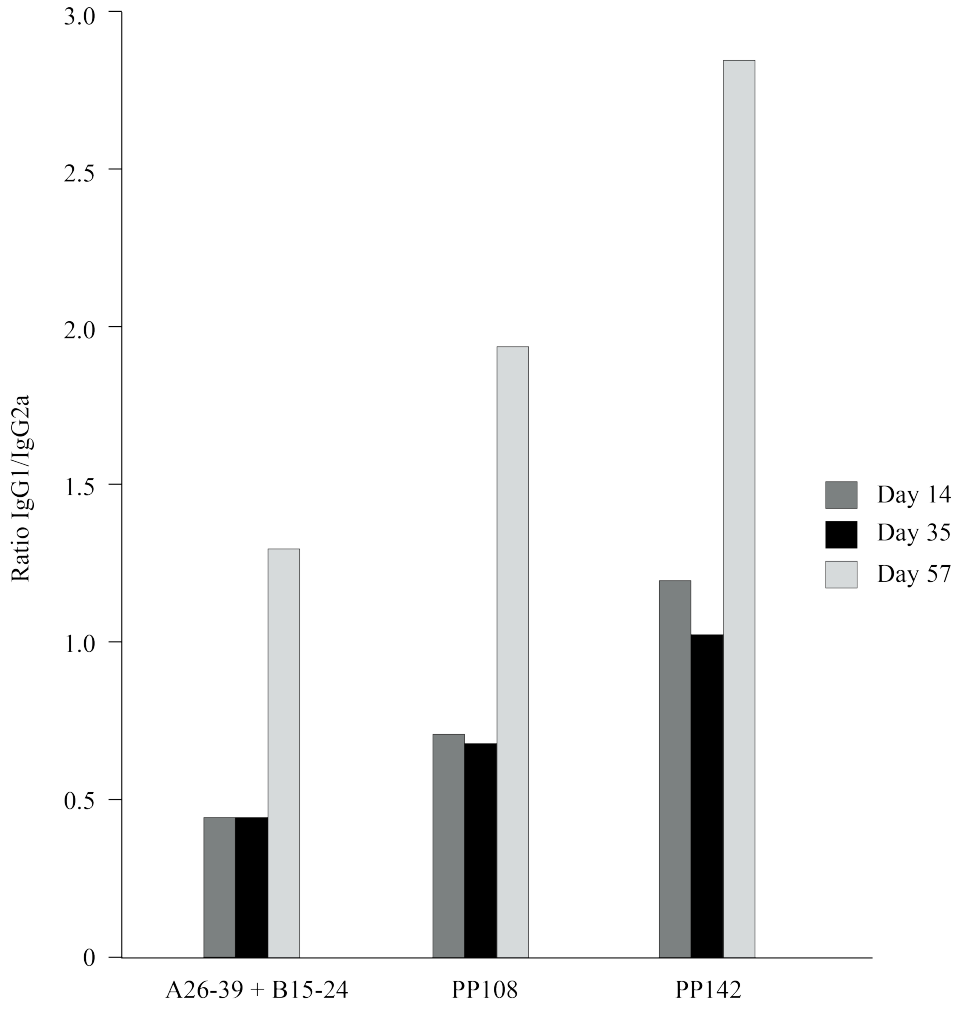


Figure 5.8 Isotype Ratios. The ratio of IgG1 to IgG2a antibodies obtained in serum obtained from mice dosed orogastrically with a protein mixture (rA26-39 + rB15-24) or with PP108 or PP142 spores is shown. IgG values come from figures 5.6 and 5.7. Increasing ratios indicate a Th1 bias.

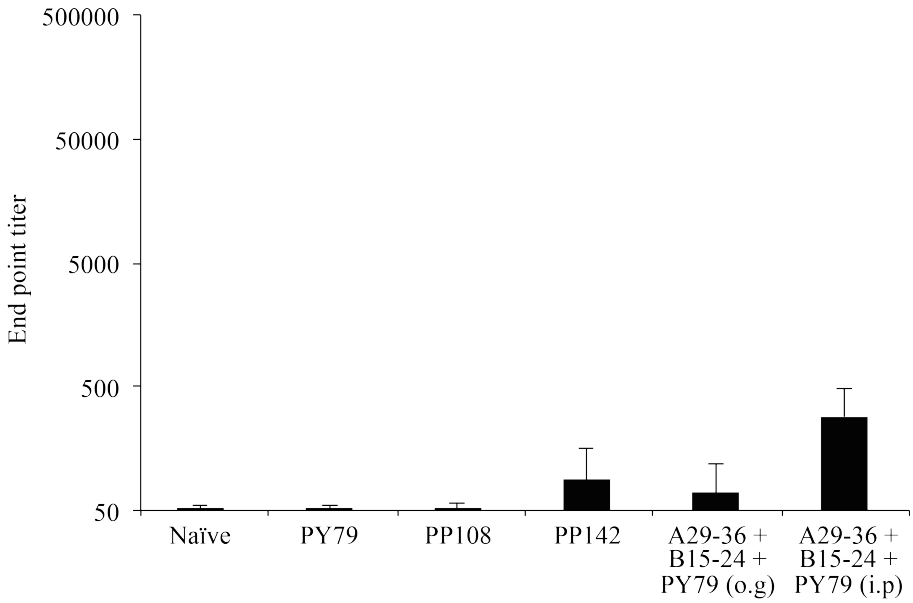


Figure 5.9 Anti-spore IgG response measurement. All groups were immunised via oral dose, with the exception of A29-36+B15-24 PY79(i.p.)(right hand column) which received the dose by inter-parentally. IgG titres determined by indirect ELISA in mice. Plates were coated with PY79 spores (Duc *et al*. 2004. Intracellular fate and immunogenicity of *B. subtilis* spores. Vaccine 22: 1873-85). The end-point IgG titer was calculated as the dilution of serum producing the same optical density as 1/40 dilution of a pooled pre-immune serum.

Secretory IgA (sIgA) was measured in fecal samples (Figure 5.10) and anti-A26-39 responses were found to be particularly high (Figure 5.10A) with clear seroconversion in animals dosed with PP108 spores ($p < 0.001$) but not with PP142 ($p > 0.05$). Anti-B15-24 sIgA responses were lower but seroconversion was found in animals dosed with both PP108 and PP142 (Figure 5.10B; $p < 0.05$). The anti-B15-24 cross-reacting IgG and sIgA responses found in animals dosed with PP108 spores expressing only the A26-39 protein further supports our finding that antibodies against the A26-39 domains are cross-reactive to B15-24.

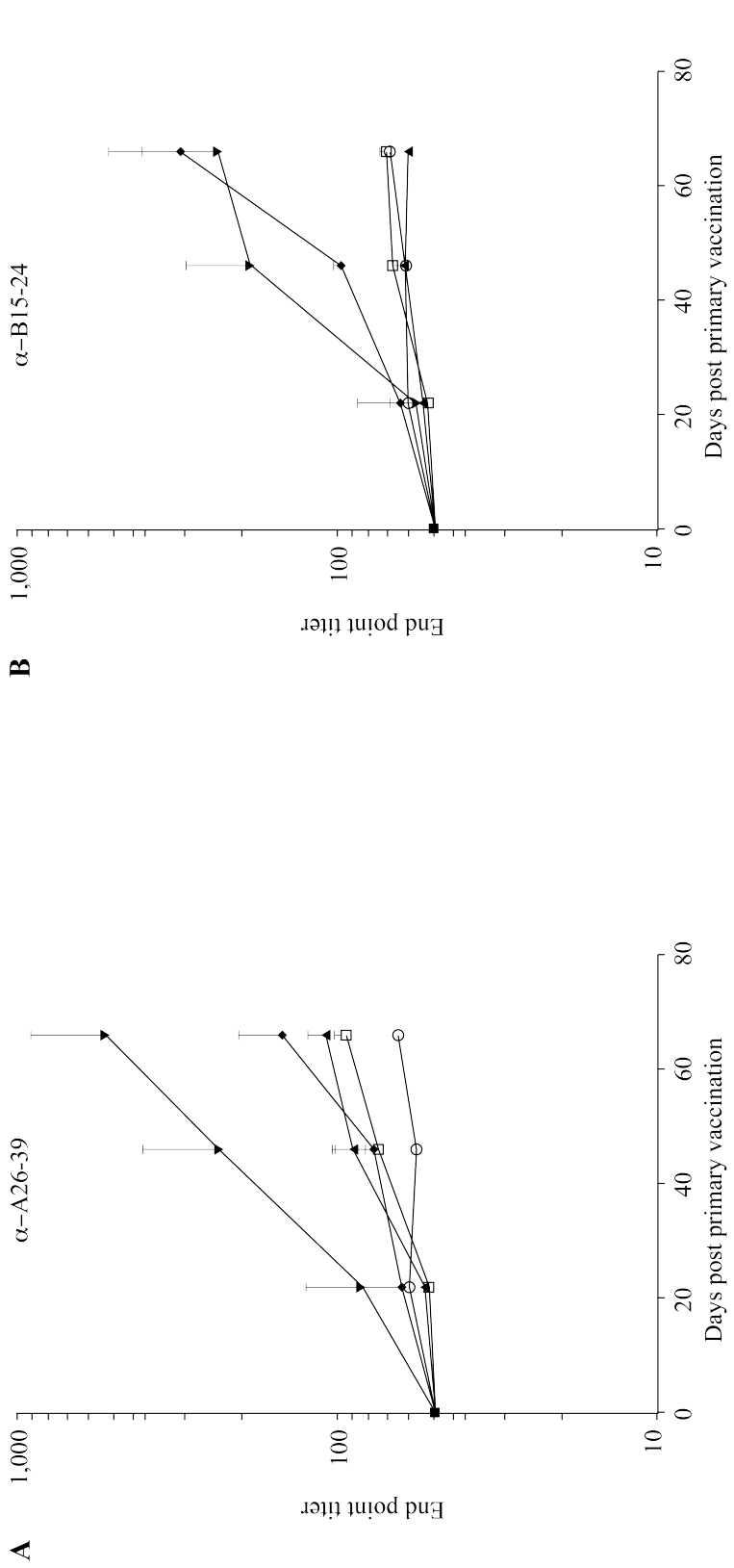


Figure 5.10 Secretory IgA responses after orogastric immunisation of mice with recombinant spores. Anti-A26-39-specific sIgA (A) and anti-B15-24-specific sIgA (B) responses. Mice were immunised with spores of strain PP108 (CotB-A26-39 CotC-A26-39) (▼), PP142 (CotB-B15-24 CotC-A26-39) (♦), nonrecombinant PY79 (□), and a mixture of the rA26-39 and rB15-24 recombinant proteins (▲) (10 μg of each). ○, naïve mice.

5.2.4 *In vitro* neutralisation of cytotoxicity

Both toxin A and toxin B exhibit cytotoxicity on cultured cells with HT29 cells being most sensitive to toxin A and VERO cells to toxin B (Torres *et al.*, 1992, Lyras *et al.*, 2009). Serum (IgG) and mucosal (fecal sIgA) antibodies from mice were assessed for their ability to neutralise toxin A and toxin B-mediated cytotoxicity using either purified toxins or partially purified supernatants from strain 630 (toxin 630) (Table 5.1). Cytotoxicity was confirmed by examination of cells over a 24-48 h period with susceptible cells showing a rounded cell morphology (Figures 5.11A and B**)**.

Antibodies from PP108 o.g. immunised mice were found to neutralise both toxin A and toxin B using either HT29 and VERO cells with higher titers shown by HT29 cells (toxin A specific). By contrast, antibodies produced by PP142 o.g. immunised animals only neutralised toxin B. These results show first, that in mice, o.g. delivery of recombinant spores expressing A26-39 or B15-24 can generate systemic and mucosal neutralising antibodies. Second, using PP108 spores, expressing only A26-39, neutralising antibodies to toxin A as well as toxin B could be elicited.

Figure 5.11A Neutralisation of cytotoxicity. HT29 and VERO cells were cultured as monolayers and their morphological phenotype examined after 24-48h of incubation. In parallel, partially purified toxin supernatants from *C. difficile* 630 (A^+B^+) cultures were pre-neutralised (37^0C, 1h) with antibodies from serum and fecal (pooled) diluted samples respectively obtained from immunised mice or naïve mice (1/10 dilution for serum samples and 1/50 [w:v] for feces) as shown in Figure 5.5 and 5.10. Naïve serum IgG or fecalsIgA being unable to neutralise toxins A and B exhibits the characteristic 'rounded cell' morphology associated with cytotoxicity.

Figure 5.11B Neutralisation of cytotoxicity. HT29 and VERO cells were cultured as monolayers and their morphological phenotype examined after 24-48h of incubation. In parallel, purified toxin A and toxin B from *C. difficile* 630 (A^+B^+) cultures were pre-neutralised (37^0C, 1h) with antibodies from serum and fecal (pooled) diluted samples respectively obtained from immunised mice or naïve mice (1/10 dilution for serum samples and 1/50 [w:v] for feces) as shown in Figure 5.5 and 5.10. Naïve serum IgG or fecal sIgA being unable to neutralise toxins A and B exhibits the characteristic 'rounded cell' morphology associated with cytotoxicity.

5.2.5 Protection in hamsters

The hamster model of *C. difficile* infection closely resembles the human disease and is the best indicator of protective immunity (Sambol *et al*., 2001, Goulding *et al*., 2009). We dosed hamsters o.g. with PP108 and PP142 spores, treated with clindamycin and then challenged them with 100 spores of *C. difficile* strain 630 (Figure 5.12). Control groups included hamsters dosed with non-recombinant PY79 spores and animals dosed with a mixture of the rA26-39 and rB15-24 proteins (10 µg of each). Spores of *C. difficile* were used instead of live cells since, as shown recently, they better mimic the natural infection process (Goulding *et al*., 2009) as well as minimising the difficulties of administering live anaerobic bacteria. All naïve animals (10/10) were susceptible to *C. difficile* and showed symptoms of disease after just 40-42h in close agreement with a previous study (Goulding *et al*., 2009). Interestingly, most of the animals dosed with PY79 spores showed delayed symptoms as did those dosed with a mixture of the rA26-39 and rB15-24 proteins but in both cases all animals succumbed. Hamsters dosed with PP142 spores showed no protection with every animal displaying symptoms. PP108 immunised animals however showed protection with six out of eight animals surviving the duration of the experiment.

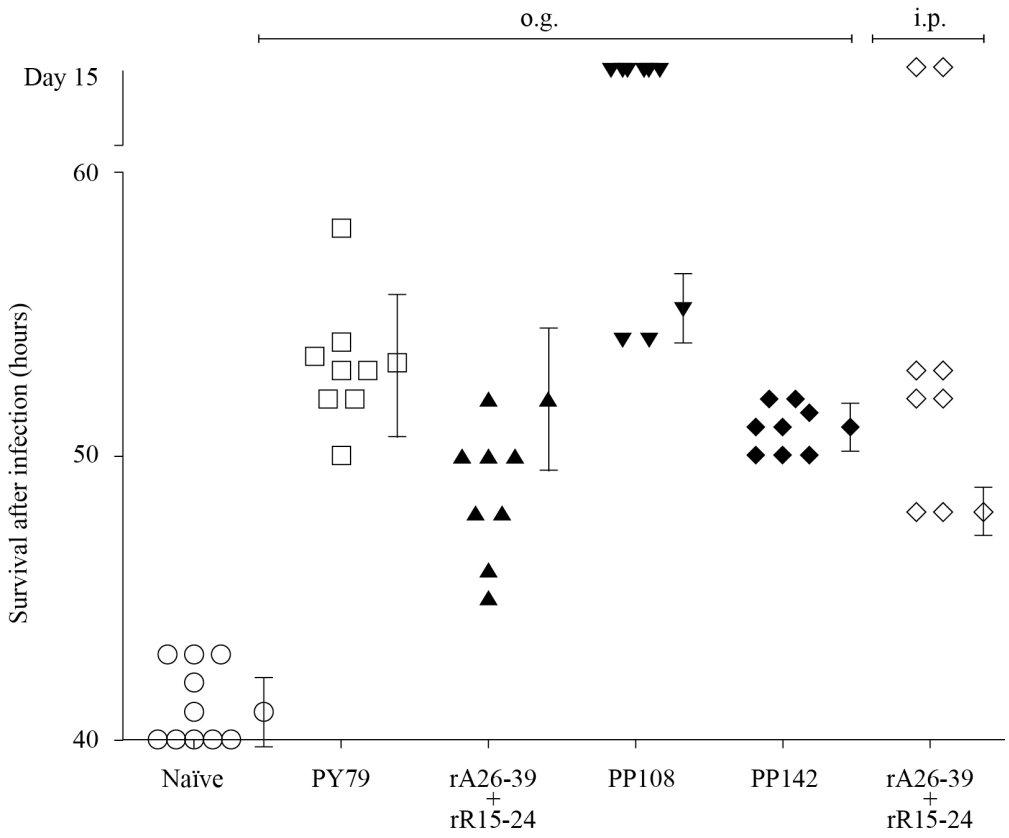


Figure 5.12 Protection in Golden Syrian hamsters. Hamsters were given four o.g. doses (days 0, 14, 35, and 57) of recombinant spores (PP108 or PP142) and then challenged with *C. difficile* 630 (A+B+). Control groups included naïve animals, a group dosed with nonrecombinant PY79 spores, and, finally, a group receiving a mixture of the rA26-39 and rB14-24 proteins (10 µg each). Further groups were hamsters dosed parenterally (i.p.) with rA26-39 plus rB14-24 (10 µg each). Colonisation of hamsters is presented as time from inoculation to signs of first symptoms, when animals were killed. Animals showing no symptoms after 14 days were considered protected (i.e., the PP108 group). For animals showing signs of infection, the mean times (±standard deviations) to visible symptoms were, for naïve hamsters, 40.9 h (±1.7 h), for PY79 hamsters, 53.2 h (±2.3 h), for PP108 hamsters, 55.6 h (±1.7 h), for PP142 hamsters, 50.9 h (±0.9 h), and for hamsters dosed o.g. with the protein mixture and for hamsters in the i.p. group, 51.8 h (±2.4 h) and 48.6 h (± 2.3), respectively.

5.2.6 Protection against reinfection

All surviving hamsters that showed no symptoms of *C. difficile* infection were then re-challenged 16 days after the first challenge using clindamycin to induce *C. difficile* colonisation. Control groups included unimmunised (naïve) hamsters and animals dosed o.g. with non-recombinant PY79 spores. We found that only PP108 hamsters were 100% protected to *C. difficile* challenge and showed no symptoms of infection (Figure 5.13). These results show that o.g. immunisation of hamsters with the A26-39 domain of toxin A displayed on the surface of *B. subtilis* spores is sufficient to confer protection against a strain of *C. difficile* that produces both toxin A and toxin B. Moreover, protected animals were fully resistant to re-infection.

5.2.7 Parenteral immunisation of the cell binding domains of toxin A and toxin B

As a comparator of oral versus parenteral delivery mice were dosed (intra-peritoneal, i.p.) with a mixture of rA26-39 (10 μg/dose) and rB15-24 (10 μg/dose). High ELISA titers of IgG antibodies specific to A26-39 (34,161 ± 9,838) and B15-24 (21,475 ± 7,152) were found two weeks after the last dose (day 45) significantly greater than naïve animals ($p < 0.001$) and higher than serum samples from PP108 immunised mice ($p < 0.001$). By contrast, no significant ($p > 0.05$) levels of sIgA were detectable in feces. Serum antibodies were shown to be able to neutralise the cytotoxic effects of both toxin A and toxin B (Table 5. 1). In the hamsters dosed i.p. with the recombinant proteins 25% protection was achieved (Figure 5.12) but animals failed to survive re-infection with *C. difficile* (Figure 5.13).

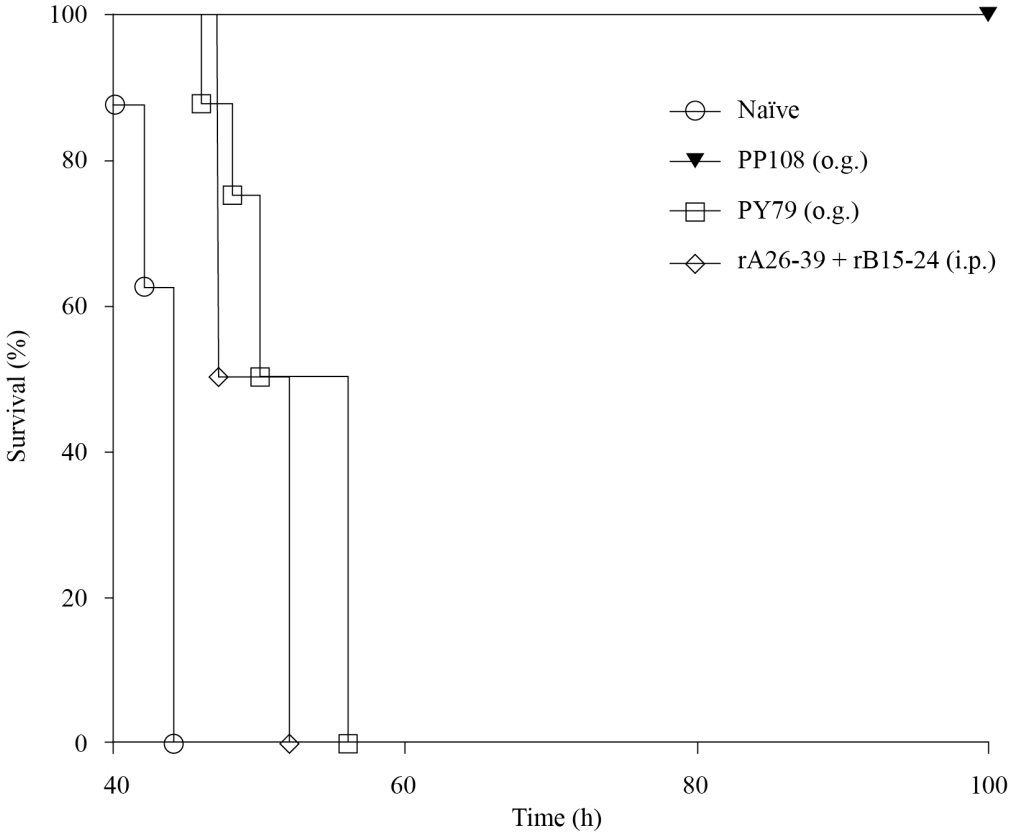


Figure 5.13 Protection against reinfection. Hamsters immunised orally with PP108 spores or parenterally with recombinant proteins that had survived challenge with *C. difficile* 630 (as shown in Figure 5.12) were then rechallenged with *C. difficile* 630 spores 16 days after the end of the first challenge. Kaplan-Meier survival estimates are shown. Results are also shown for control groups of naïve hamsters and animals dosed with nonrecombinant PY79 spores (dosed on days 0, 14, 35, and 57).

Table 5.1 Neutralisation of *in vitro* cytotoxicity and protection.

Immunised groups	Route	Toxin neutralisation titers[1]						Protection (%)[2]	
		α-A		α-B		α-(A+B)[3]		1st	2nd
		IgG	sIgA	IgG	sIgA	IgG	sIgA		
Naïve		-	-	-	-	-	-	**0**	
PY79	**o.g.**	-	-	-	-	-	-	**0**	
[rA26-39 + rB15-24]	**o.g.**	-	-	-	-	-	-	**0**	
PP108	**o.g.**	++	+++	+	+	++	+++	**75**	**100**
PP142	**o.g.**	-	-	+	+	-	-	**0**	
[rA26-39 + rB15-24]	**i.p.**	+++	-	+	-	+++	-		**0**

1 Neutralisation titers against toxin A or toxin B were measured on HT29 (α-A) or VERO (α-B) cells. End-point titers were determined as the highest dilution of sample that prevented the cytopathic cell rounding effect of the toxin in 100% of cells. Negative results are expressed as (-) where the endpoint titer dilution was <1/10 for serum samples and <1/50 (w/v) for faecal samples. Representative neutralisation data is shown in Figures 5.11A and 5.11B and summarised as follows: Serum titers; (+) 10-20, (++) 40-80, (+++) >160. Faeces titers; (+) 50-100, (++) 100-200, (+++) >200.

2 survival data is shown in Figure 5.12. Animals surviving 1st challenge were then re-challenged (2nd).

3 neutralisation against toxins partially purified from CD630, measured using HT29 cells.

5.2.8 Protection against a ToxA$^-$ ToxB$^+$ strain of *C. difficile*

The studies described so far show that antibodies to toxin A are cross-reactive and can neutralise toxin B. We wondered therefore whether PP108 spores that expressed only the cell-binding domain of toxin A could protect against a *tcdA*$^-$ *tcdB*$^+$ strain. Accordingly, we repeated the immunisation experiments in hamsters (5 animals) using four oral doses of PP108 (5 X 10^5 spores/dose). In parallel, we dosed hamsters (n = 5) 3-times (intra-muscular) with the rA26-39 protein (8 μg/dose). Hamsters were challenged with 100 spores of strain M68 (*tcdA*$^-$ *tcdB*$^+$*)* that expresses only toxin B.

All hamsters failed to be protected to M68 challenge with either immunisation regimen showing that antibodies to toxin A are unable to protect against toxin B. Figure 5.15 shows the end point IgG titres specific to rA26-39, toxin A and toxin B obtained in hamster serum. Although IgG titres were higher in animals dosed with rA26-39 these clearly fail to protect.

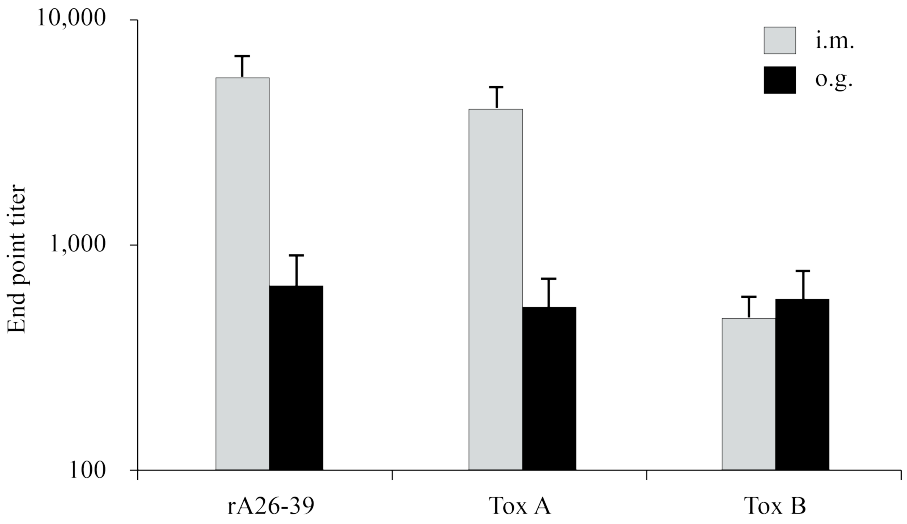


Figure 5.14 Comparision of ELISA serum IgG responses between intramuscular (i.m.) and orgastrical (o.g.) routes of administration in serum hamster model. IgG titers specific to rA26-39, toxin A and toxin B obtained in hamster serum are shown.

5.2.9 Formalin inactivation of spore vaccines

Formalin (formaldehyde in aqueous solution) is known to be effective as a method to inactivate biological entities and is used for inactivating live organisms in a number of vaccine formulations. The action of formalin has been recently shown to enhance the immunogenicity of biological particles compared to other methods of inactivation (Sander *et al.*, 2011). We inactivated PP108 spores using three treatments, autoclaving (121^{o}C, 15 psi, 30 mins), UV-C irradiation and by formalin treatment (2% formaldehyde in PBS, pH 7.4, RT 3 h). In each case 100% loss of viability was confirmed by serial dilution and plating out on agar plates. Mice (n = 4) were dosed (i.p.) 3-times (days 0, 14 and 28) with inactivated PP108 spores (1X10^{9} CFU/dose). As controls groups of mice were also dosed with rA26-39 protein (5 µg/dose) and

with live PP108 spores (1 X 10^9/dose). In these experiments one dose of PP108 spores would equate to 0.2µg of rA26-39. rA26-39-specific IgG responses were determined at day 45 and revealed that very high antibody titres were observed in animals dosed with either rA26-39 and formalin inactivated PP108 spores (Figure 5.15). Formalin inactivated PP108 spores produced the same titres of IgG as found in rA26-39 immunised mice but surprisingly, the total dose of rA26-39 delivered using PP108 spores was 25-times less than dosing by protein alone. By contrast much lower responses were observed in animals dosed with live PP108 spores and no responses in animals dosed with autoclaved or UV-C irradiated PP108 spores. These results show that formalin appears to enhance the immunogenicity of the rA26-39 domain expressed on PP108 spores, presumably, by fixing the protein in its natural conformation rather than by denaturing it. Moreover, in agreement with recent studies on the use of formalin for inactivation of microbial entities immunogenicity is enhanced (Sander *et al*., 2011).

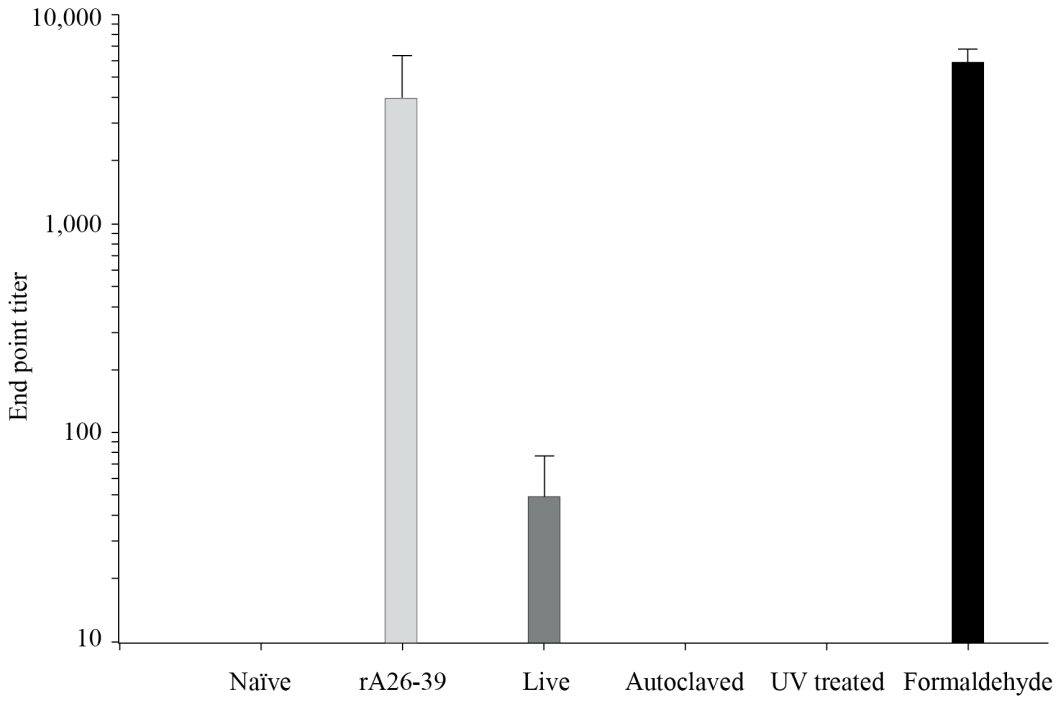


Figure 5.15 ELISA IgG responses of spores with different treatments. Serum IgG responses obtained from mice dosed orogastrically with spores of PP108 individually treated with either formaldehyde or UV-C or autoclaving compared to live spores and A26-39 proteins are shown.

5.3 Discussion

Our use of *B. subtilis* spores as a vehicle for *C. difficile* vaccination demonstrated a number of important findings that will be invaluable to the design of an effective *C. difficile* vaccine. First, that only toxin A antibodies are required for protection, second, the importance of mucosal immunity and, finally, protection against relapse. We discuss each of these in turn.

5.3.1 Toxin A antibodies and protection

Expression of the toxin A (A26-39) and toxin B (B15-24) C-terminal domains on *B. subtilis* spores was shown to generate high titers of specific IgG and sIgA antibodies. Using spores (PP108) displaying only the toxin A domain, A26-39, serum and fecal antibodies were found to be cross-reactive to B15-24. Moreover, they neutralised both toxin A and toxin B *in vitro* and hamsters immunised with A26-39 were protected against *C. difficile* disease. Further evidence of the cross-reactivity of toxin A antibodies came from Western blotting which showed that anti-A26-39 antibodies could recognize B15-24. The repeat domains of both toxins share limited sequence identity which could explain this cross-reactivity. In particular, the sequences YFAPANT, MQIGVF, AAT and YYF are conserved between the toxin A and B repeat used here and together they may contain epitopes responsible for the observed cross reactivity.

We infer that antibodies to A26-39 are of higher avidity than those to B15-24 since, in mice, ELISA antibody titers to A26-39 and B15-24 were equivalent. PP142 spores

present both toxin A and toxin B antigens, so why do these spores not provide protection against disease after administration? Although anti-B15-24 neutralising antibodies were produced, no neutralising antibodies to A26-39 were detected. This implies, that for protection, antibodies to toxin A are of greater importance than those to toxin B. PP142 spores do express the A26-39 domain yet no neutralisation of toxin A was observed. To account for this we predict that insufficient A26-29 was expressed since PP142 spores carried nine times less A26-39 than PP108 spores. Of course, we cannot completely rule out the potential contribution of anti-B15-24 antibodies in protection but rather our work shows that anti-A26-39 responses are sufficient. It is possible that our prototype vaccines simply do not elicit sufficiently high anti-toxin B neutralising titers possibly due to B15-24 being displayed in a partially denatured form that impairs the generation of appropriate neutralising antibodies. This may reflect its fusion partner, the spore coat protein CotB, although this has been used successfully previously (Duc *et al*., 2003, Hoang *et al*., 2008). Full protection to challenge using PP108 spores might be achieved either by changing the dosing regimen or by increasing the dose of heterologous protein expression (whether A26-39 and/or B15-24).

This work agrees with a number of studies linking protection against *C. difficile* infection with toxin A. For example, a passive immunisation study using oral delivery of anti-toxin A antibodies (Kink & Williams, 1998) and studies showing that there is strong association between serum antibody responses to toxin A and protection against *C. difficile* in humans (Kotloff *et al.*, 2001, Kyne *et al.*, 2000, Aboudola *et al*., 2003). Of particular note is the study of Kim *et al* (Kim *et al.*, 1987)

where hamsters immunised (sub-cutaneous) with *C. difficile* toxoid A were fully (100%) protected but no protection was observed in hamsters immunised with toxoid B. Our work does show however that a vaccine expressing only A26-39 should be protective to all known naturally virulent strains of *C. difficile* (A^+B^+ and A^-B^+). However, a challenge experiment using an A^-B^+ strain is required for confirmation this finding is important for the design of future *C. difficile* vaccines.

5.3.2 Mucosal immunity

Considerable effort has been directed towards parenteral vaccines to *C. difficile* and the generation of systemic responses. However, far less attention has been spent on examining the role of local immunity despite a number of reports demonstrating the importance of sIgA. First, a correlation between neutralising sIgA and protection in hamsters has been reported using mucosal delivery of toxoids with 100% protection using intra-nasal delivery and 40% with o.g. (Torres *et al.*, 1995). Second, and most importantly, several studies that demonstrate unequivocally that sIgA is both capable of neutralising toxin A and is superior to both IgG and monomeric serum, IgA (Johnson *et al*., 1995, Stubbe *et al*., 2000).

Oral delivery of A26-39 using PP108 spores promoted high titers of fecal sIgA with levels of anti-toxin A and anti-toxin B (cross reacting) neutralising sIgA correlating with protection. By contrast, i.p. delivery of the rA26-39 + rB15-24 proteins produced high titers of serum neutralising IgG to toxin A and toxin B, no localised immune responses (sIgA) and lower levels of protection. In another study however, Torres *et al* (Torres *et al*., 1995) used parenteral delivery (including i.p.) of toxoids A

and B and demonstrated 100% protection in hamsters. A number of factors might account for why we did not observe complete protection when using the recombinant proteins. For example, in the Torres study the complete toxoids were administered together with an adjuvant and a different virulent strain of *C. difficile* was used for challenge (Torres *et al*., 1995).

5.3.3 Relapse

PP108 immunised hamsters that survived *C. difficile* challenge were fully protected against re-challenge, a phenomenon particularly important for treatment of CDAD where patients succumb to relapse. Up to 20% of patients with CDAD two to eight weeks after discontinuation of antibiotic therapy (metronidazole or vancomycin) relapse and a further 30% of these patients may do so again after a second course of therapy (Buggy *et al.*, 1987, Jobe *et al.*, 1995). Antibiotic treatment may disrupt the normal human microflora and lead to overgrowth of toxigenic strains of *C. difficile*, a condition favoring relapse. Vaccination then may provide the only rationale treatment to control CDAD so it is encouraging that we found no symptoms of disease in rechallenged animals. This is consistent with an earlier study that has shown that antibodies to toxins A and B, administered orally, can prevent relapse (Kink & Williams, 1998) and also mucosal and parenteral delivery of toxoids A and B where 75% protection to relapse was found (Torres *et al*., 1995). In our studies, we only observed protection against relapse for hamsters dosed with PP108 spores and not with animals that had been vaccinated with the recombinant proteins using a parenteral dosing regimen. Interestingly, animals dosed parenterally all failed to generate significant sIgA responses and it is possible that production of mucosal

antibodies is important for protection from re-infection. Some probiotic formulations have been shown to significantly reduce the occurrence of relapses in double-blinded controlled trials (Surawicz *et al.*, 2000, Hickson *et al.*, 2007). Potentially, live spores may share some attributes with these 'probiotic' bacteria and it is notable that *B. subtilis* is itself used extensively as a probiotic (Hong *et al*., 2005). *B. subtilis* spores can germinate and resporulate in the GI-tract and possibly even transiently colonize (Tam *et al.*, 2006). PP108 spores may therefore be able to promote a more extensive production of anti-A26-39 sIgA sufficient to protect against re-infection. With regard to their potential probiotic attributes, it is noteworthy that even non-recombinant PY79 spores, when administered orally to hamsters provided a noticeable delay to the onset of symptoms and it is possible that this phenomenon may reflect involvement of an innate immune response such as interaction with Toll-like receptors. Althernatively, although highly speculative, we can not rule out sequestration of the toxin in the animal GI-tract since *Bacillus* spores have been shown able to adsorb *C. difficile* toxin *in vitro* (Huang *et al*., 2010).

Finally we have used *C. difficile* spores, in contrast to vegetative cells or toxins, as our challenge in the vaccine protection experiments which, as has been shown recently, best mimics the natural course of infection in humans (Goulding *et al*., 2009). Our study confirms that in the hamster model of infection, there is a correlation between neutralising antibodies and protection in hamsters with HT29 cells providing a more precise *in vitro* indicator of protection. In summary, we demonstrate a strategy for *C. difficile* vaccination that is based on mucosal administration generate antibodies to toxin A. Further, we describe a vaccine that is

particularly suitable for its end user, i.e., a vaccine that is both heat stable, can be administered orally and is in current use as a probiotic.

In summary, we show that oral delivery of *B. subtilis* spores displaying high levels of toxin A repeats can confer immunological protection to hamsters to a lethal dose of *C. difficile* spores. The basis of the vaccination strategy is induction of secretory IgA which shows cross reactivity to toxin B. Such a vaccine would be particularly attractive to the end user as it can be delivered orally, is heat stable and the vehicle is currently used as a probiotic.

5.4 Conclusion

This work shows that *B. subtilis* spores have potential as a vaccine delivery system for CDAD. The most important finding was that antibodies to the cell-binding domain of TcdA are cross-reactive to TcdB. This may be assumed based on the extensive homology between these domains but this work is the first that shows this. Although spores as a vaccine confer protection this is not 100% and to consider a fully protective vaccine we plan to construct spores with a higher level of TcdB expression of the spore surface. Our work also shows the importance of mucosal antibodies and further experiments are in progress where we are evaluating mucosal IgG responses in hamsters (note, that to date, no commercial secondary antibodies to IgA from hamsters are available). The use of formalin may provide a useful approach to vaccinating against CDAD using spores and we plan to dose hamsters with formalin inactivated spores and evaluate protection. Finally, the use of spores as an oral vaccine is attractive and could be viable and to this aim we are also evaluating spore vaccines using sub-lingual dosage. Sub-lingual delivery has been used for *Bacillus* vaccines (Amuguni *et al.*, 2011) and is an attractive route for antigen delivery since the oral immune system is targeted yet reduced levels of antigen are required and oral tolerance is avoided (Cuburu *et al.*, 2009, Song *et al.*, 2008).

CHAPTER 6

GENERAL DISCUSSION

6.1. Sporulation of *C. difficile*

Until the seminal studies by Lawley *et al* (2009) it has proven almost impossible to prepare good crops of *C. difficile* spores. Till this point the presence of spores could be assumed from the analysis of heat-resistant CFU in samples and EM pictures showing the presence of mature endospores or spores maturing within sporulating cells. The spores closely resemble endospores of other *Bacillus* and *Clostridial* species in size and shape and they all carry a thick proteinaceous coat. In the study of Lawley *et al* (2009) an exosporial layer was shown and the studies shown here also suggest that *C. difficile* spores carry an exosporium but we believe that this layer is either very fragile or is produced (or lost) under certain conditions. We assume this is because we could not always discern the exosporium and routinely produced crops of spores that lacked the exosporium. Our analysis of spore coat proteins most probably was made on spore crops where most spores lacked the exosporium and as such we may have missed proteins found in the exosporial layer. One protein we did not identify was BclA for which three structural genes are known from the genome sequence of *C. difficile* (Lawley *et al*., 2009). In other spore formers such as *B. anthracis* and *B. cereus* BclA is a collagen-like protein that has three subunits forms

filaments resembling flagella and which protrude from the surface of the spore (Thompson & Stewart, 2008, Brahmbhatt *et al.*, 2007, Bozue *et al.*, 2007, Sylvestre *et al.*, 2002). They do this by assembling in the exosporium. Since *C. difficile* carries both an exosporium and a *bclA* gene we believe our spore preps have mostly lost the exosporium and this would account for why we cannot detect the protein using Mass-spec analysis of extracted spore coat proteins. Another possibility is that the BclA protein is indeed present but is not amenable to Mass spec analysis as a result of its highly unusual structure. One question is the function of the exosporium? Our analysis of adhesion shows that spores are almost 100-times more efficient at binding to host intestinal cells than live vegetative cells. This is important and suggests that spores themselves could play an important role in host colonisation and we argue this to be more so than vegetative cells. It has always been thought that the live cell of *C. difficile* would be responsible for colonisation, that is, spores germinate, outgrow and the live cell secretes toxins and colonises the GI-tract. Further, the S-layer protein, SlpA, which encases the live cell could play an important role in adhesion. While the SlpA protein is able to adhere to host cells (Calabi *et al.*, 2002) studies using it as a decolonisation factor in vaccine formulations have been disappointing. Indeed, injecting SlpA into animal models showed no significant reduction of *C. difficile* counts in the GI-tract suggesting that antibodies raised against SlpA have little effect on colonisation (Ni Eidhin *et al.*, 2008). We reason then that *C. difficile* spores and not live cells are key to *C. difficile* colonisation and infection. The role of the exosporium could be in adhesion and the BclA-filaments could play a role. Our opinion is that the BclA filaments (which of course were not shown in this study) are not themselves key to adhesion, rather they may be involved in aggregation of spores,

rather like tethers or anchors, whether by aggregating *C. diffcile* spores or tethering to other bacteria. Our studies to date have used standard methods of adhesion using cultured cell lines and this of course is artificial and does not closely resemble the natural condition. However, long-term studies should examine the adhesion and colonisation of wild type and mutant *C. difficile* spores. A mutant could be made using the ClosTron system (Heap *et al.*, 2010) developed by the Minton lab in Nottingham and *cot* and *bclA* mutants examined for adhesion *in vitro* as well as colonisation in a mouse model. This type of analysis would reveal the importance of spore coat proteins and the exosporium in cell adhesion.

Our work also showed that the spore coats of *C. difficile* carry a number of interesting proteins of which three carry enzymatic properties. The catalase and peroxiredoxin activities are thought to be involved in reducing the toxic effects of hydrogen peroxide that are produced during spore coat biosynthesis and are necessary for cross-linking of spore coat proteins. The chitinase activity is more interesting since it does not seem logical for a dormant spore to carry an active enzyme on its surface since by definition this entity (the spore) is dormant. Rather we believe the chitinase (which may also have cellulase activity) is only rendered active when the spore germinates. In such a scenario the spore when it germinates would release an active enzyme that can digest macromolecules and make these available for the newly germinated and outgrowing cell. Perhaps even more intriguing is that chitinases and peroxiredoxins have important links with inflammatory responses. Specifically, production of these enzymes can be linked to the production of the pro-inflammatory cytokines. These molecules are often

produced during inflammation resulting from acute infection or disease and of note, in colitis and other inflammatory bowel disease pro-inflammatory responses occur. We wonder then whether CotE plays some role in inflammation and the action of this protein plays some role in the manifestations of CDI. To address this the entire CotE protein should be expressed and the protein used in an *in vitro* assay using cell culture to measure the stimulatory effects of CotE on the proinflammatory cytokines, for example, TNFa, IL-2 and IL-6. The expressed protein should also retain enzymatic activity and could be used to verify that, *in vitro*, the protein is both a peroxiredoxin and a chitinase. In addition, a *cotE* mutant made using the ClosTron system could be used to evaluate the CotE phenotype in comparison to the wild type strain, 630. A *cotE* mutant should lack both peroxiredoxin and chitinase activity. This work is currently being performed in this laboratory and initial indications show that CotE does indeed carry chitinase and peroxiredoxin activity.

In other spore forming bacteria, most notably *B. subtilis*, the genetic control of gene expression that drives sporulation has been well characterized (Errington, 1993, Errington, 2003). Here, spore formation is controlled by developmental genes normally referred to as sporulation or *spo* genes (Stragier, 1994, Stragier, 2002). Their gene products are either structural proteins or regulatory factors that further fine tune developmental gene expression. Central to this is the activation of five sporulation-specific sigma factors, σ^H, σ^E, σ^F, σ^G and σ^K, each of which directs transcription of a subset or sporulation genes (Stragier, 1991, Stragier & Losick, 1990). This ensures that temporal gene expression as well as spatial gene expression since the sigma factors, σ^F and σ^G are confined to the forespore compartment of the

developing sporangial cell. Gene expression is further refined using developmental checkpoints where each sigma factor is itself activated by signals tightly coupled with the developing cell. These post-translational events ensure a high fidelity to spore formation and are often referred to as checkpoints (Losick & Stragier, 1992). Interestingly though, with regard to σ^K, no post-translational control is present and expression of active σ^K is dependent only on transcriptional control of the *sigK* gene and its chromosomal rearrangement brought about by excision of the *skin*^{Cd} element (Haraldsen & Sonenshein, 2003, Paredes *et al.*, 2005). One important finding from this work is that we have been able to develop a protocol for synchronous sporulation. The approach requires a substantial dilution of the overnight culture before inoculation into fresh medium. Failure to extensively dilute does not stop spore formation but rather leads to asynchronous sporulation. Based on our studies it takes approximately 7-8h to form a spore in *C. difficile* which is faster than the other solventogenic *Clostridia*. Expression of the key regulatory factors in *C. difficile* appears similar to that of *B. subtilis* but interestingly differences between strains occurs. One area of work that needs to be conducted is to define why and how expression differs so markedly between strains. In our case we showed that expression of the toxin, *tcd*, genes is different between virulent and hypervirulent strains which is intriguing and we wonder whether this reflects the increased virulence of the hypervirulent.

The spore coat proteins that are assembled onto the outermost layers of the spore are also sporulation genes but normally are referred to as Cot and their genes as *cot*. Most *cot* genes are expressed in the mother cell chamber of the sporulating cell and are controlled by either σ^E or σ^K (Henriques & Moran, 2007). Temporal expression

of the spore *cot* genes ensures that these proteins can accumulate and assemble as defined layers. Evidence for self-assembly of at least some Cot proteins has been found in *B. subtilis* (Krajcikova *et al*., 2009).

Spores of *C. difficile* are similar to those of other Gram-positive spore formers and a comparison made using bioinformatics has shown that *Clostridia* and specifically *C. difficile* share similar pathways of regulatory gene expression. Based on this analysis the principal components for coordinating sporulation-specific gene expression in *B. subtilis* are also found in *C. difficile,* this includes the major sigma factors as well as the regulatory proteins such as SpoOA, SpoIIID etc (Paredes *et al*., 2005). Interestingly, though, a proteomic study of *C. difficile* spores while arriving at similar conclusions also showed some significant differences (Lawley *et al*., 2009). Specifically, of the 70 or so *cot* genes found in *B. subtilis* only 18 orthologues were found in *C. difficile*. This shows that substantial differences may occur in the structural composition of *C. difficile* spores despite their apparent similarity at a visual and genetic level.

Our analysis of *cot* gene expression showed two distinct phases of gene expression which we assume is mediated by σ^E (1^{st} phase) followed by σ^K (2^{nd} phase). In addition, some of the *cot* genes showed a biphasic pattern of gene expression which resembles that of some genes in *B. subtilis* (for example, *cotE*) which are transcribed by RNAP associated with σ^E and then by σ^K. Further work will need to be done to clarify spore *cot* gene expression. Specifically, the dependence of gene expression must be determined. To do this Clostron mutations must be made in the *sigE* and *sigK* genes and then expression of the cot genes determined again, by qPCR. If gene expression is blocked in both mutants then the *cot* gene must be dependent on σ^E

while if it is blocked only in a *sigK* mutant then it must be σ^K transcribed. These experiments are now in progress and the aim will be to define spore coat gene expression in *C. difficile* and provide a framework for understanding developmental gene expression.

6.2. The Requirements of a Better Vaccine to *C. difficile*

One of the most important findings from this work is first the demonstration that delivery of the carboxy-terminal segment of *tcdA* can confer protection in hamsters and second, that antibodies against this C-terminal domain are cross-reactive to the same C-terminal domain of *tcdB*. Although other studies have highlighted the importance of the C-terminus of toxin A (CDTA) as being important for generating neutralising antibodies (Ward *et al.*, 1999a, Ward *et al.*, 1999b) this is the first study to demonstrate this in a challenge model. We also show that using live bacterial spores we can get protection to relapse, i.e., animals surviving the first challenge were fully protected to challenge. This is in marked contrast to animals vaccinated using the parenteral route where surviving animals died. We believe that mucosal antibodies play the most important role in preventing primary infection and this may be the case for prevention to relapse. In humans IgA specific to toxin A was found in 57% of colonic aspirates in patients with colitis (Kelly *et al.*, 1992). Also, in postpartum women 64% of colostral samples had toxin A-specific neutralising IgA (Kim *et al.*, 1984). It has also been shown that intestinal IgA responses to toxin A accompany serum IgG responses in patients with CDI (Johnson *et al.*, 1992). These studies also looked at the levels of toxin A-specific IgA in patients with *C. difficile* associated diarrhea where levels of sIgA are higher than serum IgG (Johnson *et al*., 1992). However, it is well known that mucosal responses, particularly, secretory polymeric IgA are short-lived so this may be one explanation why relapse occurs. It is also possible that other factors are required for protection to relapse. Studies have shown that immunodominant antigens exist in *C. difficile* notably a 36-37 kDa surface exposed cell wall protein, SlpA (Cerquetti *et al.*, 1992, Pantosti *et al.*, 1989).

Is it possible then that full protection to both infection and relapse requires more than only an anti-toxin response. Support of this comes from work made with non-toxigenic strains of *C. difficile* that when used as a prophylactic oral treatment prevent CDI in animal models (Sambol *et al.*, 2002). In our studies we have shown that PP108 recombinant *B. subtilis* spores confer protection to infection and relapse. This is intriguing and shows that some aspect of the *Bacillus* spore itself is playing a role in protection. Is it possible that the *B. subtilis* spore shares an antigen or epitope similar to *C. difficile*. While possible we have shown here that antibodies to *B. subtilis* do not cross-react with *C. difficile* spores and visa versa. Another possibility is that live *B. subtilis* is able to colonise the gut and perhaps spores are able to bind to the mucosal epithelium preventing *C. difficile* binding. This may or may not be the case but we wonder whether the mucosa has receptors for *C. difficile* spores and whether *B. subtilis* spores could bind to the same site. Some support comes from our adhesion studies which show that *C. difficile* spores have very high binding to epithelial cells. This assay cannot be conducted though with *B. subtilis* spores since spores germinate rapidly in cell culture media, outgrow and proliferate which prevents a direct measurement of spore binding. We believe that recombinant spores could be used in a vaccine formulation. One concern may be that spores are recombinant, that is, GM (genetically modified). We have evidence that spores can be inactivated with formaldehyde and this treatment actually enhance the immunogenicity of spores. Formalin is commonly used for fixing proteins and maintains the polypeptide antigen in its natural 3D shape. Further, we have considered using spores in a sub-lingual delivery where the vaccine would be delivered under the tongue facilitating entry of spores and antigens to the buccal cells

which are part of the oral immune system. Sub-lingual delivery would also offer two advantages, reduced dose of antigen and the potential to reduce oral tolerance. We consider it possible that the reason we did not obtain 100% protection in hamsters using PP108 spores is because of oral tolerance although we have no evidence of this. Another possibility is that of modifying the dosing regimen to improve immunogenicity, for example 2-3 early doses followed by 2 at day 14 or 28. These studies are in progress currently in the laboratory.

In conclusion this thesis has addressed two aspects, the structural dissection of the spore with a first attempt at defining the composition of the spore surface, its surface properties and role in adhesion. Second, to evaluate a novel mucosal vaccine to *C. difficile*. The spore structure analysis potentially could illuminate potential candidates for vaccine design or as decolonisation factors. The identity of enzymes, particularly the chitinase could prove useful in the identification of drugs that reduce chitinase activity and therefore inflammation.

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APPENDICES

Copies of Publications

1. Permpoonpattana, P., E. H. Tolls, R. Nadem, S. Tan, A. Brisson & S. M. Cutting, (2011) Surface Layers of *Clostridium difficile* Endospores. *J Bacteriology*. 193: 6461-6470.

2. Permpoonpattana, P., H. A. Hong, J. Phetcharaburanin, J. M. Huang, J. Cook, N. F. Fairweather & S. M. Cutting, (2011) Immunisation with *Bacillus* spores expressing toxin A peptide repeats protects against infection with toxin A+ B+ strains of *Clostridium difficile*. *Infect Immun*. 79: 2295-2302.

3. Patima Permpoonpattana, H.A. Hong, R. Khaneja, S. M. Cutting. (2012) Evalution of *Bacillus subtilis* strains as probiotics and their potential as a food ingredient . Beneficial Microbes. 3: 127-135.

JOURNAL OF BACTERIOLOGY, Dec. 2011, p. 6461–6470 Vol. 193, No. 23
0021-9193/11/$12.00 doi:10.1128/JB.05182-11

Surface Layers of *Clostridium difficile* Endospores[▽]†

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Received 27 April 2011/Accepted 15 September 2011

Clostridium difficile **is an important human pathogen and one where the primary cause of disease is due to the transmission of spores. We have investigated the proteins found in the outer coat layers of *C. difficile* spores of pathogenic strain 630 (CD630). Five coat proteins, CotA, CotB, CotCB, CotD, and CotE, were shown to be expressed on the outer coat layers of the spore. We demonstrate that purified spores carry catalase, peroxiredoxin, and chitinase activity and that this activity correlates with the predicted functions of three spore coat proteins identified here, CotCB, CotD, and CotE. CotCB and CotD are putative manganese catalases, and CotE is a novel bifunctional protein with peroxiredoxin activity at its amino terminus and chitinase activity at its carboxy terminus. These enzymes could play an important role in coat assembly by polymerizing protein monomers in the coat. CotE, in addition to a role in macromolecular degradation, could play an important role in inflammation, and this may be of direct relevance to the development of the gastrointestinal symptoms that accompany *C. difficile* infection. Although specific enzyme activity has not yet been assigned to the proteins identified here, this work provides the first detailed study of the *C. difficile* spore coat.**

Clostridium difficile is the most common cause of nosocomial antibiotic-associated diarrhea in developed countries (32). Morbidity and mortality rates have been steadily increasing in recent years and probably result from the emergence of more virulent strains of *C. difficile* as well as the changing patterns of antibiotic usage. Recent estimates of the incidence of *C. difficile*-associated diarrhea (CDAD) in the United States suggest that there are as many as 500,000 cases per year, with up to 20,000 mortalities (29). *C. difficile* colonizes the intestinal tracts of infected patients, and antibiotic treatment can promote the overgrowth of this bacterium, which in turn leads to clinical symptoms of disease, from diarrhea to, in more severe cases, pseudomembranous colitis (32).

CDAD is caused by the secretion of two toxins, toxin A (TcdA) and toxin B (TcdB), both monoglucosyltransferases that are cytotoxic, enterotoxic, and proinflammatory (3). CDAD is particularly problematic to treat and to contain because of the ability of this bacterium to form robust endospores (spores) that can persist and be easily transferred from person to person in a hospital environment (9, 37). Currently, the only treatment for CDAD is the use of antibiotics such as vancomycin and metronidazole, and a relapse of CDAD (i.e., diarrhea recurring within 30 days after the first treatment) is a particular challenge in a hospital environment (9, 10). Moreover, evidence has now arisen showing that antibiotic treatment suppresses the diversity of resident intestinal microflora and promotes the growth and proliferation of highly infectious *C. difficile* spores (18). This "supershedder" state ends once antibiotic treatment is terminated, providing an important clue to both the transmission of *C. difficile* infection in humans in a hospital environment and the importance of the spore as the pathogenic agent.

With the advent of genomics and proteomics and by comparison with the extensive data available for unicellular differentiation in *Bacillus subtilis*, some invaluable information is now emerging on *C. difficile* sporulation. *C. difficile* strain QCD-32g58 has been found to contain 18 orthologues of *B. subtilis* spore coat proteins and 3 orthologues of proteins found in the exosporium of spores of *Bacillus anthracis* and *Bacillus cereus* (13). Bioinformatic analyses of the genome of *C. difficile* strain 630 (CD630) (30) coupled with recent studies of the spore proteome (19) have revealed only 18 orthologues of *B. subtilis* spore coat proteins (30). *B. subtilis* coats are comprised of about 70 different proteins, so it is probable that *C. difficile* and, indeed, other clostridial spore formers will have equivalent complexities, which in turn suggests that *C. difficile* spore coat proteins have diversified considerably.

In this work, we have made the first attempt at characterizing the spore coats of CD630. Using coat protein extractions, we have identified five coat (Cot) proteins, three of which, based on bioinformatics analysis, could have enzymatic activity (two catalases and one bifunctional peroxiredoxin-chitinase). We also show that these enzymes may be confined to the outermost coat layer, where they could play a key role in spore coat polymerization and maturation.

MATERIALS AND METHODS

General methods and strains. Methods for the preparation of *Bacillus* spores were described previously (23). *C. difficile* 630 is a pathogenic strain that produces *tcdA*$^+$ *tcdB*$^+$ and was obtained from Neil Fairweather (Imperial College, United Kingdom). CD630 was routinely grown in vegetative culture by growth (10 ml) overnight at 37°C in TGY-vegetative medium (3% tryptic soy broth, 2% glucose, 1% yeast extract, 0.1% L-cysteine) (25). *Streptococcus mutans* GB1 was

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† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 23 September 2011.

obtained from Phan Nghia (Hanoi University, Vietnam); *B. subtilis* strain PY79 is a prototrophic (Spo^+) laboratory strain and a laboratory stock, as was *Bacillus clausii* O/C. *Bacillus licheniformis* strain HU14 was obtained from the *Bacillus* Genetic Stock Center (Columbus, OH).

Sporulation of *C. difficile*. All manipulations were made in an anaerobic incubator (Don Whitley, United Kingdom). A single bacterial colony was grown on BHIS agar (brain heart infusion agar supplemented with 0.1% L-cysteine and 5 mg/ml yeast extract [31]) overnight at 37°C. One fresh single colony from the BHIS plate was inoculated into 10 ml of TGY medium (3% tryptic soy broth, 2% glucose, 1% yeast extract, 0.1% L-cysteine) (25) and incubated at 37°C overnight. One milliliter of TGY culture was then subcultured into SMC broth [90 g peptone, 5 g proteose peptone, 1 g $(NH_4)_2SO_4$, 1.5 g Tris] containing 0.1% L-cysteine (modified from methods reported previously by Wilson et al. [38]), incubated until an optical density at 600 nm (OD_{600}) of 0.4 to 0.7 was reached, and then plated onto SMC agar. After 7 days of incubation at 37°C, the sporulation efficiency was confirmed by phase-contrast microscopy and measurements of heat-resistant CFU, and spore crops were harvested immediately or after overnight incubation at 4°C.

Spore purification. The methods used for spore purification were modified from those described previously by Lawley et al. (19). Spores were washed in water two times and then suspended in phosphate-buffered saline (PBS) containing 125 mM Tris, 200 mM EDTA, 0.3 mg/ml proteinase K (catalog number E00492; Fermentas), and 1% sarcosyl and incubated with gentle shaking at 37°C for 2 h. Spores were centrifuged (8,000 rpm for 10 min), and pellets were resuspended in water and washed a further 10 times. After the final suspension in water, spores were heat treated (60°C for 20 min) to kill any residual cells; aliquots were stored at 4°C until use. To calculate the spore CFU, aliquots were serially diluted in PBS and plated onto BHIS agar supplemented with 0.1% sodium taurocholate (Sigma, United Kingdom). Plates were incubated for 24 to 48 h before CFU were enumerated.

Spore coat extractions. The spore coat extraction procedure was described previously (36), but in brief, 2×10^9 spores were suspended in 100 μl of freshly prepared sodium borate-sodium dodecyl sulfate (SDS)-dithiothreitol (DTT) buffer consisting of sodium borate (0.1 M; pH 10), 0.5% SDS, and 50 mM DTT, and then incubated at 68°C for 75 min with gentle agitation. After centrifugation (8,000 rpm for 15 min), the supernatant was removed, mixed with 4× SDS-PAGE loading buffer, and fractionated by SDS-PAGE. For *B. subtilis* and *B. clausii* spores, coat proteins were extracted by using an SDS-DTT buffer described previously (23).

Peptide fingerprinting. Spore coat proteins were fractionated on 12.5% SDS-PAGE minigels, and bands were excised and digested with trypsin before analysis by matrix-assisted laser desorption ionization (MALDI) mass spectrometry. Digestions and analysis were conducted by the University of Cambridge Protein and Nucleic Acid Chemistry Facility (PNAC) (http://www.bioc.cam.ac.uk/pnac).

Antibody production. pET28b expression vectors that express the complete *cotA*, *cotB*, *cotCB*, and *cotD* open reading frames (ORFs) were constructed by amplifying the respective DNAs by PCR from *C. difficile* 630 chromosomal DNA and ligating them into cleaved pET28b. For *cotE*, we were unable to clone the entire ORF, so a fragment encoding the N-terminal peroxiredoxin domain was cloned instead. Primers used for the construction of pET28b clones are shown in Table S1 in the supplemental material. High levels of expression were obtained upon isopropyl-β-D-thiogalactopyranoside (IPTG) induction and the purification of proteins by the passage of the cell lysate through a HiTrap chelating HP column on a Pharmacia Akta liquid chromatography system. Polyclonal antibodies were raised in mice immunized by the intraperitoneal route with 2 μg of purified recombinant proteins on days 1, 14, and 28. Antispore antibodies were made by treating spores with 2% formalin (2% [vol/vol] formaldehyde in PBS) overnight at 4°C. Spores were washed 5 times with PBS and were used to dose mice (2×10^8 spores/dose) on days 1 and 14.

Confocal microscopy. Spores were labeled with mouse anti-Cot serum (1:1,000 dilution), followed by an anti-mouse IgG-tetramethyl rhodamine isocyanate (TRITC) conjugate. Images were taken by using a Nikon Eclipse fluorescence microscope equipped with a Bio-Rad Radiance 2100 laser scanning system.

TEM. A transmission electron microscopy (TEM) methodology using suspensions of purified CD630 spores (7 days old) was described previously for *B. subtilis* spores (14).

Spore germination. Spores (1×10^8) were suspended in 100 μl of 0%, 3%, and 5% sodium taurocholate in PBS (pH 7.4). Spore suspensions were routinely agitated, and absorbance (A_{580}) readings were determined until no further change in absorbance [OD_{580} (t_n) − OD_{580} (t_0)] could be detected.

Catalase assay. A catalase assay was performed as described previously (1). Spores or vegetative cells (1×10^7 CFU) were pelleted and resuspended in 60 μl of 50 mM potassium phosphate buffer (pH 7.0). H_2O_2 (1.94 ml) was added to the mixture to start the reaction at room temperature (RT). Samples were centrifuged, and the OD_{240} of supernatants was measured immediately.

Peroxiredoxin assay. The peroxiredoxin assay was described previously (11, 20). Spores or vegetative cells (1×10^8) were pelleted by centrifugation and suspended in a reaction buffer that included H_2O_2. Reactions were made at 37°C, and after 15 min, cells were pelleted, and the OD_{340} of supernatants was measured.

Chitinase assay. The chitinase activity was determined by using a presupplied kit (catalog number CS0980; Sigma) using spore or cell suspensions (1×10^8 CFU/assay) in water. 4-Nitrophenyl-*N*-acetyl-β-D-glucosaminide (1 mg/ml) was used as a substrate, and after the reaction was stopped (200 μl of 0.04 g/ml sodium carbonate), the suspension was centrifuged, and the OD_{405} of supernatants was measured. The reaction time was 3 h at 37°C. The assay was also performed on spores that had been pregerminated or following sonication. For germination, 1×10^8 spores were suspended in 100 μl of 0%, 3%, and 5% sodium taurocholate in PBS (pH 7.4) for 30 min at 37°C, after which the chitinase activity was determined. For sonication, 1×10^8 spores were suspended in 100 μl PBS (pH 7.4). The solution was sonicated for 2 or 7 times (3-mm microtip probe and 10% amplitude for 30 s) (S-450D sonicator; Branson), after which the assay was performed.

RESULTS

***C. difficile* spore formation.** Using an empirical approach, we adapted existing methods (38) to generate high levels of spore formation on a solid medium using *C. difficile* strain 630 ($tcdA^+$ $tcdB^+$), which we refer to here as CD630. After 7 days of growth on agar, we routinely obtained >75% sporulation (see Fig. S1 in the supplemental material), with crops consisting of mature, released spores, which were then purified, further providing suspensions devoid of viable vegetative cells. TEM analysis of spores (Fig. 1A and B) revealed a structure common to those produced by the majority of Gram-positive spore formers (13), namely, an inner core surrounded by a primordial germ cell wall (peptidoglycan derived from vegetative cell walls) and a thick cortical layer (loosely cross-linked peptidoglycan specific to the spore) (Fig. 1B). Finally, above the cortex, a thick, more electron-dense layer was present on all spores, this being the spore coat. A closer examination revealed further definition to this layer, including laminations resembling the striated outer coats of *Bacillus* spores (13). In other work, spores of *C. difficile* were reported to carry an exosporium (19, 24), a loose-fitting saclike structure enveloping the mature spore (13). In our studies, we have observed an exosporial layer resembling that described by previous studies, and an example is shown in Fig. 1C. However, we found observations of this structure to be inconsistent. The exosporium was apparent only in samples harvested and processed immediately (Fig. 1C), and for those spores where it was detected, the layer was only partially attached. In contrast, the images shown in Fig. 1A and B were from CD630 spore preps that had been left overnight at 4°C before spore purification.

Identification of *C. difficile* spore coat proteins. Coat proteins were extracted from freshly prepared spores of CD630 grown on solid medium using a sodium borate-SDS-DTT buffer and fractionated by SDS-PAGE (Fig. 2). Since spores were processed immediately, we reasoned that they may carry some residual exosporial material, as shown in Fig. 1C. Eleven protein bands were excised from Coomassie-stained gels and subjected to peptide mass fingerprinting using trypsin digestion and MALDI mass spectrometry. This analysis revealed that a number of protein bands corresponded to truncated breakdown products (Table 1). One high-molecular-mass species of

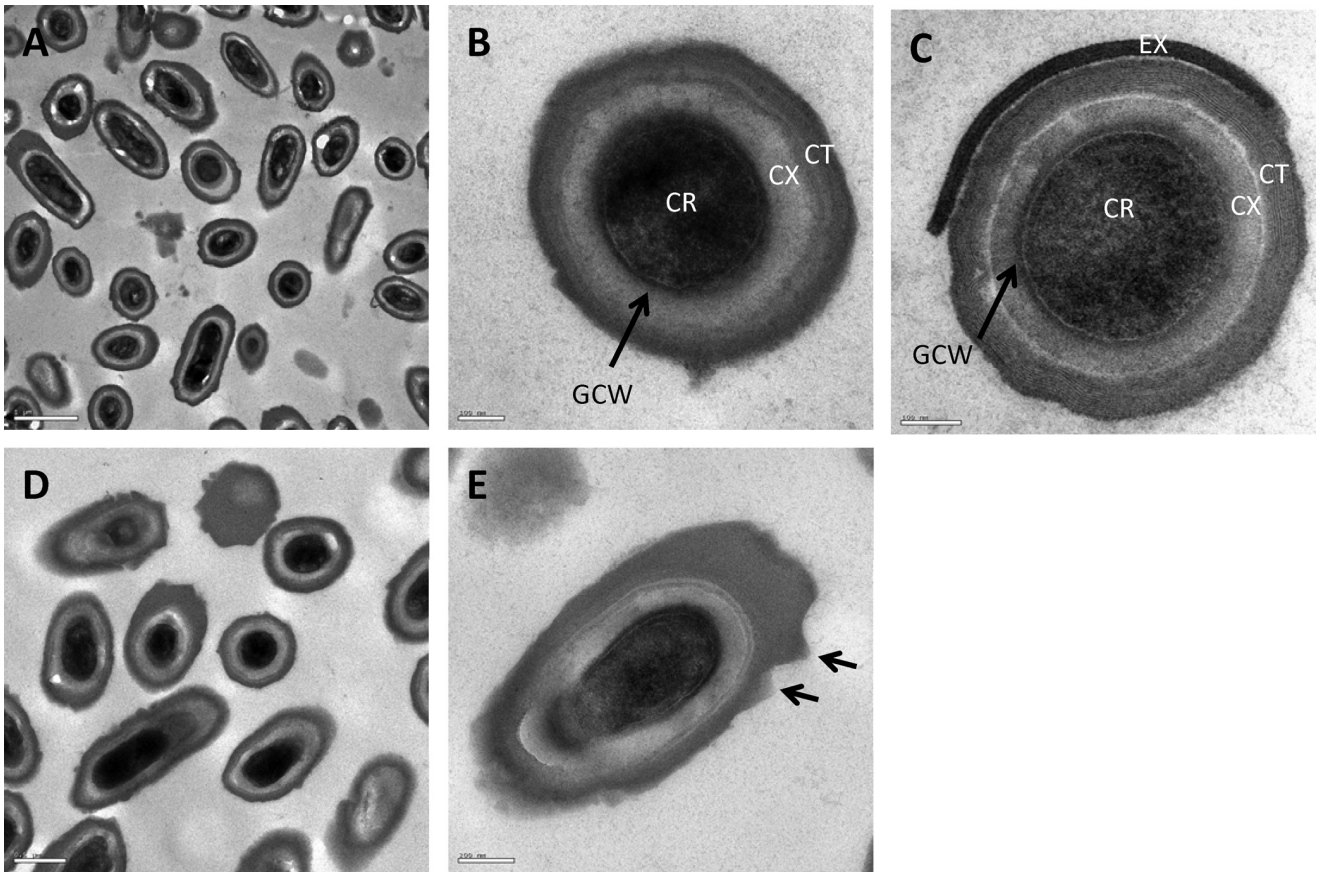

FIG. 1. Ultrastructure of *C. difficile* 630 spores. (A to C) Representative images of CD630 spores after 7 days of incubation on solid medium. Panel B shows the basic structural features found in a mature endospore. CR, core; GCW, germ cell wall; CX, cortex; CT, coats. Panel C shows a spore containing a partially attached exosporium (EX). (D and E) Seven-day-old spores subjected to 10 cycles of sonication. Panel E shows angular projections found to be more abundant in sonicated samples.

118 kDa could not be identified and may be an aggregate. Two further bands were chain E of proteinase K, which was a contaminant derived from the spore purification process. The remaining eight protein species corresponded to five different proteins, which we refer to as CotA, CotB, CotCB, CotD, and CotE, and we refer to their genes as *cotA* to *cotE* (Fig. 3), based on the nomenclature used for *B. subtilis* (13) (orthologues are shown in Fig. S2 in the supplemental material). CotA shared no homology with other proteins in existing databases, but CotB had orthologues in a number of bacilli and clostridia. CotCB and CotD were homologous with both each other (70% conserved residues) and manganese catalases, including the CotJC inner spore coat protein (and putative catalase) found in *B. subtilis* (see Fig. S3 in the supplemental material). As will be discussed below, the 25-kDa protein is most probably encoded by the second cistron of an operon, so we refer to the gene and protein as *cotCB* and CotCB, respectively. CotE, based on its amino acid sequence, corresponded to a novel bifunctional protein with amino-terminal peroxiredoxin (1-Cys peroxiredoxin) and carboxy-terminal manganese chitinase activities (Fig. S4). The predicted molecular mass of this protein was 81 kDa, although the full-length protein was not clearly discernible in our SDS-PAGE fractionations; rather, a 20-kDa truncated species was found. CotE had orthologues in a number of spore formers (Fig. S2). As a single bifunctional protein, no orthologues were found for other bacilli or clostridia, but matches were found with either the peroxiredoxin or chitinase domain carried in CotE. These included a putative peroxiredoxin, YkuU, in *B. subtilis* (BS938810) and a number of putative chitinases from exosporium-containing species, including *Bacillus anthracis*, *B. cereus*, *Bacillus thuringiensis*, *Bacillus clausii*, and *Bacillus halodurans* (Fig. S2).

Immunoanalysis of spore coat proteins. Polyclonal antibodies to recombinant Cot proteins were raised in mice. In the case of CotE, we used the amino-terminal peroxiredoxin domain of CotE to generate antibodies. Using confocal imaging of antibody-labeled *C. difficile* spores (purified after overnight incubation at 4°C), we observed uniform surface decoration using all antisera (Fig. 4), while naive serum gave no labeling (not shown). Since, as mentioned above, these spores lacked an exosporium, our data suggest that each of the five coat proteins must be exposed on the outermost layers of the spore and must be components of the spore coat rather than the exosporium.

These antibodies were used in Western blot analyses to probe spore coat protein extractions (Fig. 5A). CotA, CotB, and CotD were present as single bands of 47, 40, and 23 kDa,

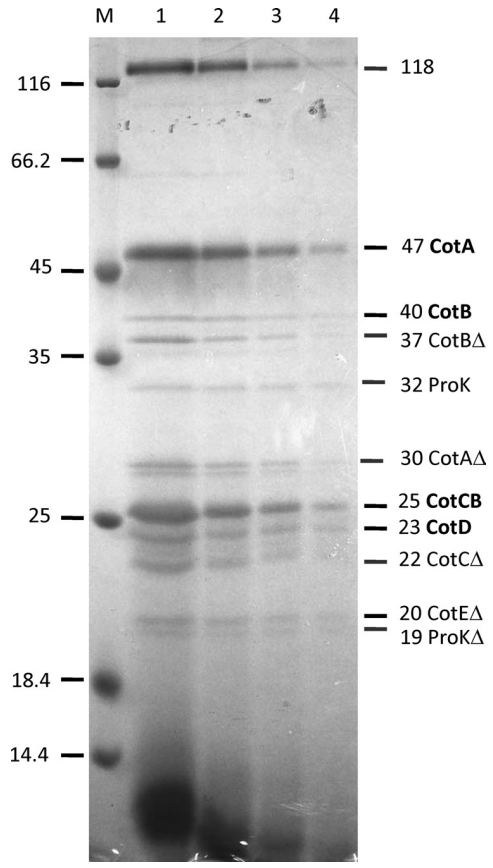

FIG. 2. Proteins extracted from CD630 spores. Proteins were extracted from CD630 spores (7-day-old cultures grown on solid medium) by using a sodium borate-SDS-DTT extraction buffer. Proteins were fractionated by SDS-PAGE (12.5% gel), and samples were loaded as dilutions. Lane 1, no dilution; lane 2, 1/2 dilution; lane 3, 1/4 dilution; lane 4, 1/8 dilution. M, markers. Alongside the gel, the identities of the bands excised and analyzed by mass spectrometry are shown. Partially truncated proteins (Δ) are indicated.

respectively, corresponding to the predicted molecular masses of each of these proteins. CotE antisera identified two strongly reacting bands of 81 and 40 kDa, but the 20-kDa species (identified as a peptide fragment in Fig. 2) was not observed.

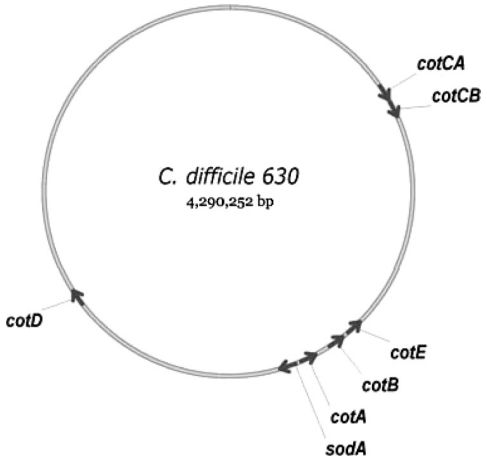

FIG. 3. Spore coat genes. The chromosomal positions of genes referred to in this work are shown.

The most likely explanation is that this 20-kDa species is a C-terminal fragment that is not recognized by the polyclonal CotE antibodies that were raised against the N terminus of CotE. For CotCB, when probed with anti-CotCB antibody, we could sometimes discern two bands of 25 and 23 kDa, although this is not apparent in Fig. 5A. Since CotCB and CotD were homologous, we wondered whether these proteins shared related epitopes. Using recombinant proteins (recombinant CotCB [rCotCB] and rCotD), we probed each one with anti-CotCB and anti-CotD sera. As shown in Fig. 5B, CotD was recognized by both anti-CotCB and anti-CotD sera. On the other hand, CotCB was detectable by using anti-CotCB antibodies but only very weakly by using anti-CotD antibody.

Using antisera raised against formalin-inactivated CD630 spores, we probed spore coat proteins extracted from CD630, *B. subtilis*, and *B. clausii* (Fig. 5C). *C. difficile* serum showed no cross-reaction against either *B. subtilis* or *B. clausii* spore coat proteins, the latter of which carries an exosporium (7, 13). Similarly, antiserum raised against formalin-inactivated *B. subtilis* spores showed no reaction against CD630 spores but some cross-reaction to *B. clausii* (Fig. 5D). These results support results from previously reported bioinformatic analyses that

TABLE 1. SDS-PAGE and MALDI peptide fingerprint analysis of *C. difficile* 630 spore coat proteins

Fragment molecular mass (kDa)	Protein description[a]	Coding sequence[b]	Predicted molecular mass (kDa)	Assigned gene
118	ND			
47	Hypothetical protein	CD1613	34	*cotA*
40	Hypothetical protein	CD1511	35	*cotB*
37	Hypothetical protein	CD1511	35	*cotB*
32	Proteinase K (contaminant from purification steps)			
30	Hypothetical protein	CD1613	34	*cotA*
25	Putative spore coat protein; manganese catalase; similar to CotJC of *B. subtilis*	CD0598	21	*cotCB*
23	Putative spore coat protein; manganese catalase; similar to CotJC of *B. subtilis*	CD2401	21	*cotD*
22	Putative spore coat protein; manganese catalase; similar to CotJC of *B. subtilis*	CD0598	21	*cotCB*
20	Putative bifunctional protein, peroxiredoxin/chitinase	CD1433	81	*cotE*
19	Proteinase K (contaminant from purification steps)			

[a] Based on peptide mass fingerprinting of tryptic digestions. ND, no determination.
[b] Coding sequences are described in reference 30.

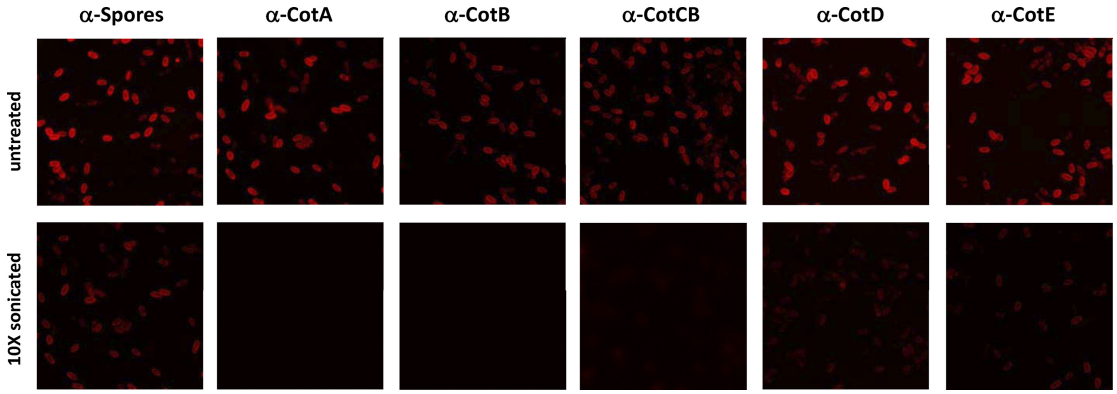

FIG. 4. Surface display of CotA, CotB, CotC, CotD, and CotE using confocal imaging of suspensions of CD630 spores (7-day-old cultures grown on solid medium) labeled with mouse serum (1:1,000 dilution) raised against each of the five Cot proteins. CD630 spores labeled with preimmune serum served as a control and showed no labeling (not shown). Spores labeled with antispore serum are also shown ("Spores"). An anti-mouse IgG-TRITC conjugate was used for secondary labeling. Images were taken by using a Nikon Eclipse fluorescence microscope equipped with a Bio-Rad Radiance 2100 laser scanning system (image size, 37 by 37 μm). The top row shows the labeling of untreated spores, and the bottom row shows the labeling of spores that had been sonicated 10 times.

have shown little conservation between *C. difficile* and *Bacillus* spores (19, 30).

Effect of sonication on spore coat proteins. An exosporium has been documented for *C. difficile* spores prepared in liquid medium (19, 24). The exosporium remains the least-understood component of the bacterial endospore (13), and harsh physical methods, such as sonication and shear stress, have been reported to remove the exosporial layer (27). Although the majority of spores present in our preparations of CD630 did not appear to carry a recognizable exosporium, we subjected CD630 spores to repeated cycles of sonication. Spore pellets and supernatants were then separated. Pellets were then extracted with Na borate-SDS-DTT buffer, and extracts were run on 12.5% SDS-PAGE gels together with supernatant fractions (Fig. 6A). Our results showed that as few as two cycles of sonication were sufficient to remove almost the entire

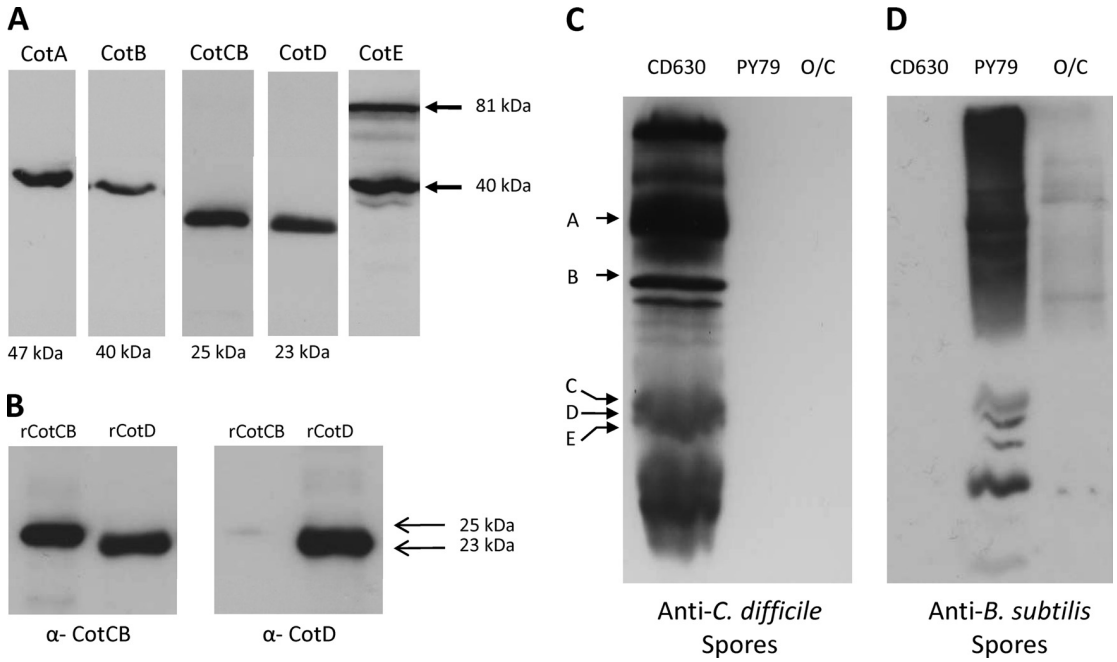

FIG. 5. Immunoanalysis of spore coats. (A) Spore coats of CD630 were extracted, and separate lanes were probed with polyclonal (mouse) antibodies to CotA to CotE. Molecular masses of the relevant bands are shown. For CotE, two principal bands of 81 and 40 kDa were found. Serum from unimmunized mice did not react with *C. difficile* spore coat proteins. (B) Purified recombinant CotC and CotD proteins (2 μg) were fractionated on SDS-PAGE gels and probed with either CotC or CotD antibodies at 1/1,500 and 1/3,000 dilutions, respectively. Positions of the CotC (25 kDa) and CotD (23 kDa) bands are indicated. (C) Coat proteins extracted from spores of CD630, *B. subtilis* PY79, and *B. clausii* O/C were fractionated and probed with antiserum to formalin-inactivated CD630 spores. Positions of CotA to CotE are shown. (D) Same as panel C except that proteins were probed with antiserum to formalin-inactivated *B. subtilis* PY79 spores.

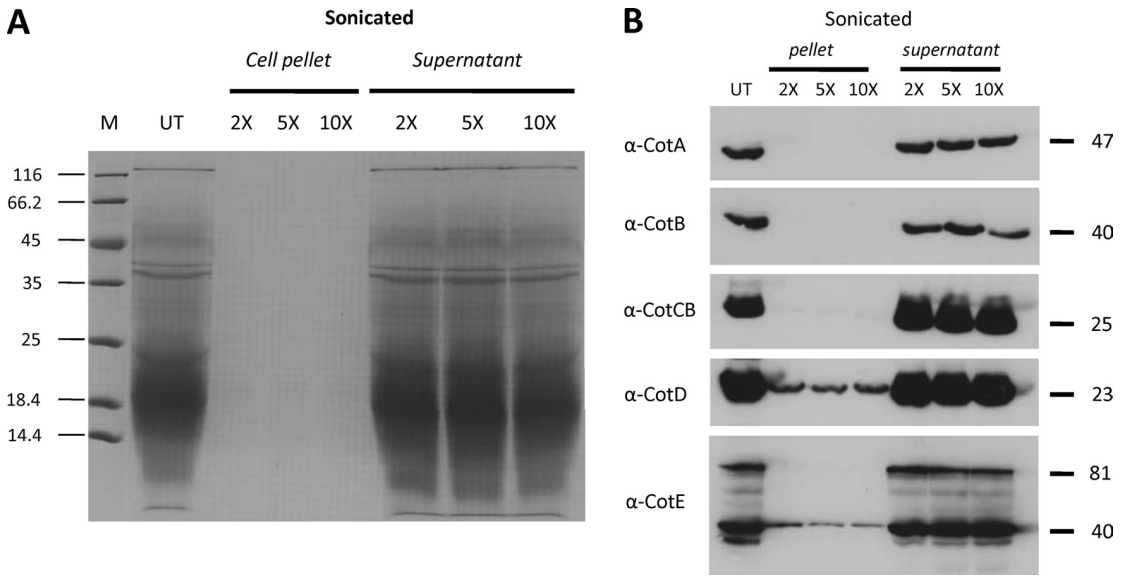

FIG. 6. Removal of Cot proteins using sonication. (A) *C. difficile* spores were sonicated (30-s cycles) 2 times, 5 times, and 10 times, and pellets and supernatants were separated. Pellets were treated with Na borate-SDS-DTT extraction buffer, and extracted proteins were mixed with SDS-PAGE loading dye (4×) and fractionated by SDS-PAGE (12.5%). Supernatants were mixed with loading dye and run directly. (B) Spores were sonicated, and pellet and supernatant fractions (from panel A) were probed with antiserum to each of the five Cot proteins. Molecular masses of Cot proteins are shown. UT, untreated spores; M, markers.

component of sodium borate-solubilized proteins, all of which were found in the supernatant fraction. Examination of the sonicated spores by phase-contrast microscopy revealed that phase-bright spores remained intact, and analyses of CFU before and after sonication demonstrated no change in viability. Analysis by TEM revealed no clear-cut differences between sonicated spores (Fig. 1C) and unsonicated spores (Fig. 1D). Although precise quantification was not possible, we observed that in sonicated samples, many spores carried angular projections on the surface layers (shown in Fig. 1D). These crystalline-like structures might indicate an underlying layer of coat resulting from sonication and the removal of one or more layers of coat material.

Following sonication, the spore pellet and supernatant fractions were probed with antiserum to CotA to CotE (Fig. 6B). CotA, CotB, and CotCB were not detectable in the spore pellets and were found only in the supernatant fractions. CotD and CotE, although not visibly apparent in Coomassie-stained gels, were present in both the spore pellet and supernatant fractions by immunoanalysis. If CotD was still present in the spore coat fraction, then why was it not detected by use of anti-CotCB serum, since CotCB and CotD share related epitopes? We reason that although the recombinant proteins, at high concentrations, could be detected, this does not reflect the composition and abundance of CotCB and CotD in the spore coat but rather the different binding strengths and specificities of the respective antibodies. In the case of CotE, only the 40-kDa CotE fragment was found in the spore pellet fraction. We used confocal imaging to examine antibody-labeled sonicated spores (Fig. 4). This analysis revealed that in each case, following sonication, surface labeling was massively reduced. Labeling correlated well with the Western blotting data (Fig. 6B), with CotA, CotB, and CotCB showing almost no labeling and CotD and CotE weak labeling, suggesting that some CotD and CotE remained in the sonicated spores. These results then showed that all five Cot proteins are located on the spore surface and can be liberated either by sonication or by use of a sodium borate-SDS-DTT extraction buffer.

Enzymatic properties of spores. Based on the amino acid sequences of CotC, CotD, and CotE and their surface location, we predict that spores carry enzymatic activity, either latent or active. Accordingly, we conducted a number of assays to measure catalase, peroxiredoxin, and chitinase activities. In each case, we used suspensions of purified spores that had been checked microscopically to confirm greater than 99.99% free spores. Catalase activity was measured (Fig. 7A) by a photometric assay of H_2O_2 breakdown using suspensions of CD630 spores and vegetative cells and, as useful comparators, *B. subtilis* spores and vegetative cells. CD630 spores had noticeable catalase activity, while vegetative cells were completely negative. In comparison, *B. subtilis* spores were catalase negative, and vegetative cells were catalase positive. We next focused on CD630 spores, and we heated the spores at different temperatures for 20 min, allowed the spore suspension to return to an ambient temperature, and then conducted the catalase assay. We found that heating at 50°C had no effect on enzyme activity but that enzyme activity was reduced by 40% at 60°C and by 60% at 70°C, showing that although spores were heat stable, the enzymatic activity was not.

The maturity of spores may affect spore-associated enzyme activity, since in other spore formers, notably *B. subtilis*, the spore coat physically changes over time, with the spore coat shrinking and forming distinctive surface corrugations (6). Spore suspensions were assessed for catalase activity at 1-day intervals postpurification, and we observed a marked decline in enzymatic activity after just 1 day of maturation (Fig. 7B).

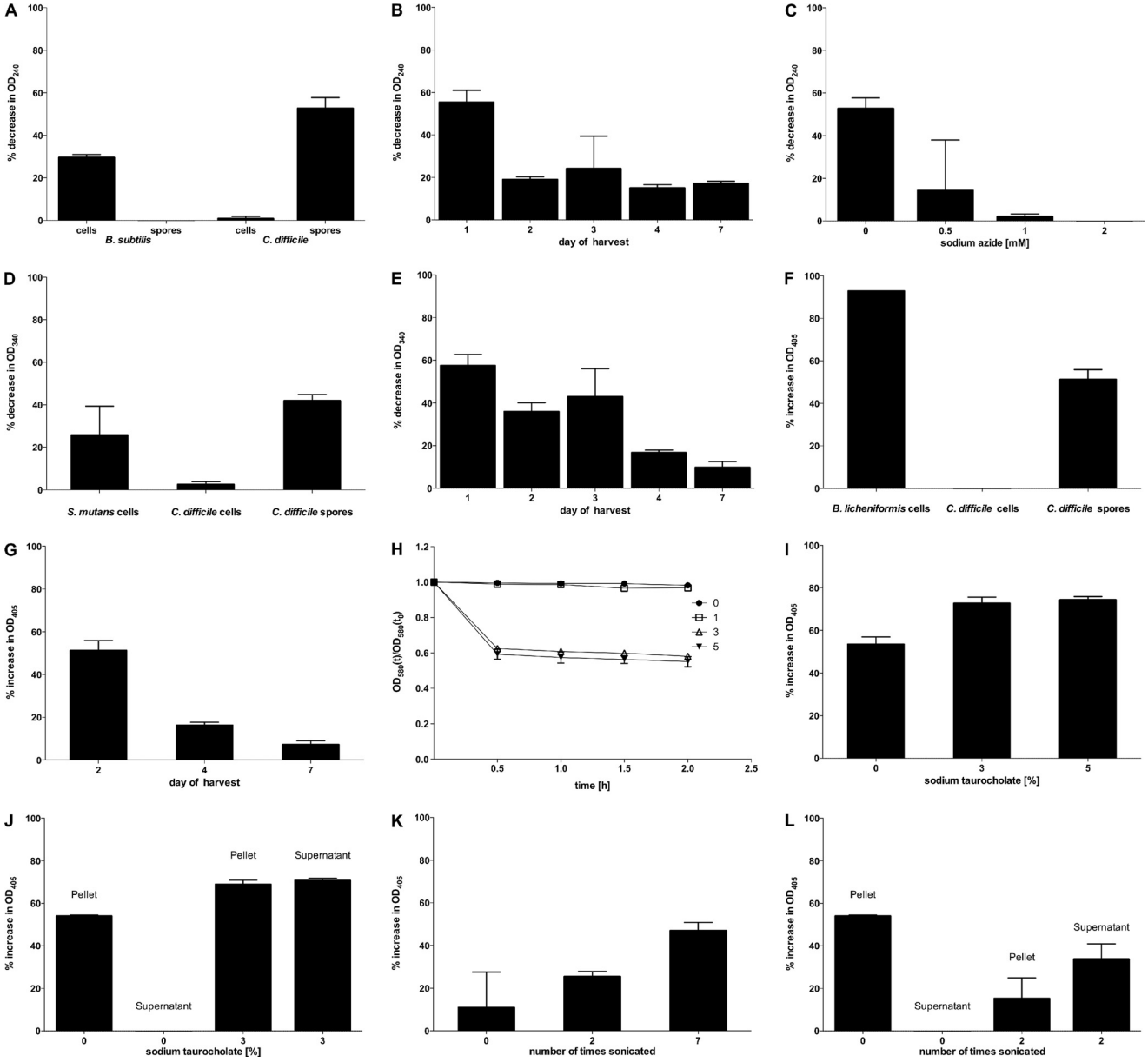

FIG. 7. Enzymatic activities of *C. difficile* 630 spores. (A) Catalase activity in CD630 and *B. subtilis* spores or vegetative cells. (B) Catalase activities of CD630 spores at different stages of maturation on solid agar. (C) Inhibition of catalase activity by sodium azide. (D) Peroxiredoxin activity of CD630 spores and vegetative cells and *S. mutans* cells. (E) Peroxiredoxin activity of CD630 spores at different stages of maturation on solid agar. (F) chitinase activity in CD630 spores and vegetative cells of CD630 and *B. licheniformis*. (G) Chitinase activity of CD630 spores at different stages of maturation on solid agar. (H) Germination of CD630 spores in sodium taurocholate solutions. (I) Chitinase activity in response to spore germination using 3% or 5% sodium taurocholate solutions. (J) Chitinase activity obtained in cell pellet and supernatant fractions of CD630 spores following incubation with 0% and 3% sodium taurocholate for 30 min. (K) Chitinase activity of CD630 spores in response to sonication (30-s cycles). (L) Chitinase activity obtained in cell pellet and supernatant fractions of CD630 spores following sonication (30-s cycles).

Since the substrate for catalase activity, H_2O_2, was the same as that used in the peroxiredoxin assay, we measured the effect of sodium azide on catalase activity, since catalase is sensitive to sodium azide, while peroxiredoxin activity is not (21). Using increasing concentrations of sodium azide, the catalase activity of CD630 spores was inhibited, demonstrating that we were measuring spore-associated catalase and not that of peroxiredoxin (Fig. 7C).

Peroxiredoxin activity was assessed by using CD630 spores and vegetative cells together with a suspension of *S. mutans* cells that are known to produce this enzyme (26). CD630 vegetative cells carried barely detectable levels of activity, while spores clearly were positive and had levels of activity equivalent to that of *S. mutans* (Fig. 7D). Peroxiredoxin activity exhibited a marked decline (60%) when spores were heated at above 60°C, and at 80°C, activity was abolished. The effects of spore maturity were also assessed, with activity gradually declining over time, with 7-day-old spores losing 48% of

the activity exhibited by 1-day-old preparations (Fig. 7E). The peroxiredoxin activity of 1-day-old spores was measured in the presence of 0.5, 1, and 2 mM sodium azide, and no decline in activity was observed, indicating that the activity was that of peroxiredoxin and was not due to catalase.

The chitinase activities of CD630 spores and vegetative cells were assessed by using vegetative cells of chitinase-producing cells of *B. licheniformis* as a positive control (34). CD630 cells had no activity, but spores carried activity equivalent to that of *B. licheniformis* (Fig. 7F). As with catalase and peroxiredoxin activities, the age of the spores had a marked effect on spore-associated activity, with 7-day-old spores carrying 20% of the activity found for 2-day-old spores (Fig. 7G). Chitinase, as an enzyme involved in macromolecular degradation, would serve no obvious benefit to a dormant spore, but this would not be the case for a germinating spore. The release and subsequent activation of a latent enzyme could provide nutrients to an outgrowing cell, so we asked whether chitinase activity might be enhanced by the disruption of the spore coat and exosporium. We measured activity first following spore germination, which would rupture the spore coat, and second following the sonication of spores, which, as shown above, would remove the surface coat layers. The spore germination of 1-day-old spores was evaluated by using different solutions of sodium taurocholate as the germinant (38). By measuring the change in the OD_{580} attributed to the phase darkening of spores, we identified 3% and 5% sodium taurocholate as being optimal for spore germination, with a 38% reduction (at 3%) to a 50% reduction (at 5%) in the OD_{580} in 30 min (Fig. 7H). Next, 30 min following germination using 3% and 5% sodium taurocholate, we measured chitinase activity. We found that compared to untreated spores, the germinant produced a marked increase (21%) in chitinase activity with both 3% and 5% solutions (Fig. 7I). Using commercially obtained chitinase (catalog number C6242; Sigma) of the same type (family 18) as that predicted for CotE, we determined that sodium taurocholate had no effect on enzyme activity (data not shown). We also found that the chitinase activity was released into the medium following spore germination. Using 3% germinant, activity was clearly detectable in the supernatant fraction following centrifugation, in contrast to spore samples that had not been germinated (Fig. 7J). In support of this finding, we subjected suspensions of 7-day-old spores to increasing cycles of sonication. Seven 3-s bursts of sonication yielded more chitinase activity than two bursts, which in turn yielded more activity than untreated spores (Fig. 7K). As was the case during spore germination, sonication was sufficient to release chitinase activity into the medium (Fig. 7L). These results demonstrate first that catalase, peroxiredoxin, and chitinase activities are associated with spores and second that activity declines as spores mature, demonstrating that the enzyme either is not required or is rendered latent (dormant). Finally, for chitinase, activity is enhanced if the spore coat is disrupted.

DISCUSSION

This study has provided an initial examination of the spore coats of *C. difficile* spores with the identification of five proteins that are exposed on the outermost layer of the spore coat. We have named these proteins Cot and their genes *cot*, since our study clearly shows that they are located in the outermost layers of the spore coat. However, it should be noted that with two distinct structures, the coat and exosporium, found on the outermost layers of the spore, the assignment of names should be approached with caution. With the exception of an unidentified 118-kDa species, these five proteins represented the major proteins extractable by using the procedures followed here. We believe, however, that the coats of *C. difficile* are far more complex and that these five proteins represent just a fraction of the total protein content of the spore coat. We base this assumption on existing bioinformatic analyses and the extraction studies performed here. In *B. subtilis*, more than 70 proteins are thought to be found in the coat layers (13), and 18 orthologues have been identified in *C. difficile* (13, 19). Using anti-CD630 serum, no cross-reaction was found with *B. subtilis* spore coat proteins, suggesting that the functional composition and organization of the coat may be very different in *C. difficile*. Interestingly, recent spore proteome studies of CD630 (19) identified five potential spore coat proteins, which included CotCB and CotD but not CotA or CotB (CotE, as a chitinase, was identified but not as a spore coat bifunctional protein). Regarding the extraction procedures, our method was based on the use of sodium borate, SDS, and DTT, used previously for *Clostridium perfringens* (36) and shown here to efficiently extract five proteins. However, the presence of a number of truncated protein species suggests that this method may be overly harsh (it should be noted that the inclusion of a proteinase K treatment step might contribute to this observed partial degradation of some proteins). It is also possible that these products arise from cleavage reactions occurring during spore maturation. SDS-PAGE analysis revealed that the sonication of spores was able to remove all five Cot proteins recovered by extraction with buffer. Interestingly, though, residual spores, which were still viable, released no additional protein when extracted with borate-SDS-DTT buffer. Sonicated spores, when examined by TEM, showed that the spore coat layers were still essentially intact, suggesting that sonication removes one component (possibly one or more layers of coat) of the spore coat and that the remaining underlying coat is impervious to further extraction or sonication. We believe that the deciphering of the inner layers of the spore coat will require the development of new extraction procedures. One additional structure of the spore is the exosporium, although note that this should not be considered part of the spore coat *per se*. We have shown here that *C. difficile* spores do carry an exosporium, but at best, this is loosely attached to the spore, and it is possible that the stability of the exosporium is linked to the conditions required to prepare (e.g., solid versus liquid medium) and/or store spores. In comparison, the exosporium of *B. anthracis* spores is reasonably well characterized, yet there are conflicting reports regarding how stable this structure is, with recent studies suggesting that the exosporium is not easily removed by either sonication or shear stress (35). If the *C. difficile* exosporium is particularly fragile, then what, if any, is the biological significance? Since our initial extraction and identification of proteins were made with spores carrying some exosporial material, we believe that one or more exosporial proteins may have been recovered and perhaps were present in low abundance in the protein extractions shown in Fig. 2. One candidate could be the collagen-rich glycoprotein BclA1

(CD0332), which has orthologues in a number of other spore formers, including *B. anthracis*, where it forms filaments that are attached to the exosporium and facilitates interactions with host cells, including enhancing spore uptake by macrophages (2). Interestingly, BclA is extracted from exosporium-containing *Bacillus* spores as a high-molecular-mass species, and its identification using conventional proteomic tools is problematic (5, 33). It will therefore be of interest to determine whether the 118-kDa species is in fact BclA.

Some of the most interesting findings of this work are the enzymatic properties of the spores and the identification of three enzymatic coat proteins (CotCB, CotD, and CotE) that most probably reside in the exosporium. Although absolute confirmation will require the inactivation of the chromosomal genes and, preferably, evidence of the enzyme activity of the purified proteins, this assumption is supported by several lines of evidence. First, vegetative cells were shown to exhibit no enzyme activity, so this is unlikely to arise from any contaminating cells. Second, a previous analysis of the spore proteome (19) revealed no additional genes that could encode these enzyme activities, although it must be emphasized that the spore proteome is incomplete.

What, then, are the functions of these putative spore-associated enzymes? The catalase (CotCB and CotD) and peroxiredoxin (CotE) activities are potential antioxidants, and at first glance, all three would reduce the cellular toxicity of H_2O_2 by conversion to oxygen and water. In the case of *C. difficile*, which is a strict anaerobe, the presence of oxygen would in turn be harmful to the cell. Since the cell is irreversibly committed to dormancy, it is conceivable that this is not actually harmful and that *C. difficile* spores can be maintained in an oxic environment. Presumably, though, there is a need to remove H_2O_2. Previous studies of *B. subtilis* sporulation showed that H_2O_2 may play a key role in spore coat synthesis and could serve as a substrate for the oxidative cross-linking of spore coat monomers (12). Here, the enzyme superoxide dismutase (SodA) is essential to the cross-linking of tyrosine-rich spore coat proteins, and in CD630, a manganese-dependent SodA orthologue has been identified in the spore proteome (CD1631). CotE, as a 1-Cys-peroxiredoxin would be expected to have the same enzymatic activity as that of a peroxidase and could participate in the cross-linking of tyrosine-rich spore coat proteins. None of the other coat proteins identified in this work are tyrosine rich, but an examination of the *C. difficile* genome has revealed at least one gene (CD0597) that would encode a tyrosine-rich protein (10.34% tyrosines). This protein is homologous to CotJB of *B. subtilis*, and in *C. difficile*, its ORF lies immediately upstream of *cotC*, which in turn encodes an orthologue of *B. subtilis* CotJC. The ORFs are separated by 61 bp and probably lie within the same operon, so we propose to name CD0597 *cotCA* and the downstream cistron *cotCB* (Fig. 2B).

For chitinase activity, the presence of this enzyme in the spore coat is intriguing, since it would be expected to be involved in the breakdown of fungi and other biological matter whether in the soil or in the intestine. However, spores are dormant, so we speculate that chitinase activity may be released (or activated) during spore germination, enabling a potential source of nutrients as the *C. difficile* cell emerges from its coats. We have evidence to support this: the chitinase activity decreased as spores matured but increased during both spore germination and following sonication, with both of these being events that would rupture the spore coat layers. Interestingly, CotE (the putative chitinase) was detectable in the supernatant fraction as either a full-length species (81 kDa) or a single 40-kDa species following sonication, and we wonder whether the smaller species is actually the active chitinase enzyme. Another interesting aspect of CotE, based solely on its sequence prediction, is its bifunctionality and its characterization as one of a growing number of "moonlighting proteins" (15) that carry multiple functions, including a mammalian protein, 1-Cys-peroxiredoxin, that carries peroxidase and phospholipase activities (4). There is possibly a more important consequence of a chitinase and a peroxiredoxin displayed on the surface of *C. difficile* spores that should not be overlooked. This relates to the potential link between peroxiredoxins, chitinases, and inflammation. Peroxiredoxin 1 (a 2-Cys-peroxiredoxin), secreted from tumor cells (22), was shown previously to induce proinflammatory cytokines in macrophages via interactions with Toll-like receptor 4 and to promote chronic inflammation, which could support tumor growth (28). Regarding chitinases, it is now clear that some inflammatory conditions of the gastrointestinal (GI) tract (inflammatory bowel disease [IBD] and ulcerative colitis [UC]) lead to the induction of host cell chitinases by triggering the increased uptake of intracellular bacteria by colonic cells (16, 17) and in potentiating the development of epithelial tumorigenesis (8). Considering that some symptoms of CDAD resemble those of both IBD and UC, *C. difficile* chitinase may play a direct role in infection and not simply in macromolecular degradation.

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INFECTION AND IMMUNITY, June 2011, p. 2295–2302 Vol. 79, No. 6
0019-9567/11/$12.00 doi:10.1128/IAI.00130-11

Immunization with *Bacillus* Spores Expressing Toxin A Peptide Repeats Protects against Infection with *Clostridium difficile* Strains Producing Toxins A and B[▽]†

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Received 8 February 2011/Returned for modification 2 March 2011/Accepted 30 March 2011

***Clostridium difficile* is a leading cause of nosocomial infection in the developed world. Two toxins, A and B, produced by most strains of *C. difficile* are implicated as virulence factors, yet only recently has the requirement of these for infection been investigated by genetic manipulation. Current vaccine strategies are focused mostly on parenteral delivery of toxoids. In this work, we have used bacterial spores (*Bacillus subtilis*) as a delivery vehicle to evaluate the carboxy-terminal repeat domains of toxins A and B as protective antigens. Our findings are important and show that oral immunization of the repeat domain of toxin A is sufficient to confer protection in a hamster model of infection designed to closely mimic the human course of infection. Importantly, neutralizing antibodies to the toxin A repeat domain were shown to be cross-reactive with the analogous domain of toxin B and, being of high avidity, provided protection against challenge with a *C. difficile* strain producing toxins A and B (A^+B^+). Thus, although many strains produce both toxins, antibodies to only toxin A can mediate protection. Animals vaccinated with recombinant spores were fully able to survive reinfection, a property that is particularly important for a disease with which patients are prone to relapse. We show that mucosal immunization, not parenteral delivery, is required to generate secretory IgA and that production of these neutralizing polymeric antibodies correlates with protection. This work demonstrates that an effective vaccine against *C. difficile* can be designed around two attributes, mucosal delivery and the repeat domain of toxin A.**

Clostridium difficile is the most common cause of nosocomial antibiotic-associated diarrhea in developed countries. Antibiotic therapy and disruption of the normal gastrointestinal (GI) microflora are the primary causes of *C. difficile*-associated disease (CDAD), and the presence of one or both of these factors is a prerequisite for colonization of the gut by this Gram-positive bacterium. Morbidity and mortality rates have been steadily increasing in recent years and probably result from the emergence of more virulent strains of *C. difficile* as well as the changing patterns of antibiotic usage. Recent estimates of CDAD in the United States suggest as many as 500,000 cases per year, with up to 20,000 deaths (32). CDAD is caused by the secretion of two toxins, toxin A (TcdA) and toxin B (TcdB), both of which are monoglucosyltransferases that are cytotoxic, enterotoxic, and proinflammatory (5). CDAD is particularly problematic to treat and contain because of the ability of the bacterium to form robust endospores that can persist and be easily transferred in a hospital environment. Currently, the only treatment for CDAD is the use of antibiotics such as vancomycin and metronidazole, possibly followed by surgery if the disease is serious and refractory to antimicrobial treatments. Recurrence of CDAD (i.e., diarrhea recurring within 30 days after the first treatment) is a particular challenge for which there is no standard, uniformly effective treatment.

Although *C. difficile* can naturally cause disease without toxin A, most clinically isolated *C. difficile* strains produce both toxin A and toxin B (A^+B^+) (28). Therefore, an effective vaccine to CDAD should target the two principal virulence factors, toxin A and toxin B, since high titers of antibodies against these toxins correlate well with protection in both hamsters and humans (1, 21, 26). Recent studies have shown that both toxins are important for disease and that recombinant, isogenic *C. difficile* strains that are A^-B^+ or A^+B^- are able to cause disease in the hamster model of infection (23). This work seemingly contradicts an earlier study suggesting that only toxin B is responsible for virulence (30) yet is supported by numerous other studies implicating both toxin A and toxin B in infection (7, 20, 29, 42). Both of the *tcdA* and *tcdB* genes, which encode toxin A and toxin B, respectively, carry limited identity at their C termini, where each carries an elaborate array of repeated domains (40). The C-terminal domain of *tcdA* has been shown to be involved in initial binding of the toxin to sensitive cells prior to its translocation across the endosomal membrane (17). Previous studies indicate that these repetitive domains may be suitable as antigens against CDAD. Some examples are, first, that toxin A cell binding repeats, and a monoclonal antibody (MAb) directed against them, prevented cytotoxicity (34). Second, a defined segment of repeats known as 14CDTA expressed in a recombinant *Salmonella* vaccine elicited local and systemic immunity and toxin A-neutralizing activity (44). Finally, human monoclonal antibodies directed

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† Supplemental material for this article may be found at http://iai.asm.org/.

▽ Published ahead of print on 11 April 2011.

against toxins A and B prevent *C. difficile*-induced mortality in hamsters (2) and reduced recurrence in humans (27).

The colon is the primary site of residency of germinated *C. difficile* spores, and luminal epithelial cells are targeted by the C-terminal regions of toxins A and B. High-avidity binding facilitates the subsequent internalization of the toxins via receptor-mediated endocytosis in clathrin-coated pits (41). Antibodies to toxin A have been shown to confer protection against *C. difficile* A^+B^+ strains, whether delivered mucosally (21) or parenterally (2, 20), although levels of protection are more complete if antibodies to both toxins are used. Such passive-immunization studies show that antibodies are the key effector molecule, and in the GI tract, polymeric secretory IgA (sIgA) may interfere with toxin binding. Despite this, current vaccination strategies are based mostly on parenteral delivery and inducing IgG, whose mechanistic action is far from clear (9). Recombinant bacterial vaccines expressing the toxin A binding domain have been shown to induce both mucosal-sIgA- and serum IgG-neutralizing antibodies following oral administration (43, 44), which prompted us to consider *Bacillus subtilis* spores as a delivery vehicle for *C. difficile* antigens.

Recombinant, heat-stable spores of *B. subtilis* have been used for mucosal delivery of heterologous antigens. In experiments using spores expressing antigens on their surface coats, they have been shown to protect mice immunized against tetanus toxin from *Clostridium tetani* (8) and *Clostridium perfringens* alpha toxin (13). In both cases, significant levels of local immunity (sIgA) were induced. Interestingly, spores appear to possess natural adjuvant properties, and coupled with their heat stability and existing use as probiotics (14), they are attractive vehicles with which to develop a vaccine to CDAD.

In the present study, *B. subtilis* spores expressing the cell-binding domains of toxin A and toxin B were evaluated for their ability to elicit neutralizing antibodies. Our findings show that antibodies to the toxin A domain are cross-reactive to the toxin B domain and can protect hamsters from challenge with spores of a toxigenic strain of *C. difficile*. Our work shows that oral immunization and the production of local immunity are the key attributes required for vaccination against CDAD.

MATERIALS AND METHODS

General methods. Methods for work with *B. subtilis* are described elsewhere (11). *B. subtilis* strain PY79 is a standard, prototrophic laboratory strain. *C. difficile* spores of strain 630 (*tcdA*$^+$ *tcdB*$^+$; lab stock) were prepared using an anaerobic incubator (Don Whitley, United Kingdom) for all manipulations as follows. A single colony was grown on BHIS (brain heart infusion supplemented with 0.1% L-cysteine and 5 mg/ml yeast extract) agar overnight at 37°C. One fresh single colony from the BHIS plate was inoculated in 10 ml of TGY medium (3% tryptic soy broth, 2% glucose, 1% yeast extract, 0.1% L-cysteine) and incubated at 37°C overnight. One milliliter of TGY culture was then subcultured into SMC broth [90 g peptone, 5 g proteose peptone, 1 g $(NH_4)_2SO_4$, 1.5 g Tris, 0.1% L-cysteine], incubated overnight, and then plated onto SMC agar and incubated for 7 days. Sporulation was confirmed by phase-contrast microscopy, and spore crops were harvested and purified as described elsewhere (25).

Recombinant *B. subtilis* vaccine strains. Detailed strain constructions are provided in the text in the supplemental material. *B. subtilis* merodiploid strains contained chimeric genes of *cotB* and/or *cotC* fused at their 3′ ends to the C-terminal domains of toxin A (A26-39; codons for Ser_{2388} to Pro_{2706} [6]) or toxin B (B15-24; codons for Glu_{2137} to Glu_{2366} [3]). The entire *cotC* gene was used for fusion to the toxin domains, while for *cotB*, a 3′ deletion was used so that the encoded CotB segment was 33 kDa. Genes were integrated into the chromosome by a stable double-crossover recombination and were placed in *trans* into the indigenous *cotB* or *cotC* gene.

PP059 (*thrC*::*cotB*-A26-39) encodes a fusion of A26-39 to the C terminus of CotB (predicted molecular mass, 69 kDa). The chimeric gene was carried at the *thrC* locus of *B. subtilis*.

PP052 (*amyE*::*cotC*-A26-39) encodes a fusion of A26-39 to the C terminus of the CotC (predicted molecular mass, 49 kDa). The chimeric gene was carried at the *amyE* locus of *B. subtilis*.

PP108 (*thrC*::*cotB*-A26-39 *amyE*::*cotC*-A26-39) was created by transforming competent cells of PP052 with chromosomal DNA of PP059, with selection for Erm^r, conferred by the *thrC*::*cotB*-A26-39 cassette.

PP132 (*thrC*::*cotB*-B15-24) encodes a fusion of B15-24 to the C terminus of CotB (predicted molecular mass, 60 kDa).

PP142 (*thrC*::*cotB*-B15-24 *amyE*::*cotC*-A26-39) was created by transforming competent cells of PP052 with chromosomal DNA of PP132 (*thrC*::*cotB*-B15-24), with selection for Erm^r, conferred by the *thrC*::*cotB*-B15-24 cassette.

Recombinant proteins. Recombinant A26-39 (36.6 kDa) and B15-24 (29.4 kDa) were produced in *Escherichia coli* BL21(DE3)(pLys) from a pET28b expression vector that separately carried the rA26-39 and rB15-24 open reading frames (ORFs) fused to a C-terminal polyhistidine tag. High levels of expression were obtained upon isopropyl-β-D-thiogalactopyranoside (IPTG) induction and purification of rA26-39 and rB15-24 by passage of the cell lysate through a HiTrap chelating high-performance (HP) column on a Pharmacia AKTA liquid chromatography system.

Protein quantification. Coat proteins were extracted from purified spore suspensions of PP108 and PP142 using two extraction procedures, SDS-dithiothreitol (DTT) and alkali extraction, as described elsewhere (8). SDS-DTT solubilizes both CotB and CotC, while alkali extraction preferentially solubilizes CotC. Quantification was made using a Bio-Rad Gel Doc imaging system.

Polyclonal antibodies. Polyclonal antibodies were raised in mice immunized by the intraperitoneal (i.p.) route with 2 μg of purified protein on days 1, 14, and 28. Dilutions used were 1:3,500 for anti-A26-39 and 1:1,500 for anti-B15-24.

Immunizations in mice. Animals used in this work were pathogen-free BALB/c mice (Charles River) for antibody production and for analysis of immune responses. In all cases, females, aged 6 to 8 weeks, were used. All animal procedures were performed under the Home Office project license PPL 70/6126. Mice were dosed orogastrically (o.g.) (0.2 ml) on days 0, 14, 35, and 57 with a dose of 5×10^{10} spores (PY79, PP108, or PP142). A naïve group of unimmunized animals was included in all experiments, as well as a group receiving a mixture of the rA29-36 (10 μg) and rB15-24 (10 μg) proteins. Intraperitoneal (i.p.) immunizations consisted of doses on days 0, 7, and 28 with the rA26-29 and rB15-24 proteins (10 μg of each).

Determination of mouse antibody titers by indirect ELISA. For analysis of responses, serum was taken on days −1, 34, and 56 (o.g. groups) or day 42 (i.p. groups), and feces were collected on days 21, 42, and 67 and kept at −80°C. Sample extractions were made at a one-fifth (wt/vol) dilution in extraction buffer (2% fetal calf serum [FCS], Dulbecco's modified Eagle's medium [DMEM] plus protease inhibitor cocktails, trypsin [0.1 mg/ml], leupeptin [1 μg/ml], benzamide [1 μg/ml], aprotinin [10 μg/ml], phenylmethylsulfonyl fluoride [1 mM], and EDTA [0.05 mg/ml]). Samples were gently shaken for 30 min at 4°C to disrupt solid material and then centrifuged (13,000 rpm for 15 min). Supernatants were filtered (0.45-μm pore size) before analysis. Antibodies from sera and feces were determined by indirect enzyme-linked immunosorbent assay (ELISA). Greiner 96-well plates (MaxiSorp) were coated with purified recombinant A26-29 (rA26-39) (10 μg/ml) or rB15-24 (50 μl/well) protein in phosphate-buffered saline (PBS) overnight at room temperature (RT). After the plates were blocked for 1 h at 30°C with 2% bovine serum albumin (BSA), 2-fold serially diluted samples were added, starting at a dilution of 1/50 in diluent buffer (0.01 M PBS [pH 7.4], 0.5% [wt/vol] bovine serum albumin [BSA], 5% [vol/vol] fetal bovine serum [FBS], 0.1% [vol/vol] Triton X-100, 0.5% [vol/vol] Tween 20). Replicate samples were used together with a negative control (preimmune serum). Plates were incubated for 2 h at RT before addition of appropriate horseradish peroxidase-conjugated anti-mouse antibodies in conjugate buffer (5% FBS [vol/vol], 1% BSA [wt/vol], 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 optical densities (ODs) were read at 450 nm. Dilution curves were created for each sample, and endpoint titers for each specific antibody were estimated at the maximum dilution of serum giving an absorbance reading of 0.1 U over the ODs of naïve samples.

Neutralization assays. The ability of the antibody samples to neutralize *C. difficile* toxins *in vitro* was determined as described recently (30), with some modifications. HT29 and VERO cells were grown in McCoy's 5A medium and Dulbecco's modified Eagle complete medium (10% [vol/vol] fetal calf serum and 1% [vol/vol] penicillin and streptomycin). Cells were cultured at 37°C with 5%

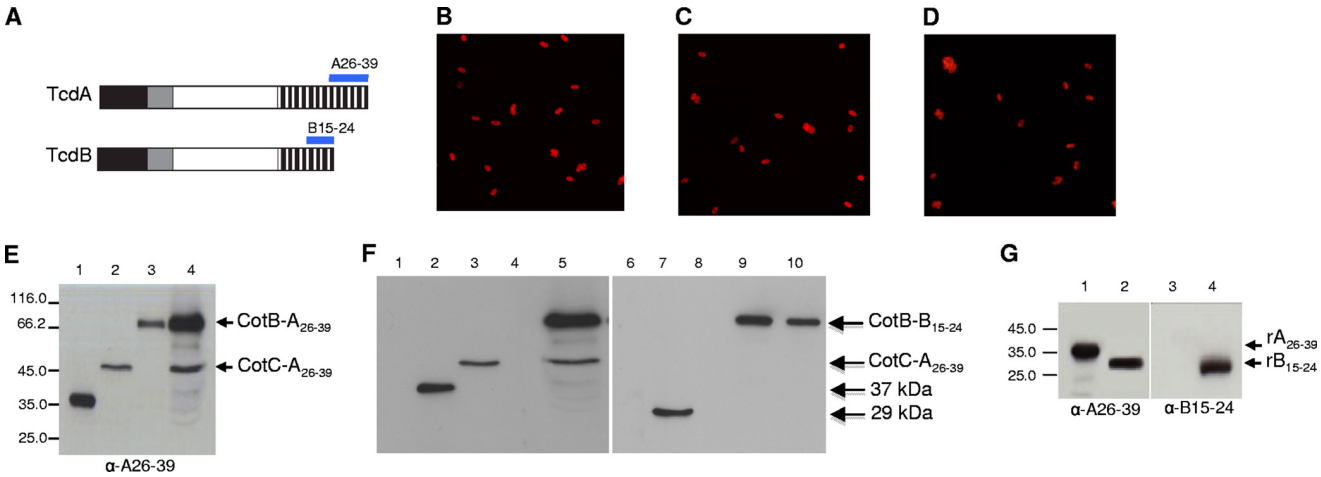

FIG. 1. Recombinant spores expressing *C. difficile* toxin domains. (A) The functional domains of toxins A and B are shown, including the glycosyltransferase (black), cysteine protease (gray), and translocation (white) domains and the repetitive sequences involved in cell binding (stripes). The regions cloned and expressed in *B. subtilis* spores are indicated. These are the A26-39 domain (formerly known as 14CDTA), which carries 14 repeat sequences and has been shown to be immunogenic and capable of inducing neutralizing antibodies in mice when delivered by a mucosal route (43), and the B15-24 domain, which carries 10 repeats and shares homology with A26-39 (see Fig. S2 in the supplemental material). (B to D) Surface display of A26-39 and B15-24, confirmed using confocal imaging of samples labeled with mouse anti-A26-39 or -B15-24 serum followed by an anti-mouse IgG-tetramethyl rhodamine isocyanate (TRITC) conjugate. Spores of PP108 were labeled with anti-A26-39 (B), and spores of PP142 were labeled with either anti-A26-39 (C) or anti-B15-24 (D). Nonrecombinant PY79 spores showed no labeling with either antiserum. Images were taken using a Nikon Eclipse fluorescence microscope equipped with a Bio-Rad Radiance 2100 laser scanning system (image size = 37 by 37 μm). (E) Expression of A26-39 on spores of PP108 (CotB-A_{26-39} CotC-A_{26-39}). Lane 4 shows PP108-extracted spore coat proteins probed with antibodies to rA26-39. Lanes 1 to 3 were also probed with anti-A26-39. Lane 1, rA26-39 protein; lane 2, coat proteins from PP052 (CotC-A_{26-39}); lane 3, coat proteins from PP059 (CotB-A_{26-39}). (F) Expression of A26-39 and B15-24 on spores of PP142 (CotB-B_{15-24} CotC-A_{26-39}) probed with anti-A26-39 (lanes 1 to 5) and anti-B15-24 antibodies (lanes 6 to 10). Lanes 1 and 6, nonrecombinant PY79 spore coat proteins; lane 2, rA26-39 protein (36.6 kDa); lanes 3 and 8, PP052 extracts (CotC-A_{26-39}); lanes 4 and 9, PP132 extracts (CotB-B_{15-24}); lanes 5 and 10, PP142 extracts; lane 7, rB15-24 protein (29.4 kDa). The positions of the 60-kDa CotB-B_{15-24} and 49-kDa CotC-A_{26-39} bands are shown. (G) Purified rA26-39 (lanes 1 and 3) (36.6-kDa) and rB15-24 (lanes 2 and 4) (29.4-kDa) polypeptides were probed with anti-A26-39 or anti-B15-24. Numbers at the left of the blots are molecular masses (in kilodaltons).

(vol/vol) CO_2 in air (100% humidity). HT29 and VERO cells were seeded in 96-well plates (0.5×10^4 cells/well and 2.5×10^4 cells/well, respectively). After 24 h, cells were washed twice in sterile PBS. Before the assay, toxins were incubated with serially diluted pooled serum or fecal samples (1:1, vol/vol) in medium containing 2% FCS and incubated for 1 h at 37°C before being added to the prewashed cell monolayer. Cells were evaluated at 24 h (HT29) and 48 h (VERO). Toxins used were toxins A and B obtained from the culture supernatant of strain 630 (toxin 630), which had been partially purified by ammonium sulfate precipitation (60%). The presence of toxins A and B was confirmed in the supernatant of 630 cultures by Western blotting (see Fig. S1 in the supplemental material). Toxin A and toxin B had been purified from cell supernatants. Appropriate toxin concentrations were determined by the highest dilutions causing 100% cytopathicity (i.e., cell rounding) (toxin 630 [120 ng/ml], toxin A [4.5 ng/ml], toxin B [6.2 pg/ml]). All assays were carried out in duplicate.

Hamster challenge. Groups of 6 to 10 female Golden Syrian hamsters (70 to 80 g; Charles River, United Kingdom), were immunized o.g. (0.2 ml) with either (i) recombinant (PP108, PP142) and nonrecombinant PY79 spores (5×10^{10} spores/dose/hamster) or (ii) a mixture of the proteins rA26-39 (10 μg) and rB15-24 (10 μg). One further group was dosed with the protein mixture delivered by the i.p. route (0.2 ml). Hamsters were dosed o.g. on days 0, 14, 35, and 57 and i.p. on days 0, 7, and 28. The challenges were performed as described by Goulding et al. (10), with some minor modifications. In brief, 14 days after the last dose, hamsters were transferred to individual sterile cages (including all food, bedding, and water) and treated with clindamycin (30 mg/kg of body weight). Twelve hours later, hamsters were o.g. infected with 100 *C. difficile* spores (strain 630). Twenty-four hours after challenge and every 2 days thereafter, hamsters were repeatedly transferred to new sterile cages. Hamsters were intensively monitored 38 to 60 h after challenge. Hamsters showing clear symptoms were killed and considered unprotected.

Statistics. The unpaired *t* test was used to compare between groups. A *P* value of >0.05 was considered nonsignificant.

RESULTS

Expression of the C-terminal domains of *C. difficile* toxin A and toxin B on *B. subtilis* spores. *B. subtilis* was engineered to express the A26-39 domain of toxin A (TcdA) and the B15-24 domain of toxin B (TcdB) on the outermost layer of the spore coat. Both A26-39 and B15-24 lie within the C-terminal repeat domains of each toxin and carry repetitive sequences (40) (Fig. 1A). Expression was achieved by fusing A26-39 and B15-24 to the C termini of the outer spore coat proteins CotB (43 kDa) and CotC (12 kDa), both of which have successfully been used for surface display and mucosal delivery of heterologous antigens (16, 31) (in the case of CotB, fusions were made to a C-terminally truncated version of CotB). Two different spore constructions were made: PP108 (*cotB*-A26-39 *cotC*-A26-39), which expressed A26-39 attached to both CotB and CotC, and PP142 (*cotB*-B15-24 *cotC*-A26-39), which coexpressed two chimeras, CotB-B_{15-24} and CotC-A_{26-39}. Surface expression of A26-39 and B15-24 on PP142 spores and A26-39 on PP108 spores was confirmed by confocal imaging of spores (Fig. 1B to D).

Antibodies to the C-terminal domain of toxin A cross-react with the reciprocal domain of toxin B. The coat proteins of PP108 and PP142 were extracted and probed with antibodies to confirm expression of the chimeric CotB and CotC proteins (Fig. 1E to F). PP108 spore coat protein extracts when probed with anti-A26-39 antibodies revealed bands of 69 kDa (CotB-

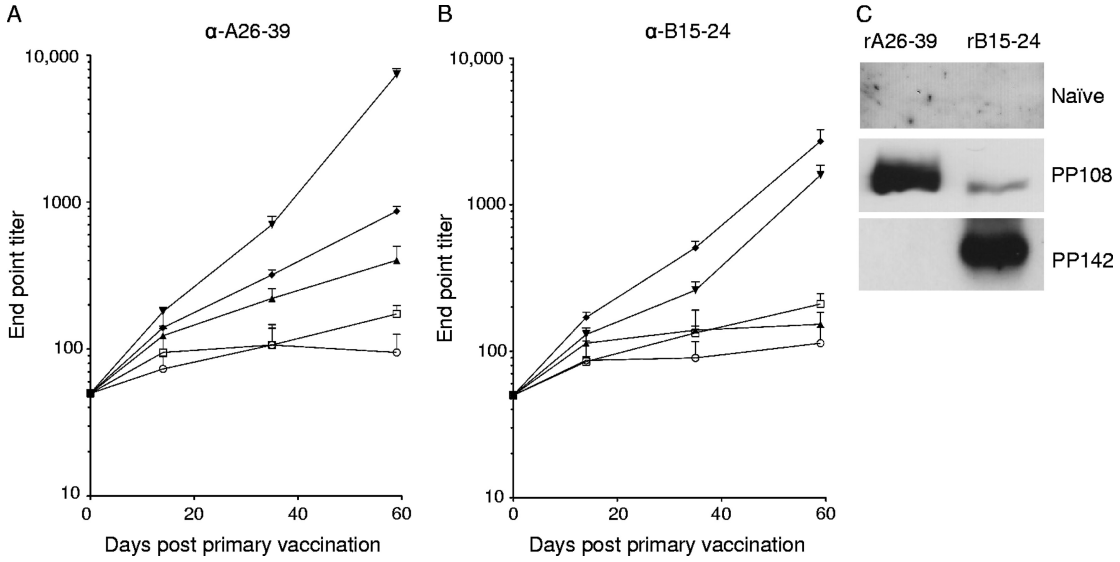

FIG. 2. IgG responses after orogastric immunization of mice with recombinant spores. Anti-A26-39-specific IgG (A) and anti-B15-24 IgG (B) responses are shown. Mice were immunized with spores of strain PP108 (CotB-$A_{26\text{-}39}$ CotC-$A_{26\text{-}39}$) (▼), PP142 (CotB-$B_{15\text{-}24}$ CotC-$A_{26\text{-}39}$) (◆), nonrecombinant PY79 (□), a mixture of the rA26-39 and rB15-24 recombinant proteins (▲) (10 μg of each) and naïve mice (○). (C) Western blots containing pooled sera from naïve and PP108- and PP142-immunized groups were used to probe rA26-39 proteins and rB15-24.

$A_{26\text{-}39}$) and 49 kDa (CotC-$A_{26\text{-}39}$). Their molecular masses were in agreement with the predicted sizes of the fusion proteins (Fig. 1E) and corresponded in size to proteins in extracts taken from spores carrying each chimera alone. Quantification revealed that PP108 spores carried 2.2×10^{-4} pg/spore of A26-39. When PP142 extracts were probed with anti-B15-24, one principal band of 60 kDa was detectable (Fig. 1F, lane 6), corresponding in size to CotB-$B_{15\text{-}24}$. Using anti-A26-39, CotC-$A_{26\text{-}39}$ was observed as a 49-kDa species; a second band of 60 kDa was also observed (Fig. 1F, lane 3). We predict that this higher-molecular-mass species is CotB-$B_{15\text{-}24}$ and could be explained by the fact that the C-terminal cell-binding domains of TcdA and TcdB share some sequence identity (30% identical residues [see Fig. S2 in the supplemental material]) and may, as shown elsewhere, carry related epitopes (40). In support of this, we demonstrated that anti-A26-39 antibodies could cross-react with both the purified-rA26-39 (36.6-kDa) and -rB15-24 (29.4-kDa) polypeptides, yet anti-B15-24 antibodies reacted only with rB15-24 (Fig. 1G). Protein expression was quantified, and each spore of PP142 was shown to contain 2.45×10^{-5} pg of B15-24 and 1.9×10^{-4} pg of A26-39. In additional work, we verified, using anti-CotB and anti-CotC polyclonal antibodies, the expected change in the molecular masses of the chimeric CotB and CotC spore coat proteins in PP108 and PP142 spores (see Fig. S3 in the supplemental material).

Oral delivery of the C-terminal domains of toxin A and toxin B displayed on spores induces systemic and mucosal antibodies. Immune responses were determined in mice dosed orogastrically (o.g.) with PP108 or PP142 spores. Control groups included naïve mice and groups dosed (o.g.) with nonrecombinant spores (PY79). In addition, we included one group dosed (o.g.) with a mixture of the rA26-39 (10-μg/dose) and rB15-24 (10-μg/dose) proteins using the same dosing regimen. Ten micrograms was chosen for each protein since this equaled or exceeded the dose of rA26-36 or rB15-24 delivered in one o.g. dose of PP108 (11 μg of A26-39) or PP142 (9.5 μg of A26-39 and 1.25 μg of B15-24) spores.

Specific antibodies (serum IgG and fecal IgA) against A26-39 and B15-24 were measured by indirect ELISA. Compared to control groups (naïve mice, mice dosed with PY79 spores, or mice receiving proteins alone), significant ($P < 0.01$) levels of A26-39 and B15-24 (cross-reacting) IgG were detected in the sera of animals dosed with PP108 (Fig. 2A and B). In mice dosed with PP142 spores, anti-A26-39 IgG responses were not significantly greater ($P > 0.05$) than in control groups, while anti-B15-24 IgG responses were significantly greater ($P < 0.01$) (Fig. 2B) and showed seroconversion. IgG isotypes were also determined (see Fig. S4 and S5 in the supplemental material), and significant ($P < 0.01$) levels of anti-A26-39 and anti-B15-24 (cross-reacting) IgG1 and IgG2a were found in the PP108 groups compared to the control groups. However, in the PP142 groups, significant levels ($P < 0.01$) of the IgG1 and IgG2a isotypes were found only against B15-24. Analysis of the IgG1/IgG2a ratios over time (see Fig. S6 in the supplemental material) showed a clear increase (3-fold) after the third dose, indicative of a Th2-biased immune response. Anti-spore IgG responses were also measured and found to be markedly low, with no responses substantially greater than those of the control groups (see Fig. S7 in the supplemental material). IgG present in the sera of PP108-immunized mice was found to bind in a Western blot to rA26-39 and rB15-24, while that present in PP142-dosed mice bound only to rB15-24 (Fig. 2C).

Secretory IgA (sIgA) was measured in fecal samples (Fig. 3), and anti-A26-39 responses were found to be particularly high (Fig. 3A), with clear seroconversion in animals dosed with PP108 spores ($P < 0.001$) but not with PP142 spores ($P > 0.05$). Anti-B15-24 sIgA responses were lower, but seroconversion was found in animals dosed with both PP108 and PP142 (Fig. 3B) ($P < 0.05$). The anti-B15-24 cross-reacting IgG and sIgA responses found in animals dosed with PP108 spores expressing only the A26-39

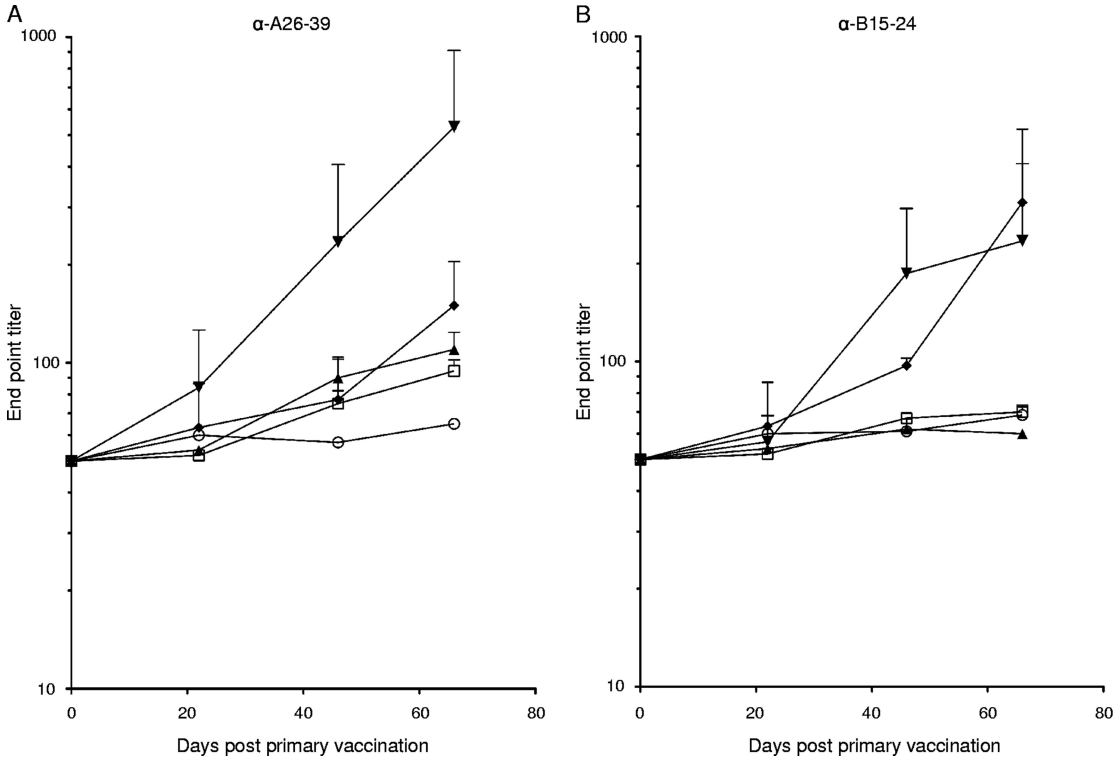

FIG. 3. Secretory IgA responses after orogastric immunization of mice with recombinant spores. Anti-A26-39-specific sIgA (A) and anti-B15-24-specific sIgA (B) responses. Mice were immunized with spores of strain PP108 (CotB-A_{26-39} CotC-A_{26-39}) (▼), PP142 (CotB-B_{15-24} CotC-A_{26-39}) (◆), nonrecombinant PY79 (□), and a mixture of the rA26-39 and rB15-24 recombinant proteins (▲) (10 μg of each). ○, naïve mice.

protein further support our finding that antibodies against the A26-39 domains are cross-reactive to B15-24.

***In vitro* neutralization of cytotoxicity.** Both toxin A and toxin B exhibit cytotoxicity on cultured cells, with HT29 cells being most sensitive to toxin A and VERO cells most sensitive to toxin B (30, 38). Serum (IgG) and mucosal (fecal sIgA) antibodies from mice were assessed for their ability to neutralize toxin A- and toxin B-mediated cytotoxicity using either purified toxins or partially purified supernatants from strain 630 (toxin 630) (Table 1). Cytotoxicity was confirmed by examination of cells over a 24- to 48-h period, with susceptible cells showing a rounded cell morphology (examples in Fig. S8 in the supplemental material).

Antibodies from PP108-immunized (o.g.) mice were found to neutralize both toxin A and toxin B when either HT29 or VERO cells were used, and higher titers were shown in HT29 cells (toxin A specific) than in VERO cells. In contrast, antibodies produced by PP142-immunized (o.g.) animals neutralized only toxin B. These results show, first, that in mice, o.g. delivery of recombinant spores expressing A26-39 or B15-24

TABLE 1. Neutralization of *in vitro* cytotoxicity and protection

Group	Route	Toxin neutralization titer[a]						Protection (%)[b]	
		Anti-toxin A		Anti-toxin B		Anti-toxins A and B[c]		1st	2nd
		IgG	sIgA	IgG	sIgA	IgG	sIgA		
Naïve		−	−	−	−	−	−	0	
PY79	o.g.	−	−	−	−	−	−	0	
rA26-39 + rB15-24	o.g.	−	−	−	−	−	−	0	
PP108	o.g.	++	+++	+	+	++	+++	75	100
PP142	o.g.	−	−	+	+	−	−	0	
rA26-39 + rB15-24	i.p.	+++	−	+	−	+++	−	25	0

[a] Neutralization titers against toxin A or toxin B were measured on HT29 (anti-toxin A) or VERO (anti-toxin B) cells. Endpoint titers were determined as the highest dilution of sample that prevented the cytopathic cell-rounding effect of the toxin in 100% of cells. Negative results are expressed as − when the endpoint titer dilution was <1/10 for serum samples and <1/50 (wt/vol) for fecal samples. Representative neutralization data are shown in Fig. S8 in the supplemental material and summarized as follows. For serum titers, + indicates a titer of 10 to 20, ++ indicates 40 to 80, and +++ indicates >160. For feces titers, + indicates a titer of 50 to 100, ++ indicates 100 to 200, and +++ indicates >200.

[b] Survival data are shown in Fig. 4. Animals surviving the first challenge were then rechallenged (2nd).

[c] Neutralization against toxins partially purified from *C. difficile* strain 630, measured using HT29 cells.

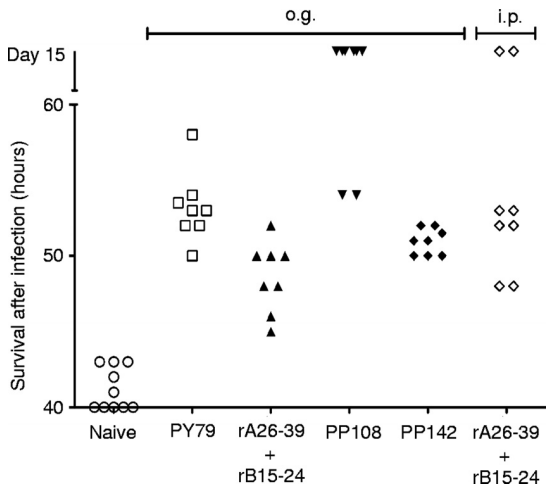

FIG. 4. Protection in Golden Syrian hamsters. Hamsters were given four o.g. doses (days 0, 14, 35, and 57) of recombinant spores (PP108 or PP142) and then challenged with *C. difficile* 630 (A^+B^+). Control groups included naïve animals, a group dosed with nonrecombinant PY79 spores, and, finally, a group receiving a mixture of the rA26-39 and rB14-24 proteins (10 μg each). Further groups were hamsters dosed parenterally (i.p.) with rA26-39 plus rB14-24 (10 μg each). Colonization of hamsters is presented as time from inoculation to signs of first symptoms, when animals were killed. Animals showing no symptoms after 14 days were considered protected (i.e., the PP108 group). For animals showing signs of infection, the mean times (±standard deviations) to visible symptoms were, for naïve hamsters, 40.9 h (±1.7 h), for PY79 hamsters, 53.2 h (±2.3 h), for PP108 hamsters, 55.6 h (±1.7 h), for PP142 hamsters, 50.9 h (±0.9 h), and for hamsters dosed o.g. with the protein mixture and for hamsters in the i.p. group, 51.8 h (±2.4 h) and 48.6 h (± 2.3), respectively.

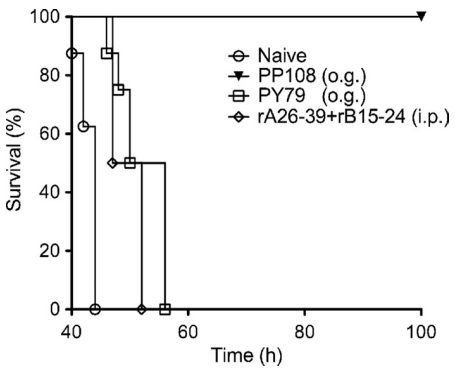

FIG. 5. Protection against reinfection. Hamsters immunized orally with PP108 spores or parenterally with recombinant proteins that had survived challenge with *C. difficile* 630 (as shown in Fig. 4) were then rechallenged with *C. difficile* 630 spores 16 days after the end of the first challenge. Kaplan-Meier survival estimates are shown. Results are also shown for control groups of naïve hamsters and animals dosed with nonrecombinant PY79 spores (dosed on days 0, 14, 35, and 57).

can generate systemic and mucosal neutralizing antibodies. Second, using PP108 spores expressing only A26-39, neutralizing antibodies to toxin A as well as toxin B could be elicited.

Protection in hamsters. The hamster model of *C. difficile* infection most closely resembles the human disease and is the best indicator of protective immunity (10, 33). We dosed hamsters o.g. with PP108 and PP142 spores, treated them with clindamycin, and then challenged them with 100 spores of *C. difficile* strain 630 (Fig. 4). Control groups included hamsters dosed with nonrecombinant PY79 spores and animals dosed with a mixture of the rA26-39 and rB15-24 proteins (10 μg of each). Spores of *C. difficile* were used instead of live cells since, as shown recently, they better mimic the natural infection process (10) as well as minimize the difficulties of administering live anaerobic bacteria. All naïve animals (10/10) were susceptible to *C. difficile* and showed symptoms of disease after just 40 to 42 h, in close agreement with a previous study (10). Interestingly, most of the animals dosed with PY79 spores showed delayed symptoms, as did those dosed with a mixture of the rA26-39 and rB15-24 proteins, but in both cases, all animals succumbed. Hamsters dosed with PP142 spores showed no protection, with every animal displaying symptoms. PP108-immunized animals, however, showed protection, with six out of eight animals surviving the duration of the experiment.

Protection against reinfection. All surviving hamsters that showed no symptoms of *C. difficile* infection were then rechallenged 16 days after the first challenge using clindamycin to induce *C. difficile* colonization. Control groups included unimmunized (naïve) hamsters and animals dosed o.g. with nonrecombinant PY79 spores. We found that only PP108 hamsters were 100% protected to *C. difficile* challenge and showed no symptoms of infection (Fig. 5). These results show that o.g. immunization of hamsters with the A26-39 domain of toxin A displayed on the surfaces of *B. subtilis* spores is sufficient to confer protection against a strain of *C. difficile* that produces both toxin A and toxin B. Moreover, protected animals were fully resistant to reinfection.

Parenteral immunization of the cell-binding domains of toxin A and toxin B. As a comparator of oral-versus-parenteral delivery, mice were dosed (intraperitoneally [i.p.]) with a mixture of rA26-39 (10 μg/dose) and rB15-24 (10 μg/dose). High ELISA titers of IgG antibodies specific to A26-39 (34,161 ± 9,838) and B15-24 (21,475 ± 7,152) were found 2 weeks after the last dose (day 45), significantly greater than titers in naïve animals ($P < 0.001$) and higher than in serum samples from PP108-immunized mice ($P < 0.001$). In contrast, no significant ($P > 0.05$) levels of sIgA were detectable in feces. Serum antibodies were shown to be able to neutralize the cytotoxic effects of both toxin A and toxin B (Table 1). In the hamsters dosed i.p. with the recombinant proteins, 25% protection was achieved (Fig. 4), but animals failed to survive reinfection with *C. difficile* (Fig. 5).

DISCUSSION

Our use of *B. subtilis* spores as a vehicle for *C. difficile* vaccination demonstrated a number of important findings that will be invaluable to the design of an effective *C. difficile* vaccine: first, that only toxin A antibodies are required for protection, second, that mucosal immunity is very important, and, finally, that vaccination provides protection against relapse. We discuss each of these in turn.

(i) Toxin A antibodies and protection. Expression of the toxin A (A26-39) and toxin B (B15-24) C-terminal domains on *B. subtilis* spores was shown to generate high titers of specific IgG and sIgA antibodies. Using spores (PP108) displaying only the toxin A domain, A26-39, serum and fecal antibodies were

found to be cross-reactive to B15-24. Moreover, they neutralized both toxin A and toxin B *in vitro*, and hamsters immunized with A26-39 were protected against *C. difficile* disease. Further evidence of the cross-reactivity of toxin A antibodies came from Western blotting, which showed that anti-A26-39 antibodies could recognize B15-24. The repeat domains of both toxins share limited sequence identity, which could explain this cross-reactivity. In particular, the sequences YFAPANT, MQ IGVF, AAT, and YYF are conserved between the toxin A and B repeat used here, and together they may contain epitopes responsible for the observed cross-reactivity.

We infer that antibodies to A26-39 are of higher avidity than those to B15-24, since in mice, ELISA antibody titers to A26-39 and B15-24 were equivalent. PP142 spores present both toxin A and toxin B antigens, so why do these spores not provide protection against disease after administration? Although B15-24-neutralizing antibodies were produced, no neutralizing antibodies to A26-39 were detected. This implies that, for protection, antibodies to toxin A are of greater importance than those to toxin B. PP142 spores do express the A26-39 domain, yet no neutralization of toxin A was observed. To account for this, we predict that insufficient A26-29 was expressed, since PP142 spores carried nine times less A26-39 than PP108 spores. Of course, we cannot completely rule out the potential contribution of anti-B15-24 antibodies in protection; rather, our work shows that anti-A26-39 responses are sufficient. It is possible that our prototype vaccines simply do not elicit sufficiently high toxin B-neutralizing titers, possibly due to B15-24 being displayed in a partially denatured form that impairs the generation of appropriate neutralizing antibodies. This may reflect the action of its fusion partner, the spore coat protein CotB, although this has been used successfully previously (8, 13). Full protection to challenge using PP108 spores might be achieved either by changing the dosing regimen or by increasing the dose of heterologous protein expression (whether of A26-39 and/or of B15-24).

This work agrees with a number of studies linking protection against *C. difficile* infection with toxin A, for example, a passive immunization study using oral delivery of anti-toxin A antibodies (21) and studies showing that there is strong association between serum antibody responses to toxin A and protection against *C. difficile* in humans (1, 22, 24). Of particular note is the study of Kim et al. (20), where hamsters immunized (subcutaneously) with *C. difficile* toxoid A were fully (100%) protected but hamsters immunized with toxoid B demonstrated no protection. Our work does show, however, that a vaccine expressing only A26-39 should be protective to all known naturally virulent strains of *C. difficile* (A^+B^+ and A^-B^+). However, a challenge experiment using an A^-B^+ strain is required for confirmation; this finding is important for the design of future *C. difficile* vaccines.

(ii) Mucosal immunity. Considerable effort has been directed toward parenteral vaccines to *C. difficile* and the generation of systemic responses. However, far less attention has been spent on examining the role of local immunity, despite a number of reports demonstrating the importance of sIgA. First, a correlation between neutralizing sIgA and protection in hamsters has been reported using mucosal delivery of toxoids, with 100% protection by intranasal delivery and 40% protection by o.g. delivery (39). Second, and most importantly, several studies demonstrate unequivocally that sIgA both is capable of neutralizing toxin A and is superior to IgG and monomeric serum, IgA (19, 35).

Oral delivery of A26-39 using PP108 spores promoted high titers of fecal sIgA, with levels of toxin A- and toxin B (cross-reacting)-neutralizing sIgA correlating with protection. In contrast, i.p. delivery of both the rA26-39 and rB15-24 proteins produced high titers of serum-neutralizing IgG to toxin A and toxin B, no localized immune responses (sIgA), and lower levels of protection. In another study, however, Torres et al. (39) used parenteral delivery (including i.p.) of toxoids A and B and demonstrated 100% protection in hamsters. A number of factors might account for why we did not observe complete protection when using the recombinant proteins. For example, in the Torres et al. study, the complete toxoids were administered together with an adjuvant and a different virulent strain of *C. difficile* was used for challenge (39).

(iii) Relapse. PP108-immunized hamsters that survived *C. difficile* challenge were fully protected against rechallenge, a phenomenon particularly important for treatment of CDAD when patients succumb to relapse. Up to 20% of patients with CDAD 2 to 8 weeks after discontinuation of antibiotic therapy (metronidazole or vancomycin) relapse, and a further 30% of these patients may do so again after a second course of therapy (4, 18). Antibiotic treatment may disrupt the normal human microflora and lead to overgrowth of toxigenic strains of *C. difficile*, a condition favoring relapse. Vaccination then may provide the only rational treatment to control CDAD, so it is encouraging that we found no symptoms of disease in rechallenged animals. This is consistent with an earlier study that has shown that antibodies to toxins A and B, administered orally, can prevent relapse (21) and also with a study in which 75% protection against relapse was found after mucosal and parenteral delivery of toxoids A and B (39). In our studies, protection against relapse was found only in hamsters dosed with PP108 spores and not in animals that had been vaccinated with the recombinant proteins by a parenteral-dosing regimen. Interestingly, animals dosed parenterally all failed to generate significant sIgA responses, and it is possible that production of mucosal antibodies is important for protection from reinfection. Some probiotic formulations have been shown to significantly reduce the occurrence of relapses in double-blind controlled trials (12, 36). Potentially, live spores may share some attributes with these "probiotic" bacteria, and it is notable that *B. subtilis* is itself used extensively as a probiotic (14). *B. subtilis* spores can germinate and resporulate in the GI tract and possibly even transiently colonize (37). PP108 spores may therefore be able to promote a more extensive production of anti-A26-39 sIgA, sufficient to protect against reinfection. With regard to their potential probiotic attributes, it is noteworthy that even nonrecombinant PY79 spores, when they were administered orally to hamsters, provided a noticeable delay in the onset of symptoms, and it is possible that this phenomenon may reflect involvement of an innate immune response, such as interaction with Toll-like receptors. Alternatively, although the suggestion is highly speculative, we cannot rule out the possibility of sequestration of the toxin in an animal's GI tract, since *Bacillus* spores have been shown to be able to adsorb *C. difficile* toxin *in vitro* (15).

Finally, we have used *C. difficile* spores, in contrast to vegetative cells or toxins, as our challenge in the vaccine protection

experiments, which, as has been shown recently, best mimics the natural course of infection in humans (10). Our study confirms that in the hamster model of infection, there is a correlation between neutralizing antibodies and protection in hamsters, with HT29 cells providing a more precise *in vitro* indicator of protection.

We show that oral delivery of *B. subtilis* spores displaying high levels of toxin A repeats can confer to hamsters immunological protection from a lethal dose of *C. difficile* spores. The basis of the vaccination strategy is induction of secretory IgA, which shows cross-reactivity to toxin B. Such a vaccine would be particularly attractive to the end user, as it can be delivered orally, it is heat stable, and the vehicle is currently used as a probiotic.

ACKNOWLEDGMENT

We thank Gill Douce for invaluable advice regarding the challenge studies.

P.P. and J.P. were supported by a scholarship from the Royal Thai Government.

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Editor: R. P. Morrison

Beneficial Microbes, June 2012; 3(2): 127-135

Wageningen Academic Publishers

Evaluation of *Bacillus subtilis* strains as probiotics and their potential as a food ingredient

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Received: 5 January 2012 / Accepted: 8 February 2012

Abstract

Spores of *Bacillus subtilis* including one strain used commercially were evaluated for their potential value as a probiotic and as potential food additives. Two isolates of *B. subtilis* examined here were HU58, a human isolate and PXN21, a strain used in an existing commercial product. Compared to a domesticated laboratory strain of *B. subtilis* both isolates carried traits that could prove advantageous in the human gastro-intestinal tract. This included full resistance to gastric fluids, rapid sporulation and the formation of robust biofilms. We also showed that PXN21 spores when administered weekly to mice conferred non-specific cellular immune responses, indicative signs of the stimulation of innate immunity. Spores mixed in wholemeal biscuits were found to survive baking at 235 °C for 8 minutes with only a 1-log reduction in viability. That spores can survive the baking process offers the possibility of using spores as probiotic supplements in a range of novel food products.

Keywords: *Bacillus subtilis,* spores, food supplements, probiotics

1. Introduction

Bacillus subtilis is a Gram-positive spore forming bacterium that has been extensively studied as a model for unicellular microbial development. Bacterial spores are of importance in the food industry where some species are involved in food-borne disease, for example, *Bacillus cereus* which is one of the most common causes of foodborne diarrhoea in developed countries. *Bacillus* spores are also being used extensively as probiotic food supplements where they are used in humans as dietary supplements and in feed for livestock and aquaculture as growth promoters and competitive exclusion agents (for reviews see Bader *et al.*, 2012; Cutting, 2011; Hong *et al.*, 2005; Leonel Ochoa-Solano and Olmos-Soto, 2006; Sanders *et al.*, 2003; Shivaramaiah *et al.*, 2011). In Europe *B. subtilis* has been granted QPS (qualified presumption of safety) status by the European Food Safety Authority (EFSA, 2008) and its use as a probiotic is likely to increase. How *Bacillus* spores exert their beneficial effects is less clear but most probably involves the induction of non-specific immune responses that prime the host against infection by bacterial or viral pathogens. Evidence showing immune stimulation in mice dosed orally with spores has been documented (Huang *et al.*, 2008) and studies in poultry and mice have shown that oral dosing with spores of *B. subtilis* can suppress infection against *Escherichia coli* 070:K80, *Salmonella enterica*, *Clostridium perfringens* and *Citrobacter rodentium* (D'Arienzo *et al.*, 2006; La Ragione and Woodward, 2003; La Ragione *et al.*, 2001).

B. subtilis has long been considered a soil organism since this is where it can be found in abundance. However, its presence is mostly as the dormant spore and a compelling case now exists that spores can inhabit the intestinal tract of humans and animals (Hong *et al.*, 2009b). In the gastro-intestinal (GI)-tract spores have been shown to germinate in the intestine, proliferate and then resporulate before being shed in the faeces (Casula and Cutting, 2002; Tam *et al.*, 2006). This intestinal residency is intriguing and implies that spore formers could actually be gut commensals and thus their use as probiotic supplements could be based on

a more scientifically rational basis. This is supported by studies that show that *B. subtilis* spores play an important role in the development of the gut-associated lymphoid tissue (GALT) in infant rabbits (Rhee *et al.*, 2004).

From a commercial perspective a probiotic supplement that can be stored at room temperature in a desiccated form is attractive and alleviates the necessity for freeze or spray-drying as well complicated encapsulation methods (Bader *et al.*, 2012; Ding and Shah, 2009; Franks, 1998). Further, the resistance properties of spores raises the possibility that they can be incorporated in a number of food products (e.g. beverages, chocolate, etc.). In the USA *Bacillus coagulans* spores are being incorporated in a number of foods including baked cakes and muffins (Cutting, 2011).

Two strains of *B. subtilis* were evaluated in this study, HU58 a human isolate (Hong *et al.*, 2009a) and PXN21 that is used in a commercial product Biokult (Protexin Ltd., Lopen Head, UK) available in the UK. The purpose of this study was threefold: first, to determine by comparison with a known natural human isolate of *B. subtilis* (strain HU58) whether PXN21 was similar to other natural isolates of *B. subtilis* and shared traits that might favour residency in the GI-tract. Second, to demonstrate that PXN21 could stimulate innate immunity. Finally, to determine whether *B. subtilis* (strains PXN21 and HU58) could be incorporated into a baked foodstuff.

2. Materials and methods

Strains

PY79 and HU58 have been described previously and are laboratory stocks (Hong *et al.*, 2009a; Youngman *et al.*, 1984). PXN21 was obtained from NCIMB Ltd. (Aberdeen, UK) as strain NCIMB 30223.

General methods

Sporulation was made in liquid Difco sporulation medium (DSM) by the exhaustion method (Nicholson and Setlow, 1990). Spore counts were determined by heating cultures at 65 °C for 45 min (to kill any vegetative cells) followed by serial dilution and plating out on DSM solid agar. Pure suspensions of spores were prepared by the exhaustion method using lysozyme treatment to remove vegetative cells as described (Nicholson and Setlow, 1990). Procedures for measurement of spore survival in simulated gastric fluid (SGF) were performed as described by Le Duc *et al.* (2003).

Physiological analysis

Surfactin was evaluated using the oil-displacement method (Youssef *et al.*, 2004). Evaluation of swarming and dendritic growth using CM, CMK and MSgg media have been described elsewhere (Fakhry *et al.*, 2009; Hong *et al.*, 2009a). Adhesion to mucin was determined as described previously (Sanchez *et al.*, 2010) by binding 1 mg of mucin (in phosphate buffered saline (PBS) at 10 mg/ml) to each well of a 96-well microtitre plate. Following the steps outlined for this assay ~10^9 cells were incubated per well for 1 h at 37 °C. Unbound cells were removed by washing and Triton-X-100 treatment used to detach bound bacteria followed by serial dilution and determination of cfu/well. The experiment was repeated two times.

Persistence in the murine gut

All experiments used Balb/c inbred mice (female, 6 weeks old). For analysis of persistence the procedure was that described previously (Barbosa *et al.*, 2005; Tam *et al.*, 2006). In brief, groups of 6 mice were each administered a single intra-gastric dose of 1×10^9 spores (0.2 ml in PBS). At time points thereafter fresh faecal pellets were recovered from individual mice and the number of heat-resistant spores (per g) determined by heat treatment (45 min at 65 °C) of homogenised faecal material followed by serial dilution and plating on DSM agar plates.

Evaluation of IFN-γ

For analysis of immune stimulation groups of mice (n=5) were each given weekly doses (intra-gastric) of 1×10^9 spores for 10 weeks (10 doses). Mice were killed 24 h after the last dose of spores. Splenocytes were recovered from spleen tissue of immunised mice and 5×10^5 splenocytes were seeded per well (using 96-well plates) and stimulated with the following antigens: *Clostridium difficile* toxoid A (CDTA; 5 μg/well; ÄKTA purified) protein, *E. coli* lipopolysaccharide (LPS; 1 μg/well; Sigma, St. Louis, MO, USA), gluteraldehyde-inactivated spores of *C. difficile* (1×10^6 spores/well) and finally two spore coat proteins, CotC and CotD that are found on the coats of *C. difficile* spores (P. Permpoonpattana *et al.*, 2011). *C. difficile* spores were inactivated by overnight incubation in 2% gluteraldehyde at 4 °C. After 4 days of incubation supernatants were analysed for IFN-γ by ELISA as described previously (Huang *et al.*, 2008).

Incorporation of spores in biscuits

Lyophilised spores of HU58 and PXN21 were mixed with 1 kg of a commercial biscuit dough using facilities at United Biscuits (High Wycombe, UK) to give a final bacterial count of ~1×10^9 spores/g of mix. The dough mixture consisted of flour (58.6%), fat (18%), sugars (13.6%), salt (0.7%), sodium bicarbonate (1.1%), malic acid (0.4%), ammonium bicarbonate (0.2%) and water (7.4%). The dough was mixed using a Hobart planetary mixer (Troy, OH, USA) in two stages. Dough was covered and allowed to rest for 25 minutes before being formed into 19 g dough pieces

using a bench scale rotary moulder. Dough pieces were baked on a 5×5 oven band at 235 °C in a static deck oven for 8 minutes and then stored in heat-sealed foil bags until analysis. The initial dough mixture had a moisture content of 14% which, after baking, was reduced to 2%. For analysis individual biscuits were homogenised in sterile PBS buffer, serially diluted and plated for cfu count on DSM agar plates. Three independent counts were made per time point.

3. Results and discussion

Sporulation efficiency

Sporulation of PXN21 and HU58 was made in parallel with a domesticated laboratory strain known as PY79 (Youngman *et al.*, 1984). Using the exhaustion method we induced sporulation of all three strains and examined heat-resistant (spore) counts. As shown in Figure 1 PXN21 most closely resembled HU58 in its kinetics of spore formation with the first detectable levels of heat resistant spores appearing 2-3 h post-induction and maximal counts at 8 h. By contrast PY79 reached maximal levels of sporulation 24 h after the initiation of development while the first detectable heat-resistant spores were found after 5-6 h. The fast and slow sporulation characteristics of HU58 and PY79 have been determined previously (Tam *et al.*, 2006) and are repeated here. Compared to PY79 it is clear that PXN21 and HU58 are fast sporulators and most closely resemble other natural isolates of *B. subtilis* (Hong *et al.*, 2009a). Although the mechanism for how this occurs is unclear, rapid sporulation is a characteristic of many natural isolates of *B. subtilis* and may reflect the necessity to adapt rapidly to movement through the GI-tract (Hong *et al.*, 2009a; Tam *et al.*, 2006).

Physiological characteristics likely to be of benefit in the gastro-intestinal tract

Many *B. subtilis* strains are known to form structured multicellular biofilms in the form of pellicle-like surface colonies on semi-solid media where different phases of colonisation can be observed (Branda *et al.*, 2001; Hong *et al.*, 2009a). Biofilm formation may enable bacteria to persist longer in the GI-tract and is a feature thought to be required for residency (Tam *et al.*, 2006). In the first phase, cells spread, or swarm, over the agar surface as dendritic branching colonies (e.g. using growth on CM agarose). If sufficient potassium ions are present, the cells enter a second rapid and profuse growth phase in which the colony can spread over the entire surface as a pellicle-like film (e.g. using CMK agarose). Dendritic growth, i.e. the first phase of colonisation, is generally considered an indication of the ability of bacteria to form surface biofilms.

Using growth on MSgg medium (Fakhry *et al.*, 2009) we found that both HU58 and PXN21 were able to form robust biofilms (Figure 2). We also examined PXN21, HU58 and PY79 on CM and CMK media (Table 1 and Appendix A) and found that on CM agar only HU58 was able to form swarming (dendritic) colonies. On CMK agarose both HU58 and PXN21, but not PY79, produced a profuse surface colony formation. This rapid surface motility has been explained earlier by a lubricating model where growth is partly due to the secretion of biosurfactants (Kinsinger *et al.*, 2003). Surprisingly, for PXN21, in contrast to PY79, we were unable to detect any significant level of surfactin (a well characterised *Bacillus* biosurfactant; Nagal *et al.*, 1996) using a simple oil-displacement method (Youssef *et*

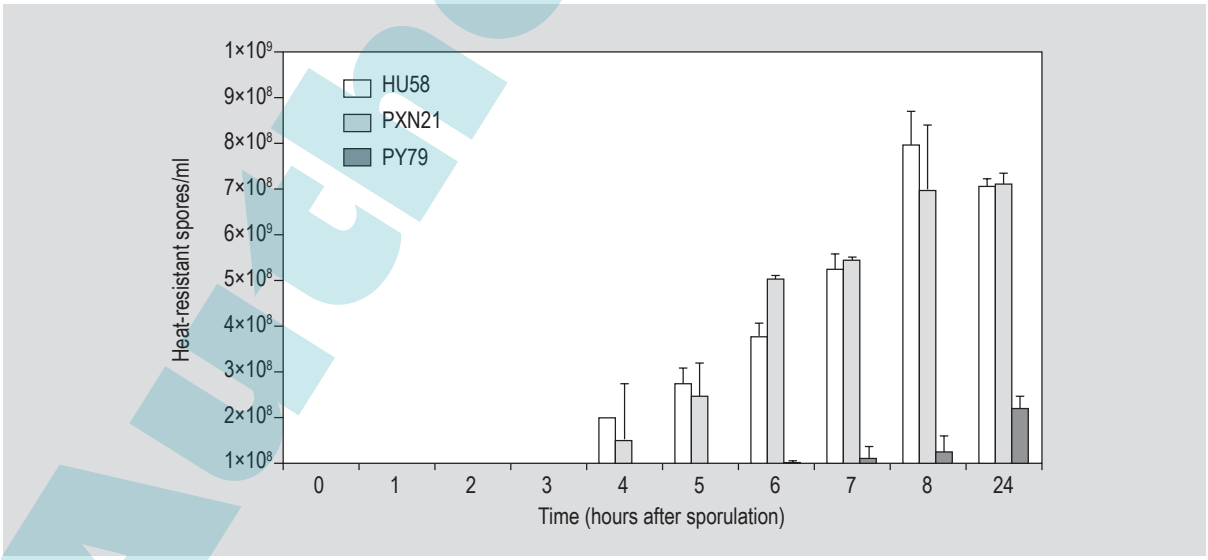

Figure 1. Sporulation efficiencies of *Bacillus subtilis* strains PY79 (a laboratory strain), HU58 a human gut isolate and PXN21 (a commercial strain) in DSM medium. Heat-resistant spore counts (cfu) were determined at time points following the initiation of sporulation.

Figure 2. Biofilm formation of *Bacillus subtilis* strains PY79, HU58 and PXN21 on MSgg medium. Both HU58 and PXN21 produced biofilms with HU58 producing the most robust growth. PY79 failed to produce biofilms.

Table 1. Physiological properties of *Bacillus subtilis* strains relevant to gut residency.

		Strain		
		PY79	**HU58**	**PXN21**
Surfactin[1]		-	+	-
Biofilms[2]		-	+	+
Surface growth[3]				
	swarming (dendritic)	-	+	-
	rapid/profuse	-	+	+
Mucin	adhesion[4]	7×10^{2}	1.3×10^{4}	7.8×10^{4}

[1] Surfactin was measured using the oil-displacement method. - no measurable activity, + represents a diameter of oil displacement of <10 mm.
[2] Overnight cultures grown on MSgg medium (see Figure 2).
[3] Swarming (dendritic growth) was determined on CM agarose; rapid profuse surface growth was determined on CMK agarose (see Appendix A): - = no dendritic or rapid/profuse growth; + = dendritic or rapid/profuse growth.
[4] Colony forming units adhered to plastic wells. Positive controls for adhesion were *Bacillus firmus* GB1 and *Bacillus indicus* HU36 as described by Manzo *et al.* (2011).

al., 2004). This does not exclude the possibility that PXN21 does not produce surfactin, but if it does, it must be at levels that we cannot detect.

We also examined the ability of all three strains to adhere to mucin, a feature which may be beneficial to intestinal bacteria enabling them to obtain nutrients and more efficiently colonise the mucosal epithelial layers (Fakhry *et al.*, 2009; Ruas-Madiedo *et al.*, 2008; Sanchez *et al.*, 2010). *Bacillus* species have been shown to adhere and degrade mucin and presumably do so by the action of a number of hydrolytic enzymes such as glycosidases, proteases, peptidases and sulfatases, all of which are required for the complete breakdown of mucin (Macfarlane *et al.*, 2005). Our analysis (Table 1) revealed that all three strains could adhere to mucin. Using strains of *Bacillus firmus* (GB1) and *Bacillus indicus* (HU36) previously characterised using the mucin-adhesion assay (Manzo *et al.*, 2011) we obtained similar binding efficiencies which in turn demonstrated that adhesion of HU58 and PXN21 was significantly higher by one (HU58) to two (PXN21)-logs, respectively, than PY79, GB1 or HU36.

Resistance to intestinal fluids

B. subtilis spores have been shown to be resistant to intestinal fluids, specifically gastric and bile salts (Barbosa *et al.*, 2005; Le Duc *et al.*, 2003). Passage through the stomach dictates that the spore must survive intact if it is to have any effect on immune stimulation or gut health. We evaluated the resistance of spores (PXN21, HU58 and PY79) in SGF at pH 2, pH 3 and pH 4. As shown in Table 2 spores exhibited almost no loss in viability after 1 h of incubation. By contrast vegetative cells were labile at pH 2 showing a 4-5 log reduction in cfu count after 1 h incubation. Interestingly, we found that at pH 4, most closely resembling the gastric pH after a full meal, vegetative cells of all three strains showed no sensitivity to SGF and total cfu count was unaffected. This implies that if live *B.*

Table 2. Survival to intestinal conditions of *Bacillus subtilis* strains.

Strains	**Initial cfu**	**cfu after 1 h in PBS[1]**	**cfu after 1 h in SGF[2]**		
			pH 2	**pH 3**	**pH 4**
PY79 spores	9.8×10^{8}	9.8×10^{8}	6.5×10^{8}	7.2×10^{8}	8.8×10^{8}
HU58 spores	1.2×10^{9}	1.1×10^{9}	9.0×10^{8}	9.3×10^{8}	9.9×10^{8}
PXN21 spores	7.6×10^{8}	7.6×10^{8}	5.2×10^{8}	6.0×10^{8}	7.0×10^{8}
PY79 vegetative cells	1.5×10^{8}	1.3×10^{8}	1.2×10^{4}	6.0×10^{6}	1.0×10^{8}
HU58 vegetative cells	8.5×10^{8}	7.0×10^{8}	4.0×10^{4}	7.1×10^{7}	7.8×10^{8}
PXN21 vegetative cells	3.0×10^{9}	2.9×10^{9}	1.4×10^{5}	2.0×10^{8}	2.6×10^{9}

[1] PBS: phosphate buffered saline.
[2] SGF: simulated gastric fluid.

subtilis vegetative cells are consumed after a full meal, the majority of bacilli should pass through the gastric barrier unscathed.

Persistence of spores in the gastro-intestinal tract

The passage of spores through the murine GI-tract can provide a useful insight into the intestinal residency of *B. subtilis*. Studies have shown that following a single inoculum of *B. subtilis* spores the first detectable counts of spores in the faeces are found after 3 h. This indicates that the minimum time taken to transit the murine gut is 3 h (Hoa *et al.*, 2001). Despite this, following a single large inoculum (~10^9 spore cfu) spores are still being shed from the gut 7-21 days post-dosing (Barbosa *et al.*, 2005; Tam *et al.*, 2006). This suggests that spores must be residing in the GI-tract either by adhering to the gut mucosa or by germinating and establishing biofilms. Interestingly, *B. subtilis* shows low levels of adhesion to intestinal cells and it is probable that the formation of biofilms may play an important role in maintaining gut residency (Hong *et al.*, 2009a). Molecular studies have shown that spores, orally administered to mice can germinate, proliferate and then re-sporulate as they pass through the GI-tract (Casula and Cutting, 2002; Tam *et al.*, 2006). In poultry evidence for mass germination of spores has also been observed (Cartman *et al.*, 2008). This implies that spores adapt to entry into the GI-tract and, by germinating, outgrowing and establishing themselves in the gut are able to reside, albeit transiently. Spore counts are eventually reduced suggesting that a natural process of competition with other members of the microflora maintains a stable population of bacilli in the gut. In humans, the resident population of *Bacilli* is approximately 10^4 spores/g of faeces (Hong *et al.*, 2009b). One interesting observation that has been made is that the transit period for *B. subtilis* varies considerably between different strains. For example, with PY79, a laboratory strain, following a dose of 10^8 spores no significant counts are detected after 7 days, while for natural isolates such as HU58 counts are still detectable 18-21 days post-dosing. This extended transit time may reflect inherent characteristics of natural strains of *B. subtilis* enabling them to maintain a gut residency, for example, the ability to adhere and/or to establish robust biofilms. We therefore evaluated PXN21, HU58 and PY79 in parallel experiments using groups of inbred mice (Figure 3). Following a single oral dose of 1×10^9 spores PXN21 was found to exhibit a profile of shedding similar to HU58 with spores still present in the faeces 18 days post-dosing. By contrast, PY79 spores were cleared after just 7 days. We interpret this as evidence that PXN21, like HU58, is better adapted to gut residency than laboratory strains and this correlates with the ability of both strains to produce biofilms.

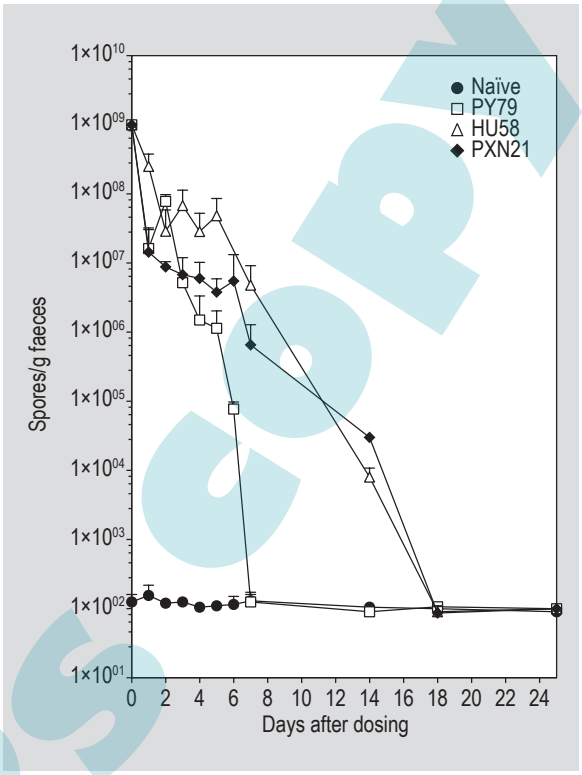

Figure 3. Persistence of *Bacillus subtilis* strains PY79, HU58 and PXN21 in the gastro-intestinal tract of inbred Balb/c mice dosed with 1×10^9 spores. Naïve animals received only PBS buffer. The detection limit was ~10^2 spores/g.

Induction of non-specific immune responses

A principal mechanism for how probiotic bacteria might produce a beneficial effect is immune stimulation. This would be the innate immune system (which is not a memorised response) and, although short-lived, repeated dosing with probiotic bacteria could stimulate the immune system enabling protective immune responses. This assumption is supported by studies which show that innate immunity can provide protection against a number of important pathogens (Korbel *et al.*, 2008; Trinchieri and Sher, 2007). Strikingly, evidence is now emerging that innate immunity can carry some memory perhaps as long as six months (Gillard *et al.*, 2011). The ingestion of probiotic bacteria could lead to their interaction with certain types of immune cells such as macrophages and dendritic cells lying in the lymphoid tissues of the GALT. Phagocytosis then leads, in turn, to a number of signals that sensitise the immune system, for example, cytokine secretion (e.g. IFN-γ), activation of the complement cascade and certain lymphocytes such as natural killer (NK) cells. Innate immunity also serves to prime the adaptive (memorised) immune response. Bacterial spores of PY79 and HU58 have been shown to stimulate the expression of Toll-like

receptors TLR2 and TLR4 and this is a key step in signalling the innate immune response (Huang *et al.*, 2008; Trinchieri and Sher, 2007). We next set out to establish whether PY79 and PXN21 spores, delivered orally, could stimulate non-specific innate immune responses using a dosing regimen that would resemble that of probiotic usage in humans. HU58 was not examined here since its ability to stimulate innate immunity has been described previously (Huang *et al.*, 2008). Groups of inbred mice were dosed orally with 1×10^9 spores of PY79 or PXN21 every 7 days for 10 weeks. At the end of the dosing regimen splenocytes were cultured and stimulated with a variety of exogenous antigens: *E. coli* LPS, CDTA of *C. difficile* (Dove *et al.*, 1990), gluteraldehyde-inactivated *C. difficile* 630 spores, as well as two *C. difficile* spore coat proteins, CotC and CotD. Measurement of IFN-γ in stimulated splenocytes showed clear differences between PY79 and PXN21 spores (Figure 4). IFN-γ was abundantly produced in splenocytes from PXN21-immunised mice showing that PXN21 must sensitise spleen cells such that stimulation with an exogenous antigen leads to production of IFN-γ, the primary cytokine involved in macrophage activation and mediating the host's defences to bacterial and viral pathogens. By contrast, splenocytes from PY79-dosed mice produced little to no detectable levels of IFN-γ when stimulated with CDTA or LPS and a low level of IFN-γ (approximately 10-times less than in PXN21 dosed mice) when stimulated with inactivated *C. difficile* spores. These results demonstrate the ability of PXN21 spores to stimulate non-specific (innate) immunity and the differences in magnitude between mice dosed with a domesticated strain of *B. subtilis* and PXN21 are both intriguing and unexpected. The low levels of IFN-γ produced by splenocytes from PY79-dosed mice, when stimulated with *C. difficile* spores, may reflect shared antigens between spores of *B. subtilis* and *C. difficile* spores and thus could imply an adaptive immune response. This study suggests that non-specific immune responses induced by *B. subtilis* should be assessed on a strain-by-strain basis and any conclusions regarding immune modulation cannot be considered universally to apply to all members of the taxonomic group.

Incorporation in food products

Bacterial spores typically are resistant to maximum temperatures of 60-85 °C. Prolonged exposure to temperatures above these limits results in loss in viability although this is not immediate and for a population of spores there is steady decline in viability. Spores of *B. coagulans* are being incorporated commercially in baked foods, e.g. cakes and muffins (Cutting, 2011) and presumably the temperatures used in baking do not inactivate spores. We designed an experiment to assess whether *B. subtilis* spores could survive the temperatures used in baking biscuits. Our analysis revealed that baking at 235 °C for 8 min reduced the count of live spores by just over 1 log (Figure 5). After this, counts remained stable for a period of three months demonstrating that live spores of *B. subtilis* can be incorporated into baked foods. The initial reduction in spore counts is most likely due to those spores that are present on the surface layers of the biscuit where temperatures were highest or alternatively some spores may have germinated during the initial preparation of the biscuit dough. The biscuit mix is presumably able to shield spores from the extremes of temperature. Untreated biscuits were found to contain a basal level of heat resistant spores

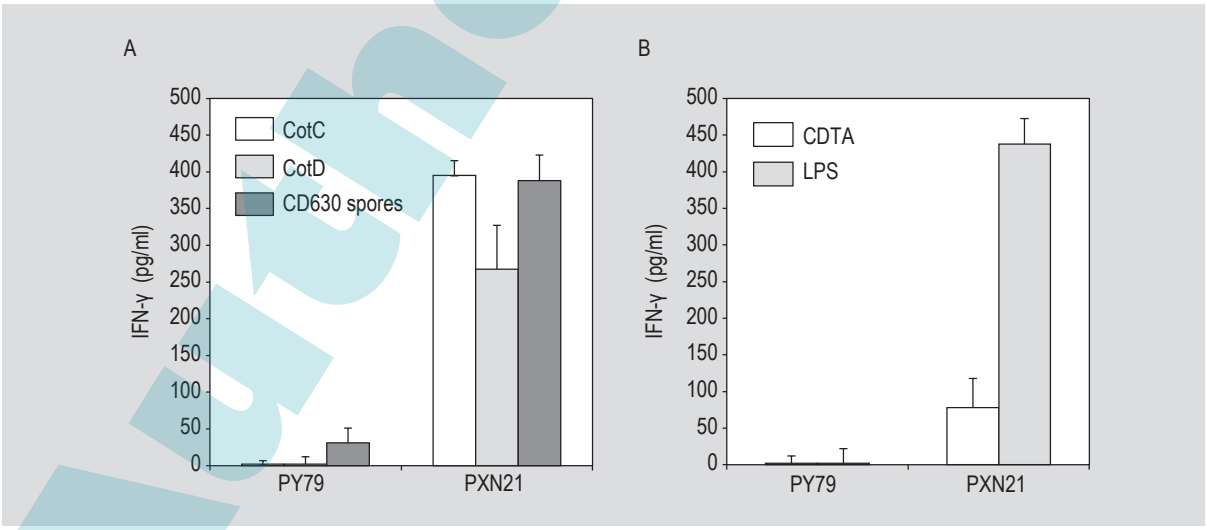

Figure 4. Induction of non-specific immunity by *Bacillus subtilis* strains PXN21 or PY79 in inbred mice dosed with 1×10^9 spores every 7 days for 10 weeks. (A) IFN-γ levels following stimulation with two *Clostridium difficile* spore coat proteins, CotC and CotD, as well as with gluteraldehyde inactivated *C. difficile* 630 spores. (B) IFN-γ levels following stimulation with the C-terminus of *C. difficile* toxin A and *Escherichia coli* lipopolysaccharides.

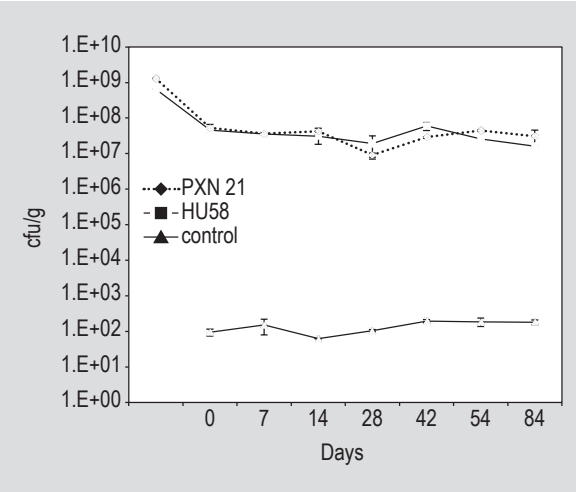

Figure 5. Survival of spores *Bacillus subtilis* strains HU58 or PXN21 in biscuits baked at 235 °C for 8 minutes. The detection limit was ~10^2 spores/g.

(~10^2/g). We conducted preliminary characterisation of these bacteria and found all to contain spores, they grew aerobically and were not strains of *B. cereus,* a *Bacillus* species commonly found in wheat-based products. Since the biscuit mix was not sterilised we assume this basal level of spores originates from one or more of the ingredients (e.g. wholemeal flour).

4. Conclusions

This work shows that bacterial spores can be incorporated into baked food products and can survive baking for 8 minutes at 235 °C with no more than a 1-log reduction in viability. This demonstrates that *Bacillus* spores can have applications as probiotic additives into a number of food products (e.g. chocolate, cakes, etc.) that could not be considered for the more common lactobacilli and bifidobacteria probiotics. We have also demonstrated that PXN21, a *B. subtilis* strain found in a commercial product carries a number of attributes common in human isolates of *B. subtilis* that support its use as a probiotic. This includes prolonged persistence in the GI-tract, the formation of robust biofilms, rapid sporulation and the stimulation of innate immune responses. *B. subtilis* currently carries QPS status in Europe and there is likely to be increased interest in using this heat-stable bacterium as a food ingredient.

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Appendix A. Growth of *Bacillus subtilis* strains PXN21, HU58 and PY79 on CM and CMK media

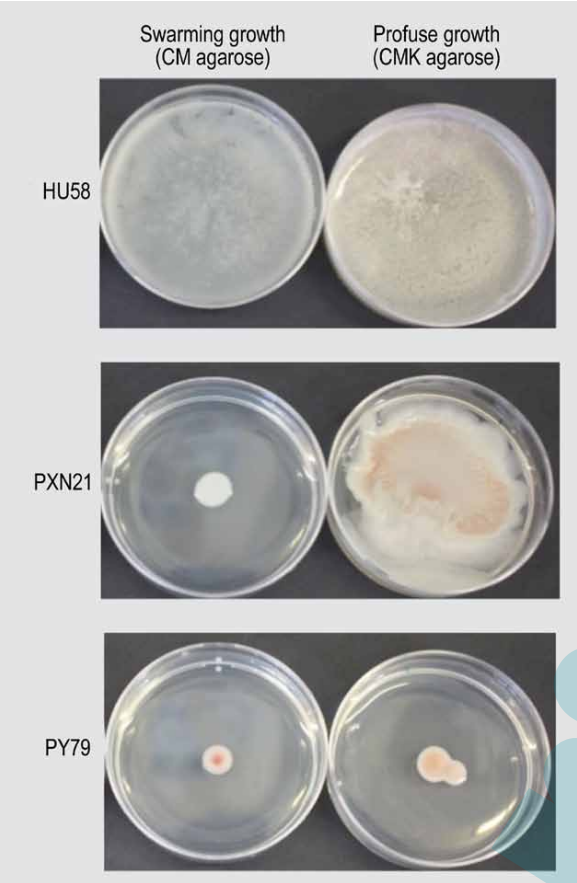

Figure A1. Swarming and profuse growth evaluated on CM and CMK media.