The role of *Clostridium difficile* spore surface proteins in mammalian cell interactions

A thesis submitted for the degree of Master of Philosophy

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Declaration of Authorship

I, Saba Anwar 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: _____

Date: _____

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Abstract

Clostridium difficile (*C. difficile)* is a Gram positive, anaerobic spore-forming bacterium, which is estimated to be responsible for about one-quarter of all hospital acquired infections. *C. difficile* is the main cause of pseudomembranous colitis (PMC) and antibiotic associated colitis and diarrhoea known as *C. difficile* associated disease (CDAD). CDAD is difficult to treat and contain due to the ability of *C. difficile* to form robust spores that can persist and be easily transferred from one individual to another in a hospital environment. More than 90% of *C. difficile* infection (CDI) occurs after or during antibiotic treatment. Antibiotics act by disrupting the normal colonic flora allowing the germinating *C. difficile* spore to outgrow and colonize the host's intestinal tract. During the course of CDI, *C. difficile* undergoes sporulation and releases spores to the colonic environment. The elevated relapse rates of CDI suggest that *C. difficile* spores has mechanisms to efficiently persist in the host colonic environment however little is known about the role of proteins in the outermost layer of the spore has to play in persistence.

This thesis investigates the role of the spore surface proteins in mammalian cell interactions. Results suggest that the spore coat protein CotE could have a role to play in the adherence of the spore to intestinal epithelial cells and mucus. The BclA protein may have a similar role to that of BclA in *Bacillus anthracis* (*B. anthracis*) where BclA has been shown to promote tropism towards macrophages and a reduction in adherence to lung epithelial cells.

Little is known about the interaction of the *C. difficile* spore and innate immune cells, therefore the interaction of *C. difficile* spores with the murine macrophage cell line J774.1

has been examined. Results suggest that BclA may act as a Pathogen Associated Molecular Pattern (PAMP) which is recognized by Pattern Recognition Receptors (PRRs) of macrophages leading to efficient phagocytosis of the *C. difficile* spore. Spores are able to remain dormant and are able to survive within macrophages and produce cytotoxic effects to J774.1 cells.

Persistence of *C. difficile* in the host might be mediated through the adherence of spores to the host's intestinal epithelium and surviving attacks of phagocytic cells. Investigating mechanisms of persistence of the spore in the host is of great importance in understanding the pathogenesis of CDI and will lead to effective diagnosis, treatment and prevention of the infection.

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Abbreviations

AAD – Antibiotic Associated Diarrhoea

BclA – *Bacillus* Collagen Like protein of *Anthracis*

BHIS – Brain Heart Infusion Supplemented with yeast extract and L-cysteine

BHISS – BHIS supplemented with sodium taurocholate

Ca-DPA – Calcium- Dipicolinic Acid

CbpA – Collagen Binding Protein A

CCP – Classical Complement Pathway

CDAD – *Clostridium difficile* associated disease

CDI – *Clostridium difficile* Infection

CEC – Colonic Epithelial Cells

CFU – Colony Forming Unit

CLR – C-Type Lectin Receptor

CWP – Cell Wall Protein

CXCL1 - CXC motif ligand 1

DAPI - 4’6-Diamidino-2-phenylindole

DC – Dendritic Cell

DMEM - Dulbecco’s Modified Eagle Medium

DPA - Dipicolinic Acid

DPBS - Dulbecco’s Phosphate-Buffered Saline

ELISA – Enzyme-Linked Immunosorbent Assay

ERM - Erythromycin

FBS – Foetal Bovine Serum

GI – Gastrointestinal

GluNAc - *N*- acetylglucosamine

IBD – Inflammatory Bowel Disease

IESC - Intestinal Epithelial Stem Cells

kDa – Kilodalton

LBP - Lipopolysaccharide Binding Protein

LPS - Lipopolysaccharide

LTA - Lipoteichoic Acid

MOI – Multiplicity of Infection

MSCRAMM – Microbial Surface Components Recognizing Adhesive Matrix Molecules

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

MyD88 - Myeloid Differentiation Primary Response 88

NAP1 - North American Pulsed Field type 1

NOD – Nucleotide-binding Oligmerization Domain

OD – Optical Density

PAMP – Pathogen Associated Molecular Pattern

PCR – Polymerase Chain Reaction

PG – Peptidoglycan

PMN - Polymorphonuclear Granulocytes

PRR – Pattern Recognition Receptor

PMC - Pseudomembranous Colitis

RIG - Retinoic Acid-inducible Gene

RLR - (RIG)-1-like receptors

ROS – Reactive Oxygen Species

SASP - Small Acid Soluble Proteins

SATH – Spore Adhesion to Hydrocarbon

SCLE – Spore Cortex Lytic Enzyme

SLP – Surface Layer Protein

SMC – Sporulation Medium supplemented with L-cysteine

SOD – Superoxide Dismutase

TcdA – Toxin A

TcdB – Toxin B

TEM – Transmission electron microscopy

TGY – Tryptic Glucose-yeast Extract Medium

TIR – Toll Interleukin Receptor

TLR – Toll-Like Receptor

TMB - 3, 3′, 5, 5′-tetramethylbenzidine

UC – Ulcerative Colitis

UV - Ultraviolet

Chapter 1

Introduction

1.1 *Clostridium difficile*

C. difficile is a Gram-positive spore forming anaerobic bacterium. It is the main cause of approximately 20% of antibiotic associated diarrhoea (AAD) cases (Pizarro-Guajardo et al., 2014). The disease ranges in severity ranging from mild diarrhoea, through to moderately severe disease with watery diarrhoea, abdominal pain, to life threatening pseudomembranous colitis (PMC) and toxic megacolon that carry mortality rates of approximately 5% (Paredes-Sabja et al., 2014) (Borellio, 1998).

In 1935, Hall and O'Toole first isolated *C. difficile* from the stool of a healthy infant (Hall et al., 1935). *C. difficile* was initially named *Bacillus difficilis* to reflect the difficulties they encountered in its isolation and culture. PMC was first described in 1893 however it was not until 1978 that George and colleagues discovered that *C. difficile* was the organism responsible for the majority of cases of AAD (Heinlen et al., 2011). During the mid and late 1990s the reported incidence of CDI in acute care hospitals in the United States remained stable at 30-40 cases per 100,000 population. This number rose in 2001 to approximately 50 cases with an increase of 84 cases reported in 2005. There was also the reports of increases in severe or fatal infection and in England CDI was listed as the primary cause of death for 499 patients in 1999. This number increased to 1,998 in 2005 and 3,393 in 2006. At this point sporadic outbreaks had been reported in many hospitals worldwide. In Quebec,

Canada, the overall incidence of CDI was relatively stable during the period from 1991-2002, in 2003 the population incidence had increased 4-fold compared to 2002. All major acute care hospitals in this region were affected, causing much concern for the general population. The major increase in incidence of CDI in Quebec was also associated with an increase in disease severity and mortality (Kelly et al., 2008). Similar increases in the incidence, severity and mortality related to CDI occurred in the United States. McDonald et al. examined *C. difficile* isolates from eight health care facilities across six states during outbreaks of the infection between 2000-2003. Isolates from a single strain accounted for at least half of the isolates from five facilities and 82% of stool samples from Quebec outbreak were positive from the same strain (McDonald et al., 2005). This epidemic strain was identified in the 1980s by restriction endonuclease analysis and was named BI but it is currently referred to as North American Pulsed Field type 1 (NAP1) and PCR ribotype 027 (i.e. B1/NAP1/027) (Kelly et al., 2008). In the early stages of *C. difficile* research the role of the hospital in CDI was not acknowledged. It was observed that hamsters which inhabited a new facility would not get CDI unless they were given *C. difficile*, however once the pathogen was established in the animal facility there was no need to again give *C. difficile* to hamsters. This leads to the assumption that the hospital is a central point of infection, which is due to the large number of patients and a large proportion of those who are elderly and undergoing antibiotic therapy. *C. difficile* was associated with an epidemic of antibiotic-associated colitis at the long-term care facility of Barnes Hospital in 1974 and in the surgical ward at a General Hospital in Birmingham, in 1978 (Bartlett., 2008).

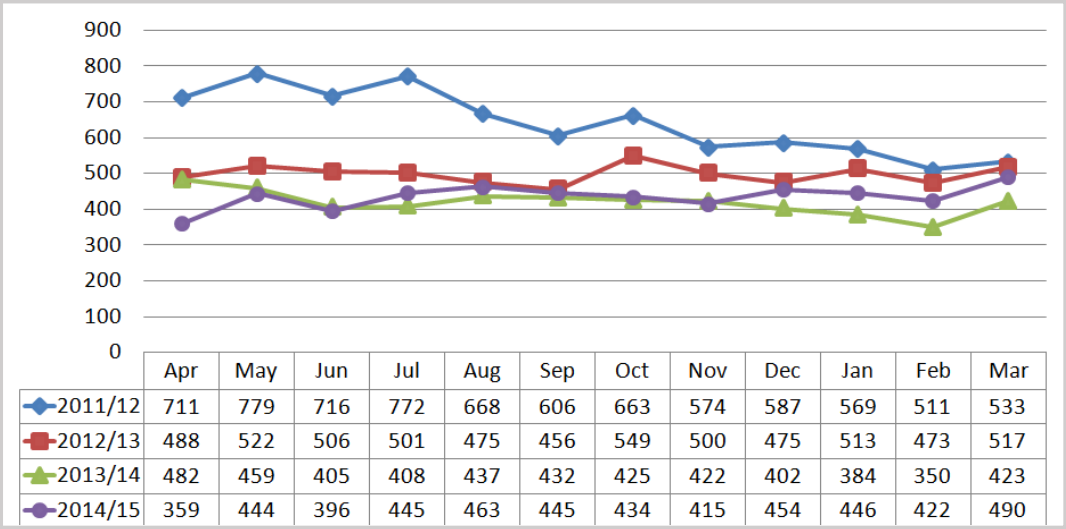
Three bacterial factors have been associated with outbreaks of CDI caused by the virulent BI/NAP1/027 strain, increased production of toxins A and B,

fluoroquinolone resistance and production of binary toxin. Toxin A and B are the major virulence factors of *C. difficile*, they are transcribed from a pathogenicity locus that encompasses five genes: two toxin genes *tcdA* (toxin A) and *tcdB* (toxin B) and three regulatory genes one of which, *tcdC* encodes a negative regulator of toxin transcription. TcdC proteins inhibit toxin transcription during the early, exponential growth phase of the bacterial life cycle. B1/NAP1/027 carries deletion mutations in *tcdC* inhibitory gene that have been associated with an increase by more than a factor of 10 in the production of toxins (Warny et al., 2005). Resistant strains may have a competitive advantage in a hospital environment where fluoroquinolone use is widespread, limiting fluoroquinolone use may help to contain the outbreaks caused by BI/NAP1/027. Another possible virulence factor of BI/NAP1/027 strain is the production of binary toxin that is unrelated to the pathogenicity locus. Binary toxin has enterotoxic activity *in vitro* but its role, if any, in the pathogenesis of CDI is unclear (Geric et al., 2006). *C. difficile* strains that produce binary toxin but not toxins A and B do not appear to be pathogenic. Findings that BI/NAP1/027 epidemic strains produce binary toxin has caused speculation that this toxin could act synergistically with toxins A and B causing severe colitis (Kelly et al., 2008).

Soon after the first descriptions of CDI in the late 1970s effective therapy with either metronidazole or vancomycin was reported. These are the two treatments still used today, however since 2000 higher failure rates have been reported for metronidazole, for example, in the outbreak of CDI in Quebec 26% of patients did not respond to metronidazole treatment. The two agents showed similar efficacy in mild infection, in patients with severe infection vancomycin was significantly more effective. For this reason metronidazole remains the treatment of choice for mild infection because

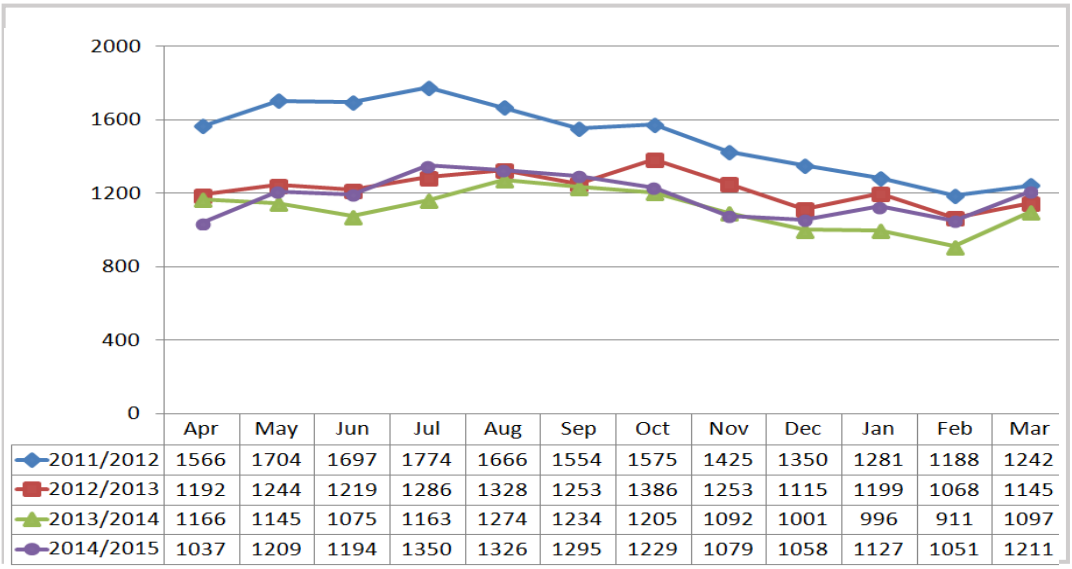
of its low cost and fears of over proliferation of vancomycin resistant nosocomial bacteria (Wilcox et al., 1998).

C. difficile is proving to be a major burden on healthcare services across Europe and North America as the incidence of *C. difficile* is significantly increasing in healthcare environments and is the leading cause of healthcare-associated infections (Giel et al., 2010). However recent epidemiologic data suggest that *C. difficile* is also becoming an important pathogen in the community with a number of reports identifying a significant proportion of CDI symptoms developing outside the healthcare setting (Fig. 1.2) (Evans et al., 2015). Infectious Diseases Society of America proposed guidelines for the classification of CDI. CDI is defined as community acquired if symptom onset occurs in the community or within 48 h of admission to hospital after no hospitalisation in the past 12 weeks. Defined as hospital acquired if onset of symptoms occurs more than 48 h after admission to or less than 4 weeks after discharge from a healthcare facility (Gupta et al., 2014). Figure 1.1 and 1.2 show that the rates of both hospital and community acquired CDI is on the rise. Of the 20,899 reported cases 73% were recorded as outside of the hospital setting over the last 13 months (MRSA action UK).



	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar
2011/12	711	779	716	772	668	606	663	574	587	569	511	533
2012/13	488	522	506	501	475	456	549	500	475	513	473	517
2013/14	482	459	405	408	437	432	425	422	402	384	350	423
2014/15	359	444	396	445	463	445	434	415	454	446	422	490

Figure 1.1 Graph showing the trends in hospital apportioned *C. difficile* infections in patients aged 2 years and over April 2011-March 2015.



	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar
2011/2012	1566	1704	1697	1774	1666	1554	1575	1425	1350	1281	1188	1242
2012/2013	1192	1244	1219	1286	1328	1253	1386	1253	1115	1199	1068	1145
2013/2014	1166	1145	1075	1163	1274	1234	1205	1092	1001	996	911	1097
2014/2015	1037	1209	1194	1350	1326	1295	1229	1079	1058	1127	1051	1211

Figure 1.2 Graph showing the trends in community apportioned *C. difficile* infections in patients aged 2 years and over April 2011 – March 2015.

A number of risk factors are associated with CDI which include hospitalisation or prolonged stays in hospital, exposure to antimicrobial agents including clindamycin, cephalosporin and fluoroquinolones. Older age is also a known risk factor for development of CDI, CDI rates have been shown to be several-fold higher in persons aged >65 years than in younger age groups. Therefore, individuals at highest risk

from CDI are those aged >65 years of age, have recently undergone antibiotic treatment and are hospitalized.

More than 90% of CDI occurs during or after antibiotic treatment, this is because antibiotics act by disrupting the normal protective flora of the gut allowing spores of *C. difficile* to germinate outgrow and disseminate in the gut environment. Higher incidences of infection in the elderly is due to the deterioration of the normal protective flora of the gut and therefore leading to poorer colonization resistance allowing *C. difficile* to colonize the gut (Borellio., 1998).

C. difficile is difficult to treat and contain due to the ability of the bacterium to form robust spores that are easily transferable from one individual to another in a hospital environment. Current treatment strategies for CDI involve administering vancomycin and metronidazole however, these antibiotics also disrupt the flora of the gut and therefore 10-40% patients whose symptoms improve also suffer relapse of CDI (Sorg et al., 2010).

1.1.1 Structure of spores

Spores are dormant and resistant to many environmental stresses such as wet and dry heat, ultraviolet (UV) light and γ- radiation, desiccation and toxic chemicals that rapidly kill growing bacteria. As a consequence spores are able to survive for long periods of time. Although many factors contribute to spore resistance and long term survival, minimization of damage to spore DNA is essential (Setlow., 2007). Spore resistance is due to a variety of factors including spore coat and impermeability of inner spore membranes. The spore consists of many layers, the inner most layer is known as the core and contains the spore DNA, RNA and most enzymes. The core

also contains properties which contributes to the spore's resistance properties. The most important factors that contribute to the spores resistance properties is the low water content and the elevated levels of dipicolinic acid (DPA) chelated with calcium (Ca-DPA) and saturation of DNA with α/β type small acid soluble proteins (SASP). The core is surrounded by a compressed inner membrane protein, which has a similar phospholipid composition to growing bacteria however shows low permeability to small molecules. This quality protects the core from DNA damaging molecules. The germ cell wall surrounds the spore inner membrane and becomes the cell wall of outgrowing bacterium. Enclosing the germ cell wall is a thick peptidoglycan layer. The peptidoglycan layer is surrounded by an outer membrane derived from the mother cell that is crucial for spore formation but has no resistance properties. A proteinaceous coat surrounds the outer membrane, in *Bacillus subtilis* (*B. subtilis*) the coat is composed of approximately seventy proteins unique to spores and provides spore resistance to environmental stresses (Setlow., 2006). It is probable that *C. difficile* has similar quantities (Permpoonpattana et al., 2011). The spore coat is the outermost layer in some species whereas in others the exosporium is the outermost layer and consists of collagen-like glycoproteins. The exosporium has been shown to play a role in pathogenesis of *Bacillus anthracis* (*B. anthracis*) spores (Bozue et al., 2007) (Oliva et al., 2008). The ultrastructure of *C. difficile* 630 spores is markedly different in ultrastructure compared to *B. anthracis* and *Bacillus cereus* (*B. cereus*) spores. *C. difficile* 630 spore exosporium lacks the distinctive paracrystalline basal layer and hair-like outer layer typical of the exosporium of *B. anthracis* and *B. cereus* spores (Escobar-Cortes et al., 2013). In contrast, spores derived from *C. difficile* strain R20291 have an exosporium-like structure with hair-like nap structure similar to that of *B. anthracis* (Paredes-Sabja et al., 2014).

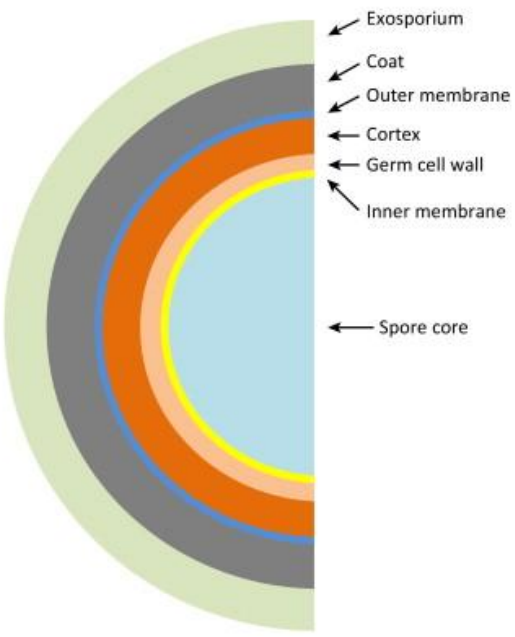


Figure 1.3. Structural layers of *C. difficile* spores. Figure reproduced with permission from (Sorg et al., 2014).

1.1.2 *C. difficile* spore surface proteins

Studies have identified spore proteins that localize to the surface of *C. difficile* strain 630. Several of these proteins have been shown to have manganese catalase activity, these include CotCB, CotD and CotG. Additional *C. difficile* spore surface localized proteins with enzymatic activity include SodA, a homolog of the *B. subtilis* superoxide dismutase (SodA), and CotE, a bifunctional protein with peroxiredoxin (amino-terminal) and chitinase (carboxy-terminal) activity (Permpoonpattana et al., 2013). The catalase and peroxiredoxin activities are potential antioxidants and would reduce the cellular toxicity of hydrogen peroxide by conversion to oxygen and water. In *B. subtilis*, hydrogen peroxide plays a role in spore coat assembly by serving as a substrate for the SodA enzyme to cross link tyrosine rich spore coat proteins together (Henriques et al., 1998). In regards to the presence of chitinase, it would be expected that this enzyme would have a role to play in the breakdown of fungi and other

biological matter whether in the soil or the intestine. Spores are dormant and therefore chitinase activity is activated during spore germination, enabling a potential source of nutrients as *C. difficile* cell emerges from its coat. This has been evidenced by a decrease in chitinase activity as spores mature but increased during spore germination and following sonication, events that both lead to the rupture of the spore coat layers (Permpoonpattana et al., 2011). An additional consequence of a chitinase and a peroxiredoxin displayed on the surface of *C. difficile* relates to the potential link between peroxiredoxins, chitinases and inflammation. Peroxiredoxin secreted from tumour cells was previously shown to induce proinflammatory cytokines in macrophages through interactions with Toll-like receptor (TLR) 4 and to promote chronic inflammation, which could support tumour growth (Riddell et al., 2010). Regarding chitinases, some inflammatory conditions of the gastrointestinal (GI) tract lead to the induction of host cell chitinases by triggering the increased uptake of intracellular bacteria by colonic cells (Kawada et al., 2007) (Kawada et al., 2008). As some of the symptoms of CDAD resemble those of inflammatory bowel disease (IBD) and ulcerative colitis (UC), *C. difficile* chitinase may play a direct role in infection and not just macromolecule degradation (Permpoonpattana et al., 2011).

Further spore surface localized proteins have also been identified and characterized, these include CotA, CotB and CotF. *C. difficile* CotA mutant spores have an unusual phenotype, approximately half of the spores examined by transmission electron microscopy (TEM) showed major morphological defects and lacked an electron dense coat layer. These observations suggest that CotA plays an essential role in spore coat and exosporium assembly. No detectable differences were observed in spore ultrastructure of *C. difficile* CotB spores and the role of CotF is yet to be investigated.

Spore coat layers are not only enzyme rich but are also quite fragile. When examining spores of wild type 630Δ*erm* prepared with or without purification clear differences were observed with unpurified spores showing an abundance of material detached from the spore. In unpurified spore preparations, a diffuse material associated with the outer layer of the spore coat was nearly always seen. It is believed that the diffuse surface material is the exosporium (Permpoonpattana et al., 2013). Suggesting that spore purification methods can lead to removal of the exosporium.

In *B. anthracis*, BclA (*bacillus* collagen-like protein of *anthracis*) is a glycoprotein and a major component of the hair-like projections that cover the exosporium (Brahmbhatt et al., 2007). Three paralogs of the *B. anthracis* collagen-like protein BclA are encoded in *C. difficile* 630 genome BclA1, BclA2 and BclA3. These proteins contain three domains i) an N-terminal domain of variable size, ii) a collagen-like domain and iii) C-terminal domain. Precise roles of BclA paralogs remain unclear. It is thought that BclA1 has a role to play in the initial stages of infection and host susceptibility (Phetcharaburanin et al., 2014).

Barra-Carrasco et al, demonstrated that the cysteine-rich exosporium protein CdeC which is unique to *C. difficile* is essential for the proper assembly of the exosporium of *C. difficile* spores. A mutation in *cdeC* considerably reduced the abundance of the exosporium layer leading to defective coat and exosporium assembly. TEM analyses of *cdeC* mutant spores revealed that although the outer coat layer was thinner than that of wild type spores, the inner coat was thicker. This observation suggests that CdeC could act as an anchoring protein at the interface of the spore coat and exosporium layers (Barra-Carrasco et al., 2013).

1.1.3 Exosporium of *C. difficile* spores

Stability of the exosporium is under much debate, several reports suggest that this layer is fragile and easily lost (Permpoonpattana et al., 2011) (Permpoonpattana et al., 2013) whereas other reports suggest that it is a reasonably stable layer that is only removed by proteases and sonication treatments (Escobar-Cortes et al., 2013). The stability of the exosporium could be related to strain type or the use of proteases in purification procedures. Recent evidence indicates that proteinase K can remove the exosporium layer while leaving the spore coat intact (Escobar-Cortes et al., 2013). The morphology of the exosporium appears to be strain dependent. *C. difficile* 630 spores have an electron dense, compact exosporium layer whereas spores derived from R20291 have a hair-like exosporium layer similar to that of *B. anthracis* (Paredes-Sabja et al., 2014).

The role of the spore coat and the exosporium on CDI and pathogenesis are not known, however recent research has shown that the spore surface of *C. difficile* was shown to interact with unidentified surface receptors of intestinal epithelial cells (Paredes-Sabja et al., 2012). *In vitro* infection of macrophages with *C. difficile* spores showed that spores are cytotoxic to macrophages (Paredes-Sabja et al., 2012). The exosporium also has been shown to give hydrophobicity to spore surface affecting the adherence of spores to surfaces (Joshi et al., 2012). Removal of the exosporium also increases the ability of *C. difficile* spores to outgrow into colonies *in vitro* therefore spores lacking an exosporium are better able to germinate more efficiently (Escobar-Cortes et al., 2013).

1.1.4 Sporulation of *C. difficile* spores

Sporulation is the process by which bacterial species are able to form highly resistant spores. The sporulation cycle of *B. subtilis* has been examined in great detail and offers a useful model to determine the stages of sporulation for *C. difficile*. *C. difficile* exists in two states, a vegetative growth state or a dormant spore state. In the vegetative state *C. difficile* is able to use nutrients to grow and divide. However, when the bacterium is subjected to unfavourable conditions such as starvation the bacterium is able to enter a latent state and form a dormant spore (Setlow, 2003).

Sporulation consists of several stages firstly vegetative cells proliferate by cell division which leads to the production of two identical sister cells. Starvation initiates sporulation which leads to unequal cell division producing a smaller forespore cell and a larger mother cell. The mother cell engulfs the forespore leading to the formation of a double membrane bound forespore. Coat assembly begins after the initiation of engulfment and continues throughout sporulation. In the final step the mother cell lyses releasing the mature spore into the environment. Spores are able to return to vegetative growth when conditions become favourable.

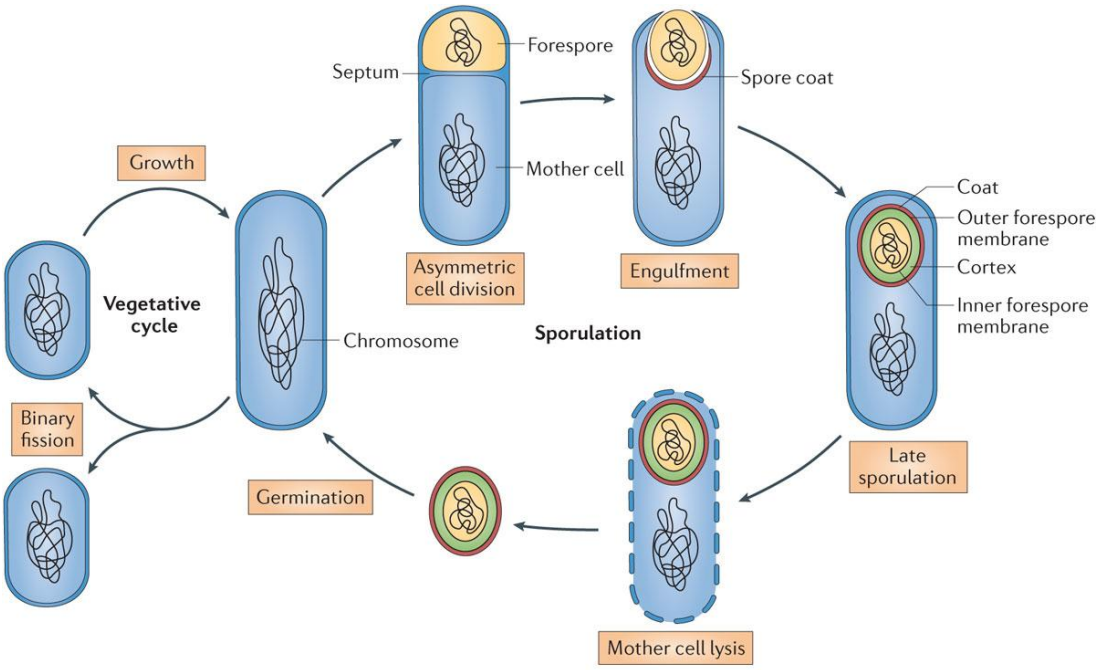


Figure 1.4. Sporulation cycle and germination cycle of *B. subtilis*. Sporulation begins when a sporangium divides asymmetrically to produce two compartments; the mother cell and the forespore, which is separated by a septum. The mother cell engulfs the forespore, and following membrane fusion at the opposite pole of the sporangium, a double membrane bound forespore is formed. Coat assembly begins just after initiation of engulfment and continues throughout sporulation. The peptidoglycan cortex between the inner and outer forespore membranes is assembled during late stage sporulation. In the final stage, the mother cell lyses to release a mature spore. In response to nutrients spores are capable of quickly germinating and continuing vegetative growth. Figure reproduced with permission from (McKenney et al., 2013).

In many *Bacillus* and *Clostridium* species the decision to enter sporulation is controlled by several orphan histidine kinases that can phosphorylate the master transcriptional regulator Spo0A. Histidine kinases are a large family of signal transduction enzymes that autophosphorylate on a conserved histidine residue. Histidine kinases catalyse the transfer of phosphate from ATP to a unique histidine residue (Wolanin et al., 2002). The *C. difficile* strain 630 genome encodes five orphan histidine kinases CD1352, CD142, CD1579, CD1949 and CD2492. The mechanism by which Spo0A is phosphorylated during the initiation of *C. difficile* sporulation is not known. It is proposed that the phosphoryl group is transferred directly from the histidine kinase to Spo0A as seen in *Clostridium acetobutylicum*

(*C. acetobutylicum*). Currently the only *C. difficile* orphan histidine kinase shown to autophosphorylate and transfer a phosphate directly to Spo0A is CD1579. The ability of the other orphan histidine kinases to phosphorylate Spo0A remains unclear (Paredes-Sabja et al., 2014).

1.1.5 Germination of *C. difficile* spores

Even though spores are metabolically dormant they interact with the environment and germinate when conditions become favourable for vegetative growth.

Endospore production is necessary for the spread of CDI, however in order to cause disease, these spores must germinate and return to vegetative cell growth. Spore germination is defined as the irreversible loss of spore-specific properties and it is this process which allows for vegetative cell growth and subsequent toxin production (Burns et al., 2010). Current mechanistic knowledge of germination is based on research carried out on *B. subtilis*. Germination is initiated when a spore senses specific effectors known as germinants. *B. subtilis* can germinate through the binding of germinants to specific receptors located in the inner membrane of the spore. The spore is then committed to germination and following events comprise of the release of monovalent cations (H^+, Na^+ and K^+) and a large store of Ca-DPA. Next, hydrolysis of the spore peptidoglycan cortex begins during which the formerly low water content of the spore is returned to that of a vegetative cell and the core is able to expand and, in turn, allowing for enzyme activity, metabolism and vegetative cell outgrowth (Burns et al., 2010).

In *B. subtilis,* germinant receptor mediated germination can be divided into two stages. Stage I is triggered when germinant receptors in the inner spore membrane

respond to germinants such as L-alanine. The interactions of germinant with GerAA-AB-AC germinant receptor leads to release of Ca-DPA from the core most likely through the SpoVA channel in exchange for water. Release of Ca-DPA from the core completes stage I. Stage II is activated by the abundance of exogenous Ca-DPA. The core is degraded by the spore cortex lytic enzymes CwlJ and SleB. The mechanism of action of SleB is unknown, CwlJ is activated by DPA leading to cortex hydrolysis which leads to swelling of the germ cell wall and core expansion. Core expansion results in further hydration of the core and complete Ca-DPA release. Upon completing stage II spores have lost most of their resistances and are no longer considered dormant and the germinated spore prepares for the outgrowth of a vegetative cell.

C. difficile spore germination is stimulated by a combination of cholic acid derivatives and glycine. While many of the ultrastructural features of the spore are conserved between *B. subtilis* and *C. difficile* there are many differences. A major difference is that *C. difficile* does not encode the classical ger-type germinant receptor. Also *C. difficile* encodes a single spore cortex lytic enzyme (SCLEs), SleC. *C. difficile* is synthesized in the mother cell during spore formation as a preproprotein. The pre-sequence is cleaved off and the proprotein remains inactive in the dormant spore until it is cleaved by a germination specific protease CspB. In *C. difficile cspB* is encoded as a fusion to *cspA*. Upon translation of *cspBA* mRNA, CspBA undergoes interdomain cleavage to generate both CspB and CspA proteins. A third protein, CspC is encoded downstream of CspBA. This protein has been identified as the bile acid germinant receptor (Francis et al., 2013). It is presumed that CspC directly activates CspB, which in turns processes pro-SleC into active SleC leading to the complete degradation of the spore peptidoglycan cortex.

The *C. difficile* germinant receptor complex (CspA, CspB, CspC and SleC) is likely located in or near the spore cortex while the *B. subtilis* germinant receptors are located in the spore's inner membrane. This indicates that there could be fundamentally different mechanisms of germinant receptor mediated *C. difficile* spore germination and *B. subtilis* spore germination. It has recently been shown that cortex hydrolysis precedes DPA release during *C. difficile* spore germination (Francis et al., 2015).

Recent research show that bile salts and glycine act as co-germinants of *C. difficile* spores, whereas the secondary bile salt deoxycholate prevented vegetative cell growth (Sonenshein et al., 2008). Bile is produced by the liver and sorted in the gall bladder. To aid in digestion the gall bladder secretes bile into the duodenum where it helps to absorb fat and cholesterol. The primary bile produced in the liver is comprised of mainly cholate and chenodeoxycholate conjugated with either taurine or glycine. During passage through the distal ileum, bile is actively reabsorbed and recycled to the liver. However some bile passes from the ileum into the cecum where it becomes the substrate for biotransforming reactions by the normal bacterial microflora. Unconjugated primary bile salts are taken up by a small quantity of bacterial species in the colon where they transport unconjugated primary bile salts into the cytosol and by a series enzymatic reactions, converts cholate and chenodeoxycholate to secondary bile salts deoxycholate and lithocholate (Sonenshein et al., 2008).

It is hypothesised that in a healthy host spores can survive the passage though the stomach and pass through the duodenum and into the jejunum where they germinate in response to high concentrations of cholate derivatives and glycine. Germinated

spores pass through the ileum to the anaerobic environment of the cecum. However cholate derivatives that did not undergo enterohepatic circulation are metabolised into secondary bile salt deoxycholate. The deoxycholate produced prevents vegetative growth in *C. difficile* and therefore the host remains uncolonized. Upon antibiotic treatment the normal microflora of the gut is disrupted and the species of bacteria capable of biotransformation of primary bile salts are largely reduced. This reduction leads to an increase in the concentration of primary bile salts and a decrease in the concentration of secondary bile salts in the cecum. The decrease in secondary bile salts may provide an environment in which *C. difficile* can grow and colonize. Therefore the protective role the normal microbial flora plays may be that of metabolising cholate derivatives to deoxycholate, an inhibitor of *C. difficile* growth (Sonenshein et al., 2008).

1.1.6 Virulence factors of *C. difficile*

CDI is caused by the disruption of the normal microbiota of the gut after antibiotic treatment. On exposure of the gut to antibiotics the microbiota becomes disrupted and colonization resistance is compromised. The gut is then susceptible to colonization by *C. difficile* (Poxton et al., 2001).

Principal virulence factors of *C. difficile* have been identified as toxins A and B, which target the Ras superfamily of GTPases for modification via glycosylation, ultimately leading to destruction of intestinal epithelial cells (Voth and Ballard, 2005).

Toxins have a range of biological activities, they have shown the ability to act on intestinal epithelial cells, alter the actin cytoskeleton causing epithelial cell damage

and increased permeability of tight junctions. Toxin A is an enterotoxin that stimulates fluid secretion and intestinal inflammation when administered in animal intestine. It also possesses cytotoxic activity against cultured cells as evidenced by rounding of intestinal and non-intestinal cells in culture. Toxin B does not cause intestinal effects in animals probably due to the absence of receptors for this toxin in the animal intestine. Toxin B is a potent cytotoxin causing rounding of cells at very low concentrations. Although toxin B is inactive in the animal intestine it is cytotoxic to human intestinal cell lines. Both toxins can directly activate human monocytes to release proinflammatory cytokines such as IL-1β, TNF-α and IL-6 and toxin A stimulates human neutrophils as shown by increased neutrophil levels and chemotaxis (Voth and Ballard, 2005).

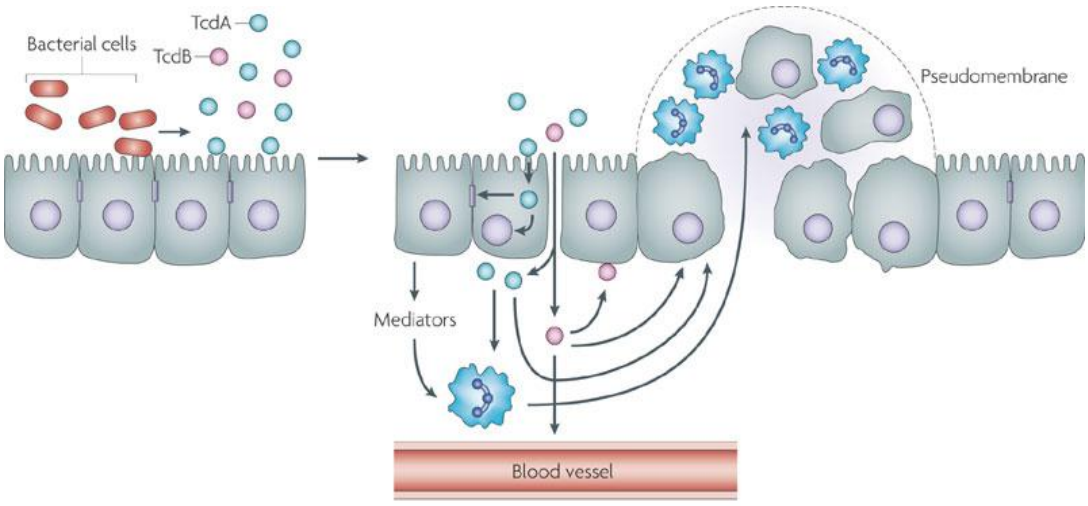


Figure 1.5. Pathogenesis of CDI. Toxin A binds to the epithelium causing disruption of the actin cytoskeleton and loosening of tight junctions of the epithelial barrier. Disruption of tight junctions allows toxin A and B to cross the epithelium. Both toxins are cytotoxic and induce the release of inflammatory cytokines from mast cells and macrophages leading to fluid secretion and intestinal inflammation. Figure reproduced with permission from (Rupnik et al., 2009).

Binding of *C. difficile* toxins to specific surface receptors appears to be an important step in the expression of biologic actions of *C. difficile* toxins. After receptor binding

C. difficile toxins are internalized into the cytosol by endocytosis via coated pits. Once in the cell the main toxin effect is cell rounding caused by disaggregation of filamentous actin. This effect is caused by a direct enzymatic modification of the Rho family of proteins, small GTPases of the Ras superfamily which regulate assembly of actin microfilaments. *C. difficile* toxins possess glucosyltransferase activity against Rho proteins. This modification leads to the disassembly of actin stress fibres, disruption of actin-associated adhesion plaque proteins, and cell detachment and rounding. Disaggregation of filamentous actin may be responsible for the dysfunction of tight junctions in animal and human intestinal epithelium following exposure to *C. difficile* toxins (Pothoulakis., 2000).

Although toxins are regarded as the primary virulence factors, additional factors have a role to play in the pathogenic process. Factors that can improve sporulation/germination, adhesion and persistence of *C. difficile* in the gut can play a role in *C. difficile* pathogenesis (Janoir et al., 2013). Adhesion to host tissues is considered a crucial step which allows pathogenic bacteria to persist in specified niches and it has been proposed that surface-associated determinants are likely to be important in *C. difficile* pathogenesis (Tulli et al., 2013).

1.2 Structure of the intestinal epithelium

The intestinal epithelium is the largest of body's mucosal surfaces, covering approximately 400m^2 of surface area with single layer of cells organized into crypts and villi. Crypts are invaginations of the intestinal epithelium. Lining the base of the crypts are small intestinal Paneth cells which produce antimicrobial proteins and stem cells which repeatedly divide and give rise to the entire intestinal epithelium. Villi are projections of the intestinal lumen into the small intestine which have an outer layer which contain mature absorptive enterocytes, mucus-secreting goblet cells and enteroendocrine cells. The intestinal epithelium is repeatedly renewed by pluripotent intestinal epithelial stem cells (pluripotent IESCs) that are located in the base of the crypts. The majority of cells which border the intestinal lumen are absorptive enterocytes which are adapted for metabolic and digestive function. Secretory intestinal epithelial cells which include enteroendocrine cells, goblet cells and Paneth cells are specialized for maintaining the digestive or barrier function of the epithelium. Luminal secretion of mucins by goblet cells and antimicrobial proteins by Paneth cells creates a physical and biochemical barrier to microbial contact with the surface of the epithelium and underlying immune cells (Peterson et al., 2014).

Human intestinal epithelium is composed of two major cell phenotypes which are enterocytes (absorptive cells) and goblet cells that store and secrete mucin glycoproteins that form mucus. The number of goblet cells varies along the GI tract from 10% in the small intestine to 24% in the distal colon where they form a thick layer of mucus (Hilgendorf et al., 2000). HT29 cells are heterogeneous in that they contain a small proportion, less than 5% of mucus-secreting cells and columnar

absorptive cells. HT29 cells after undergoing treatment with methotrexate results in the elimination of undifferentiated cells and favours the development of a stable differentiated epithelial cell population (Bocker et al., 2000). HT29-MTX cells exhibit a differentiated goblet cell-like phenotype secreting low amounts of MUC2 mucins which are mostly expressed in the small and large intestine (Gagon et al., 2013).

1.2.1 Intestinal epithelium barrier function

The intestinal microbiota functions as an initial stimulus for the development of both innate and acquired components of the mucosal immune system. The endogenous microbial flora of the gut acts as an innate defence mechanism against CDI. The diverse and complex microbial flora likely plays a role in preventing CDI by a process known as colonization resistance. Colonization resistance is a defence mechanism by which the normal, endogenous GI flora prevents the growth and establishment of potentially pathogenic bacterial species. Disruption of the endogenous gut microflora is one of the prerequisites for establishing infection. The mucus barrier also provides an innate defence mechanism against CDI. The mucus gel layer which covers the intestinal epithelium is the location which the host first encounters gut bacteria. The concept of the mucus layer functioning as a defensive barrier is suggested by studies showing altered mucus-related indexes in germfree animals and from consistent evidence of enhanced mucus secretion in response to intestinal microbes (Deplancke et al., 2001). Mucus degradation would compromise epithelial barrier function, leading to bacterial translocation into the lamina propria and subsequent recruitment of inflammatory cells.

1.2.2 Mucins

The mucus layer is an integral structural component of the intestine, acting as a medium for protection, lubrication and transport between the luminal contents and the epithelial lining. The viscoelastic, polymer-like properties of mucus are derived from the major gel-forming glycoprotein components called mucins. Mucins consist of a peptide backbone containing alternating glycosylated and nonglycosylated domains, with *O*-linked glycosylated regions comprising 70-80% of the polymer. *N*-acetylglucosamine, *N*-acetylgalactosamine, fucose and galactose are the four main mucin oligosaccharides. Mucin oligosaccharide chains are frequently terminated with sialic acid or sulfate groups, which account for the polyanionic nature of mucins at a neutral pH. Secretory mucins are secreted from the apical surface of specialized columnar epithelial cells known as goblet cells. A continuous mucus gel layer that varies in thickness covers the epithelial lining of the stomach and large intestine. The mucus layer can reach up to 450μm in the stomach. In the colon, mucus thickness increases gradually from the ascending colon, reaching 285μm in the rectum. The small intestine is covered with a thinner or discontinuous mucus layer, for example the Peyer's patches are not covered in mucus. Mucins are classified into neutral and acidic subtypes, neutral mucins are the predominant subtype expressed in the gastric mucosa and acidic mucins are expressed throughout the intestinal epithelium and dominate in the large intestine. Little is known about the physiological relevance of distinct mucin subtypes however it is suggested that acidic mucins protect against bacterial translocation this is because these are less degradable by bacterial glycosidases and host proteases. This also agrees with the observation that goblet cells in intestinal regions which are largely populated by microbes, express predominately acidic mucins. Evidence suggesting that intestinal microbiota alters

mucin composition originates from histochemical studies where germfree animals were compared to conventionally raised controls or were inoculated with mixed microbial populations. Goblet cells of germfree rodents are fewer in number and smaller in size than those of conventionally raised mice. As a result, the mucus layer may be up to twice as thick in conventionally raised than in germfree rodents, indicating greater mucus production (Kandori et al., 1996).

Some pathogens have the ability to enzymatically degrade mucus which is known as microbial mucolysis. Enzymatic digestion of mucus supplies readily available sources of carbon and energy and in turn allows bacteria to reach the epithelial surface. Degradation of mucin by bacteria involves a multistep process that first starts with proteolysis of the nonglycosylated regions of the mucin glycoproteins by host and microbial proteases. This steps leads to a reduction in mucin gelation and viscosity and leads to the accumulation of highly glycosylated subunits that are resistant to further proteolytic attack. Mucin glycopeptides are then degraded by different bacterial enzymes related to the nature of their oligosaccharide chains. It is unknown whether goblet cell numbers or mucus secretion is increased in response to bacterial mucolysis however it would be a logical defence response for the host to attempt to reconstitute or reinforce the mucus barrier to prevent bacterial translocation (Deplancke et al., 2001).

In the healthy human intestine, MUC2 is the main secreted mucin making up the mucus layer. Under the mucus layer, the cell surface mucins are a major feature of the apical surface of all mucosal epithelial cells. Cell-surface mucins are thought to have a role to play in mucosal defence as they provide a barrier. The intestine

produces the MUC1, MUC3, MUC4, MUC12, MUC13 and MUC17 cell surface mucins (Linden et al., 2008).

Cell surface mucins have an important role in mucosal defence as they provide a barrier and reporting function. Mucins comprise of a group of molecules known as the MUC genes. Presently there are nineteen known members and these can further be divided into two major groups. The secreted mucins which form viscous and largely gel forming extracellular mucus bilayers and the membrane-associated mucins that possess typical membrane spanning domains. The viscous and largely gel forming extracellular mucus bilayers has a number of qualities enabling protection from and interaction with the external environment in the GI lumen. It creates a network which is able to bind water, exchange ions, act as a filtration medium and provides a scaffold for the attachment and storage of growth factors, is an optimal medium for the enteric microflora to interact with the host at the mucosal surface and to repel pathogens. The mucus bilayer provides a medium for many proteins which play roles in mucosal defence. These include secretory IgA, lactoferrin, lysozyme, defensins and protease inhibitors. These proteins allow for antibacterial action (Corfield, 2007).

1.3 Thesis objectives

1) To determine the role of spore coat protein CotE and exosporial proteins BclA1-3 of *C. difficile* in adherence to components of the gut.

Before CDI develops it is assumed the spore has a role to play by attaching to the gut mucosa. Little is known about which aspects of the spores are involved in this process. Therefore ClosTron mutants of *C. difficile* spores devoid of N- and C-terminal of CotE protein (*cotEn*$^-$ and *cotEc*$^-$) and *C. difficile* spores devoid of BclA proteins (*bclA1*$^-$, *bclA2*$^-$ and *bclA3*$^-$) and isogenic wild type strain 630Δ*erm* have been assessed for their binding efficiency to monolayers of intestinal epithelial cell lines HT29 and HT29-MTX and to the main structural components of the ECM of the gut, collagen and fibronectin.

2) Interaction of *C. difficile* spore exosporial proteins BclA1-3 and macrophages

There is limited information regarding the recognition of *C. difficile* by the immune system and the immune response elicited following exposure to this organism. Therefore *C. difficile* spores devoid of BclA proteins (*bclA1*$^-$, *bclA2*$^-$ and *bclA3*$^-$) and isogenic wild type strain 630Δ*erm* have been assessed for their interactions with murine macrophage cell line J774.1.

Chapter 2
Materials and Methods

2.1 General Methods

2.1.1 *C. difficile* strains

For ClosTron mutagenesis and mutant analysis an erythromycin-sensitive derivative 630Δ*erm* was used. 630Δ*erm*, is a spontaneously cured derivative of 630 (*tcdA*$^{+}$ *tcdB*$^{+}$) (Hussain et al., 2005). Insertional mutations in the *bclA* and *cotE* genes were made using the ClosTron system developed at the University of Nottingham (Heap et al., 2010). Using this technique, the three *bclA* genes were inactivated in strain 630Δ*erm* creating the mutants *bclA1*$^{-}$ (*bclA1::CT1050a*), *bclA2*$^{-}$ (*bclA2::150a*) and *bclA3*$^{-}$ (*bclA3::125s*) (Phetcharaburanin et al., 2014). In the case of *cotE,* which carries two enzymatic domains (peroxiredoxin and chitinase), two mutations, inactivating the amino-terminal (*cotE*::*CT220s*) and carboxy-terminal (*cotE*::*CT1203s*) domains were made (Permpoonpattana et al., 2013).

2.1.2 Mammalian cell lines

HT29 (American Type Culture Collection, HTB-38) is an intestinal epithelial cell line. HT29-MTX (Public Health England, HT29-MTX-E12) is a mucus-secreting intestinal epithelial cell line. J774.1 (Sigma-Aldrich, UK) is a murine macrophage cell line.

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

All procedures were carried out using an anaerobic incubator (Don Whitley, UK). Bacterial colonies was grown on BHIS agar (brain heart infusion agar supplemented with 0.1% L-cysteine and 5mg/ml yeast extract) overnight at 37ºC. For ClosTron mutant strains bacterial colonies were grown on BHIS agar containing 5µg/ml of erythromycin. One fresh single colony from a BHIS plate was inoculated into 10ml of TGY medium (3% tryptic soy broth, 2% glucose, 1% yeast extract and 0.1% L-cysteine) and incubated at 37ºC overnight. 1ml of TGY culture was subcultured into SMC broth (90g peptone, 5g proteose peptone, 1g $(NH_4)_2SO_4$, 1.5g Tris and 1% L-cysteine). Incubated until an optical density (OD) at 600nm (OD_{600}) of 0.4-0.7 was reached (approximately 8 h) and then plated onto SMC agar. After 7-days of incubation at 37ºC spores were harvested from agar plates with a spreader using cold sterile water. Spore suspensions were washed at least 10-times with cold sterile water. Sporulation efficiency was confirmed by phase-contrast microscopy and spore yield was determined by counting colony forming units (CFU) of heat treated (60ºC, 20 min) aliquots on BHISS plates (brain heart infusion supplemented with 5mg/ml yeast extract, 0.1% L-cysteine and 0.1% sodium taurocholate). Plates were incubated for 48 h before CFU enumeration.

2.1.4 *C. difficile* spore purification

Spores were purified using HistoDenz (Sigma-Aldrich, UK) as follows. Crude spore suspensions were washed with cold sterile water. Aliquots were re-suspended in 500µl of 20% HistoDenz and layered over 1ml of 50% HistoDenz in a 1.5ml tube.

Tubes were centrifuged at 10,000g for 15 min at RT. The spore pellet was recovered and washed 5-times with cold sterile water. Spore purity was assessed using phase contrast microscopy and spore yield was determined by CFU of heat treated (60ºC, 20 min) aliquots on BHISS plates. Plates were incubated for 48 h before CFU enumeration.

2.1.5 Statistical analysis

Data was analysed using Student's *t*-test. Error bars represent standard deviation. All analyses were performed using GraphPad Prism 6 software (GraphPad).

2.2 Chapter 3 – Role of adhesion in pathogenesis of CDI

2.2.1 Germination of *C. difficile* spores in sodium taurocholate

Spore germination was carried out in a 96-well plate (Greiner Bio-One, UK) and germination of spores was measured by the percentage change in OD_{600}. HistoDenz purified and unpurified water washed spores at an OD_{600} of ~0.8–1.0 (~1 x 10^8/ml) were pelleted by centrifugation, (10,000g, 1 min) and suspended in 1ml of BHIS supplemented with 0.1% sodium taurocholate. The initial OD_{600} was recorded and then measured at 1 min intervals over 30 min using a microplate reader (Molecular Devices, UK). Plates were shaken in between readings to keep spores in suspension. % fall in OD was determined as (OD at time point/initial OD_{600}) x 100.

2.2.2 Germination of *C. difficile* spores in cell culture media

Spore germination was carried out in a 96-well plate and germination of spores was measured by the percentage change in OD_{600}. Unpurified water washed spores at an OD_{600} of ~0.8–1.0 (~1 x 10^8/ml) were pelleted by centrifugation, (10,000g, 1 min) and suspended in 1ml of either McCoy's 5A media (Sigma-Aldrich, UK) or Dulbecco's modified Eagle medium (DMEM) (Life technologies, UK). The initial OD_{600} was recorded and then measured at 1 min intervals over 2 h using a microplate reader. Plates were shaken in between readings to keep spores in suspension. The % fall in OD was determined as (OD at time point/initial OD_{600}) x 100.

2.2.3 SATH (spore adhesion to hydrocarbon) assay

HistoDenz purified spores and water washed unpurified spores were washed in 1M NaCl and then suspended in 0.1M NaCl for assay. 500µl of spore suspension was added to 800µl *n*-hexadecane (Sigma-Aldrich, UK) and vortexed for 1 min. Samples were then incubated for 10 min at 37°C with mild agitation, vortexed (30sec) and OD_{600} recorded. Hydrophobicity was determined from the OD of the original spore suspension (A1) and the OD of the aqueous phase after incubation with hydrocarbon (A2) using the equation: H = [(A1 - A2)/A1].

2.2.4 Cell culture of HT29 cells

HT29 cells (ATCC, Manassas), a colon epithelial cell line was seeded at 2 x 10^6 cells in 75cm^2 flasks and grown in McCoy's 5A medium supplemented with 1% L-glutamine (v/v) (Sigma-Aldrich, UK), 1% penicillin and streptomycin (v/v) (Sigma-Aldrich, UK), and 10% foetal bovine serum (FBS) (v/v) (Sigma-Aldrich, UK). Cells were incubated at 37°C in an atmosphere of 5% CO_2. Once cells reached 70-80% confluency the cells were passaged and reseeded.

2.2.5 Cell culture of HT29-MTX cells

HT29-MTX cells (PHE, UK), a mucus-secreting colon epithelial cell line was seeded at 2 x 10^6 cells in 75cm^2 flasks and grown in DMEM supplemented with 1% L-glutamine (v/v), 1% penicillin and streptomycin (v/v), 1% non-essential amino acids (v/v) (Sigma-Aldrich, UK) and 10% foetal bovine serum (FBS). Cells were incubated at 37°C in an atmosphere of 5% CO_2. Once cells reached 70-80% confluency the cells were passaged and reseeded.

2.2.6 Alcian blue staining of HT29 and HT29-MTX cells

To confirm the presence of mucus on HT29-MTX cells, 4 x 10^4 HT29 and HT29-MTX cells were seeded onto cell culture treated coverslips (Nunc, UK) in 24-well plates (Corning, UK). Monolayers were left to grow for either 7-days or 14-days with regular changes of cell culture media. Cell culture was removed and monolayers washed with 3% acetic acid for 3 min. 1% Alcian blue solution (Sigma-Aldrich, UK) pH 2.5 was added to monolayers for 30 min at RT. Monolayers were briefly rinsed with 3% acetic acid to remove excess Alcian blue solution. Monolayers were washed with distilled water and Nuclear fast red solution (Sigma-Aldrich, UK) was added to monolayers for 5 min. Excess Nuclear fast red solution was removed by washing with distilled water. Coverslips were mounted and sealed using clear nail varnish. Slides were visualized using an EVOS FL digital microscope (Life technologies, UK).

2.2.7 Adhesion of *C. difficile* spores to HT29 and HT29-MTX cells

HT29 and HT29-MTX cells were seeded at 4 x 10^4 cells/well in 24-well plates and left to grow for either 7-days or 14-days with regular changes of culture media. Cell monolayers were washed once with Dulbecco's Phosphate-Buffered Saline (DPBS) (Life technologies, UK) and monolayers inoculated with water washed unpurified spores at multiplicity of infection (MOI) 100:1. Wells were incubated with spores in appropriate culture media (DMEM for HT29-MTX cells or McCoy's 5A media for HT29 cells) for 2 h at 37ºC, 5% CO_2 (500µl/well). Wells were washed 5-times with DPBS to remove non-adherent bacteria. Cells were lysed for approximately 10 min at 37ºC, 5% CO_2. Control wells to determine the total number of *C. difficile* spores

were not washed and were directly lysed with 0.5% Triton-X-100 (Sigma-Aldrich, UK) in DPBS (v/v). To determine the adherence of *C. difficile* spores to HT29 and HT29-MTX cell monolayers, samples were serially diluted and plated onto BHISS plates. To determine the number of heat resistant cells, samples were heated at 60ºC for 20 min before serially diluting and plating onto BHISS plates. Plates were incubated anaerobically for 48 h. The number of CFU was determined and the percentage of adherence calculated using the following formula (final CFU/initial CFU) x 100.

2.2.8 Adhesion of *C. difficile* spores to human collagen type I

96-well flat bottom microtitre plates were coated with 10µg ml^{-1} of purified human collagen type I (Merck Millipore, UK) in PBS overnight at 4°C. Plates were washed 3-times with PBS and 0.05% Tween-20 (v/v) (Sigma-Aldrich, UK) and wells were blocked with PBS containing 5% BSA (w/v) (Sigma-Aldrich, UK) for 2 h at 37°C. Wells were washed 3-times with PBS and 0.05% Tween-20 and 1 x 10^7 HistoDenz purified and water washed unpurified *C. difficile* spores in PBS were added to wells (100µl per well) in triplicate for 2 h at 37°C. Plates were washed 5-times with PBS and 0.05% Tween-20 to remove any non-adherent bacteria. Subsequently, 1:5000 dilution of mouse antiserum against *C. difficile* was added to each well for 1 h at 37°C. Plates were washed 3-times with PBS and 0.05% Tween-20. 1:2000 dilution of horseradish-peroxidase labelled goat anti-mouse IgG (Sigma-Aldrich, UK) was added to wells for 1 h at 37°C. Plates were washed 3-times with PBS and 0.05% Tween-20 and 3, 3′, 5, 5′-tetramethylbenzidine (TMB) (Biolegend, UK) was added to wells. The reaction was stopped with the addition of 2M H_2SO_4 and plates were

read on microplate reader at 450nm. Wells with no spores added and wells coated with BSA served as negative controls.

2.2.9 Adhesion of *C. difficile* spores to human plasma fibronectin

96-well flat bottom microtitre plates were coated with 10µg ml^{-1} of purified human plasma fibronectin (Merck Millipore, UK) in PBS overnight at 4°C. Plates were washed 3-times with PBS and 0.05% Tween-20 (v/v) and wells were blocked with PBS containing 5% BSA (w/v) for 2 h at 37°C. Wells were washed 3-times with PBS and 0.05% Tween-20 and 1 x 10^7 HistoDenz-purified and water washed unpurified *C. difficile* spores in PBS were added to wells (100µl per well) in triplicate for 2 h at 37°C. Plates were washed 5-times with PBS and 0.05% Tween-20 to remove any non-adherent bacteria. Subsequently, 1:5000 dilution of mouse antiserum against *C. difficile* was added to each well for 1 h at 37°C. Plates were washed 3-times with PBS and Tween-20 and a 1:2000 dilution of horseradish-peroxidase labelled goat anti-mouse IgG was added to wells for 1 h at 37°C. Plates were washed 3-times with PBS and TMB was added to wells. The reaction was stopped with the addition of 2M H_2SO_4 and plates were read on microplate reader at 450nm. Wells with no spores added and wells coated with BSA served as negative controls.

2.2.10 Immunofluorescence of *C. difficile* spore adherence to HT29 and HT29-MTX cells

HT29 and HT29-MTX cells were seeded at 4 x 10^4 cells/well on coverslips and left to grow for either 7-days or 14-days with regular changes of culture media. Cell monolayers were washed once with DPBS and monolayers inoculated with water

washed unpurified spores at MOI 100:1. Wells were incubated with spores in appropriate culture media (DMEM for HT29-MTX cells or McCoy's 5A media for HT29 cells) for 2 h at 37ºC, 5% CO_2 (500µl/well). Wells were washed 5-times with DPBS to remove non-adherent bacteria. Cells were fixed with 250µl of 4% paraformaldehyde (w/v) (Alfa Aesar, UK) in PBS for 15 min at RT. Cells were washed twice with DPBS and permeabilized with 200µl of 0.5% Triton-X-100 in PBS (v/v) for 10 min at RT. Cells were washed twice with DPBS. Cells were incubated with 200µl 1:100 dilution of mouse antiserum against *C. difficile* in 1% BSA in PBS for 1 h at 37ºC, 5% CO_2. Cells were washed 3-times with DPBS. Cells were incubated with 200µl of 1:1000 dilution of anti-mouse IgG-FITC antibody (Sigma-Aldrich, UK) in 1% BSA in PBS for 1 h at 37ºC, 5% CO_2. Cells were washed 3-times with DPBS. Cells were incubated with 200µl of 1:1000 dilution of 4'6-Diamidino-2-phenylindole (DAPI) (1µg/ml), for 5 min in the dark. Cells were washed 3-times with DPBS. Coverslips were mounted using Vectashield mounting medium (Vectorlabs, UK) and sealed using clear nail varnish. Slides were visualized using an EVOS FL digital microscope (Life Technologies, UK).

2.3 Chapter 4 – Interaction of *C. difficile* spores with macrophages

2.3.1 Cell culture of J774.1 cells

J774.1 cells (Sigma-Aldrich, UK), a murine macrophage cell line was seeded at 2 x 10^6 cells in 75cm^2 flasks and grown in DMEM supplemented with 1% L-glutamine (v/v), 1% penicillin and streptomycin (v/v) and 10% FBS (v/v). Cells were incubated at 37°C in an atmosphere of 5% CO_2. Once cells reached 70-80% confluency the cells were passaged and reseeded.

2.3.2 *C. difficile* spore phagocytosis assay

J774.1 cells were seeded at 5 x 10^5 cells/well in 24-well cell culture plates in DMEM supplemented with 1% L-glutamine (v/v), 1% penicillin and streptomycin (v/v) and 10% FBS (v/v) for 48 h at 37ºC, 5% CO_2. Cell monolayers were washed once with DPBS and subsequently infected with 200µl *C. difficile* spores in DMEM at MOI 10:1 for 30 min at 37ºC, 5% CO_2. Wells were washed 3-times with DPBS to remove any spores that had not been phagocytosed. Cells were lysed with 0.01% Triton-X-100 in PBS (v/v). To determine the number of spores that had been phagocytosed, samples were serially diluted and plated onto BHISS plates. To determine the number of heat resistant cells, samples were heat treated at 60ºC for 20 min before serially diluting and plating onto BHISS plates. Plates were incubated anaerobically for 48 h. % of phagocytosis was calculated using the following formula: (CFU count / Initial number of spores added) x 100

2.3.3 *C. difficile* spore survival assay

J774.1 were seeded at 5 x 10^5 cells/well in 24-well cell culture plates in DMEM supplemented with 1% L-glutamine (v/v), 1% penicillin and streptomycin (v/v) and 10% FBS (v/v) for 48 h at 37ºC, 5% CO_2. Cell monolayers were washed once with DPBS and monolayers were infected with 200µl of *C. difficile* spores in DMEM at MOI 10:1 for 30 min at 37ºC, 5% CO_2. Wells were washed 3-times with DPBS to remove any spores that had not been phagocytosed. Monolayers were resuspended with 500µl DMEM supplemented with 1% L-glutamine (v/v). Survival of *C. difficile* spores was determined at 12 h and 24 h post infection by lysing infected macrophages with 0.01% Triton-X-100 (v/v). To determine the number of surviving *C. difficile* spores, samples were serially diluted and plated onto BHISS plates. To determine the number of heat resistant cells, samples were heated at 60ºC for 20 min before serially diluting and plating onto BHISS plates. Samples were serially diluted and plated onto BHISS plates. Plates were incubated anaerobically for 48 h. % of spore viability was calculated using the following formula: (CFU count / Number of spores phagocytosed) x 100

2.3.4 MTT cell viability assay

J774.1 were seeded at 5 x 10^4 cells/well in 96-well cell culture plates (Corning, UK) in DMEM without phenol red (Life technologies, UK) supplemented with 1% L-glutamine (v/v), 1% penicillin and streptomycin (v/v) and 10% FBS (v/v) for 48 h at 37ºC, 5% CO_2. Cell monolayers were washed once with DPBS and monolayers were infected with 100µl *C. difficile* spores in DMEM without phenol red at MOI 10:1 and 100:1 for 30 min at 37ºC, 5% CO_2. Wells were washed 3-times with DPBS to

remove any spores that had not been phagocytosed. Monolayers were re-suspended with 100µl of DMEM without phenol red supplemented with 1% L-glutamine (v/v) for 24 h at 37ºC, 5% CO_2. After 24 h incubation media was removed and replaced with 100µl of fresh DMEM without phenol red supplemented with 1% L-glutamine (v/v). 10µl of 5mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) stock solution added to each well. Plates were incubated at 37ºC, 5% CO_2 for 4 h. Following incubation all but 25µl of medium was removed from wells and 50µl of DMSO (Sigma-Aldrich, UK) was added to each well and mixed thoroughly. Plate incubated for 10 min at 37ºC, 5% CO_2. Samples were mixed again and the absorbance was read on microplate reader at 540nm. Controls included wells containing no spores, J774.1 cells and media only wells.

2.2.5 Cell surface biotinylation and *C. difficile* spore pull down

J774.1 cells were cell surface labelled by using EZ-Link Sulfo-NHS-LC-Biotin (Pierce, UK) according to the instructions of the manufacturer. Briefly J774.1 cells were harvested to a concentration of ~25 x 10^6 cells/ml. Cells were washed 3-times with ice-cold PBS (pH8) to remove amine containing media from cells. Cells were suspended in 1ml of PBS and 1mg/ml of Sulfo-NHS-LC Biotin added to cells. The reaction mixture was incubated at RT rotating for 30 min. Cells were washed 3-times with PBS and 100mM glycine to quench and remove excess biotin. *C. difficile* spores were added at a MOI of 25:1 in 1ml of PBS for 45 min rotating at RT. Sample was centrifuged at 1,500rpm for 5 min and the pellet was re-suspended in lysis buffer containing 1% Triton-X-100 and protease inhibitor (Pierce, UK) for 30 min at 4ºC. Sample washed three times in PBS. Spore-bound proteins were released with

sample buffer and analysed by western blot with HRP-conjugated streptavidin (ThermoFisher Scientific, UK).

2.2.6 Western blot of spore bound proteins

Spore bound proteins were run on a 18% SDS-PAGE gel then were transferred using semi-dry transfer method to PVDF membrane (Merck Millipore, UK). The membrane was Ponceau S stained to determine efficiency of transfer and the membrane was dehydrated in methanol and dried by incubating the membrane at RT. HRP-conjugated streptavidin was prepared at dilution 1:15,000 in PBS with 3% semi-skimmed milk powder (w/v) (Sigma-Aldrich, UK) and 0.01% Tween-20 (v/v) for 1 h at RT. The membrane was washed 3-times in PBS and developed using the Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, UK).

2.2.7 Silver staining

Silver staining of SDS-PAGE gels was carried out using ProteoSilver Plus Silver Stain Kit according to the manufacturer's instructions (Sigma-Aldrich, UK).

Chapter 3

The potential role of *C. difficile* spore surface proteins in adherence to the gut

3.1 Introduction

3.1.1 Role of adhesion in establishing infection

Adhesion is the first step in initiating bacterial infection, several adhesins known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) contribute to this step. MSCRAMMs are located on bacterial surfaces and mediate adhesion by binding to a variety of ECM proteins such as fibronectin, laminin, collagen, elastin and proteoglycan on host surfaces (Lin et al., 2011). Adhesion is also necessary for full expression of a pathogens virulence factors (Borriello et al., 1990).

The ECM is a biologically active tissue composed of a complex mixture of macromolecules. Cell adhesion, migration, proliferation and differentiation are examples of biological processes influenced by the composition and structural organization of surrounding extracellular matrices. Interactions with eukaryotic cells and ECM components mediated by integrins appear to play an integral role in cellular communications with the ECM. The ECM may serve as a substrate not only for the adhesion of the cells of the host organism but also for the attachment of

colonizing microorganisms. First, to be classified as an MSCRAMM, the molecule of interest must be localized to the microbial cell surface, secondly the microbial component must recognize a macromolecular ligand that can be found within the ECM. Finally MSCRAMMs interaction with ECM component should be of high affinity and exhibit a high degree of specificity (Patti et al., 1994).

A small number of *C. difficile* proteins involved in host-pathogen interactions have been characterized including S-layer proteins (Calabi et al., 2002), the flagellar components FliC and FliD (Tasteyre et al., 2001), the GroEL chaperone (Hennequin et al., 2001), the Cwp66 adhesin (Waligora et al., 2001), the fibronectin binding protein Fbp68 (Hennequin et al., 2003) (Lin et al., 2011) and cysteine protease Cwp84 (Janoir et al., 2007).

Fibronectin is a dimeric glycoprotein which is present in a soluble form in plasma and in an immobilized form on cell surfaces and in the ECM. It is an important target for bacterial attachment for many pathogens such as *Streptococcus pyogenes* (*S. pyogenes*), *Streptococcus pneumonia* (*S. pneumonia*) and *Listeria monocytogenes* (*L. monocytogenes*) where fibronectin binding proteins are important virulence factors (Barketi-Klai et al., 2011).

Collagen is the major insoluble fibrous protein in the ECM and in connective tissue. There are at least twenty different collagen types identified currently, however 80-90% of collagen in the body consists of type I, II and III (Lodish et al., 2000). Collagens are the major structural components of the ECM and participate in many cellular processes such as cell attachment, differentiation and migration (Gelse et al., 2003). A cell wall anchored protein of *C. difficile*, Collagen binding protein A (CbpA) has been identified which show typical features of MSCRAMMs, including

bacterial adhesins that specifically interact with ECM components (Tulli et al., 2013).

The interaction of bacterial pathogens with the ECM is an important virulence mechanism contributing to host colonization. Since the ECM is not accessible to pathogens under normal conditions it could be hypothesized that the ECM might be exposed to pathogens as a consequence of tissue damage. Damage to the intestinal mucosa would allow for access of *C. difficile* to the ECM. This interaction may permit bacteria to use the submucosal matrix as a molecular bridge to increase adherence to host tissues.

In the case of *C. difficile*, before CDI can develop it is thought that the spore adheres to a component of the gut epithelial wall allowing the spore to colonize the gut in a new host and germinate releasing toxins A and B and therefore causing symptoms of infection. During the infection cycle, *C. difficile* begins a sporulation process where it is thought that some spores remain adhered to the colonic surface and the rest are released into the environment through faeces. Studies in a mouse model have demonstrated that sporulation deficient strains are incapable of producing recurrence and persistence of *C. difficile* in the infected mouse and transmission to neighbouring mice, indicating spore formation during CDI is essential for persistence and transmission (Deakin et al., 2012). This is consistent with the fact that once antibiotic treatment for CDI has ended, the majority of patients continue shedding *C. difficile* spores for up to two weeks. *C. difficile* spores are considered the morphotype of infection and persistence and it has been suggested that the persistence of *C. difficile* in the host may be mediated through adherence of *C. difficile* spores to the host's intestinal epithelium however there is scant knowledge

about the role of proteins on the outermost layer of *C. difficile* spores and the role they play, if any, in persistence.

3.1.2 Factors affecting adhesion

The attachment of bacterial spores to surfaces is influenced by several factors such as hydrophobicity and the presence of surface structures such as appendages and the outer spore layer known as the exosporium. The exosporium is a loose outer layer which surrounds spores produced by some *Bacillus* and *Clostridium* species and is thought to play a major role in the attachment of spores to environmental and cellular surfaces (Joshi et al., 2012).

It has been shown that comparing spores from two different isolates of *C. difficile* that isolates containing an exosporium were more hydrophobic than isolates not containing an exosporium (Joshi et al., 2012). Also spores containing an exosporium showed an increase adherence to seven-day old HT29 cells compared to spores without. It has been previously been observed that spores of *C. difficile* and *Clostridium sporogenes* (*C. sporogenes*) attach to the apical microvilli of human epithelial cells by use of an exosporium-like structure. This suggests that the hydrophobic nature of the spore is due to the presence of an exosporium and this structure also has a role in aiding the adherence of spores to epithelial cells (Joshi et al., 2012). Precise factors that mediate spore adherence have not yet been confirmed however results suggest that components of the spore surface play a role in attachment to epithelial cells.

The suggestion of a link between spore hydrophobicity and the presence of an exosporium was first suggested by Takubo et al. who showed that *Bacillus*

magaterium (*B. megaterium*) spores with a defective or absent exosporium showed reduced affinity for hexadecane (Takubo et al., 1988). Also spores of *B. subtilis*, *Bacillus licheniformis* (*B. licheniformis*) and *Bacillus macerans* (*B. macerans*) do not have a distinct exosporium and are less hydrophobic than exosporium containing *B. cereus*, *Bacillus brevis* (*B. brevis*) and *Bacillus thuringiensis* (*B. thuringiensis*) spores (Koshikawa et al., 1989).

Correlation has been demonstrated between spore hydrophobicity and the ability of *B. cereus* strains containing an exosporium to adhere to human colonic adenocarcinoma cells (Andersson et al., 1998).

In a recent study of adherence of *C. difficile* spores to epithelial cells a similar correlation between spore hydrophobicity and cell surface binding to undifferentiated five-day old Caco-2 cells has been observed (Paredes-Sabja et al., 2012).

3.1.3 Hydrophobicity

Breslow states 'the hydrophobic effect is the tendency of non-polar species to aggregate in water solutions so as to decrease the hydrocarbon-water interface area' (Breslow et al., 1991). Proteins which exist in aqueous environments but may have areas of hydrophobic amino acids in a sequence. These areas of hydrophobic residues could have a role in the adhesive reactions between microorganisms and surfaces. Hydrophobic bonding is not a true bond but rather a greater affinity for apolar molecules to associate with other apolar molecules than to water. A microorganism may adhere to a surface by a hydrophobic effect if the surface it wishes to bind to has high densities of apolar areas. Bacteria and other

microorganisms have evolved ways to use hydrophobicity to adhere to surfaces and there is reason to believe that hydrophobic interactions participate in the initial adhesion of pathogens to tissues, leading to colonization, invasion or tissue destruction (Doyle., 2000). Very few microbes have entirely hydrophobic or charged surfaces however the spore coat is a structure which contributes to the hydrophobicity of *C. difficile* spores. It has been suggested that the increased hydrophobicity of bacterial spores compared to vegetative cells is due to the relative abundance of protein in the outer coats and the exosporium compared to the peptidoglycan on Gram-positive vegetative cell surfaces (Wiencek et al., 1990).

3.1.4 Experimental objectives

C. difficile spores are recognized as the persistent and infectious morphotype as well as the channel of transmission of CDI. However there is little knowledge on how *C. difficile* spores interact with the host's gut. Therefore the objective of this chapter was to investigate the potential of *C. difficile* spore surface proteins in adherence to the gut. Mutant strains of *C. difficile* created by the ClosTron system have been assessed for their ability to adhere to epithelial cells and ECM proteins of gut. ClosTron inactivation of the *bclA* genes and the amino and carboxy-terminal of *cotE* results in the absence of these proteins from the spore surface and therefore allows for investigation of how removal of these proteins can affect adhesion. As adhesion has a critical role in the infection process, bacterial adherence to host tissues represents a potential target for the development of agents that can block adherence and therefore help in combating infectious disease.

3.2 Results

3.2.1 Germination of *C. difficile* spores using sodium taurocholate

HistoDenz purified and water washed unpurified *bclA*$^{-}$, mutant CotE spores lacking either N- or C-terminal domain mutant and wild type 630Δ*erm* spores were assessed for their ability to germinate in BHI medium supplemented with 0.1% sodium taurocholate as a germinant (Figure. 3.1 and 3.2). Spores at an OD_{600} of ~0.8–1.0 (~1 x 10^{8}/ml) were suspended in 1ml of BHI medium supplemented with 0.1% sodium taurocholate. The initial OD_{600} was recorded and then measured at 1 min intervals over 30 min using a microplate reader. Germination correlates to a loss in OD_{600} as spores rehydrate and become phase dark due to the release of Ca-DPA and rehydration of the spore. Results show that purified spores germinated faster compared to water washed unpurified spores (Figure 3.1 and 3.2). Purified *bclA1*$^{-}$ and *bclA3*$^{-}$ spores germinated faster than the 630Δ*erm* wild type strain with 34% and 36% loss in OD_{600}, respectively over 30 min (Figure 3.1D). With regard to unpurified spores *bclA2*$^{-}$ and *bclA3*$^{-}$ spores germinated faster than 630Δ*erm* wild type strain with unpurified *bclA2*$^{-}$ spores showing the highest germination rate with a 15.8% loss in OD_{600} (Figure 3.1E). Results also show that unpurified *bclA1*$^{-}$ spores germinated slowest (Figure 3.1E). For *cotE*$^{-}$ mutant spores, purified *cotEn*$^{-}$ and *cotEc*$^{-}$ spores germinated faster than 630Δ*erm* wild type strain with *cotEn*$^{-}$ spores showing the highest germination rate with a 20% loss in OD_{600} (Figure 3.2C). Unpurified *cotEn*$^{-}$ spores germinated faster than 630Δ*erm* wild type strain with a 10% loss in OD_{600} (Figure 3.2D).

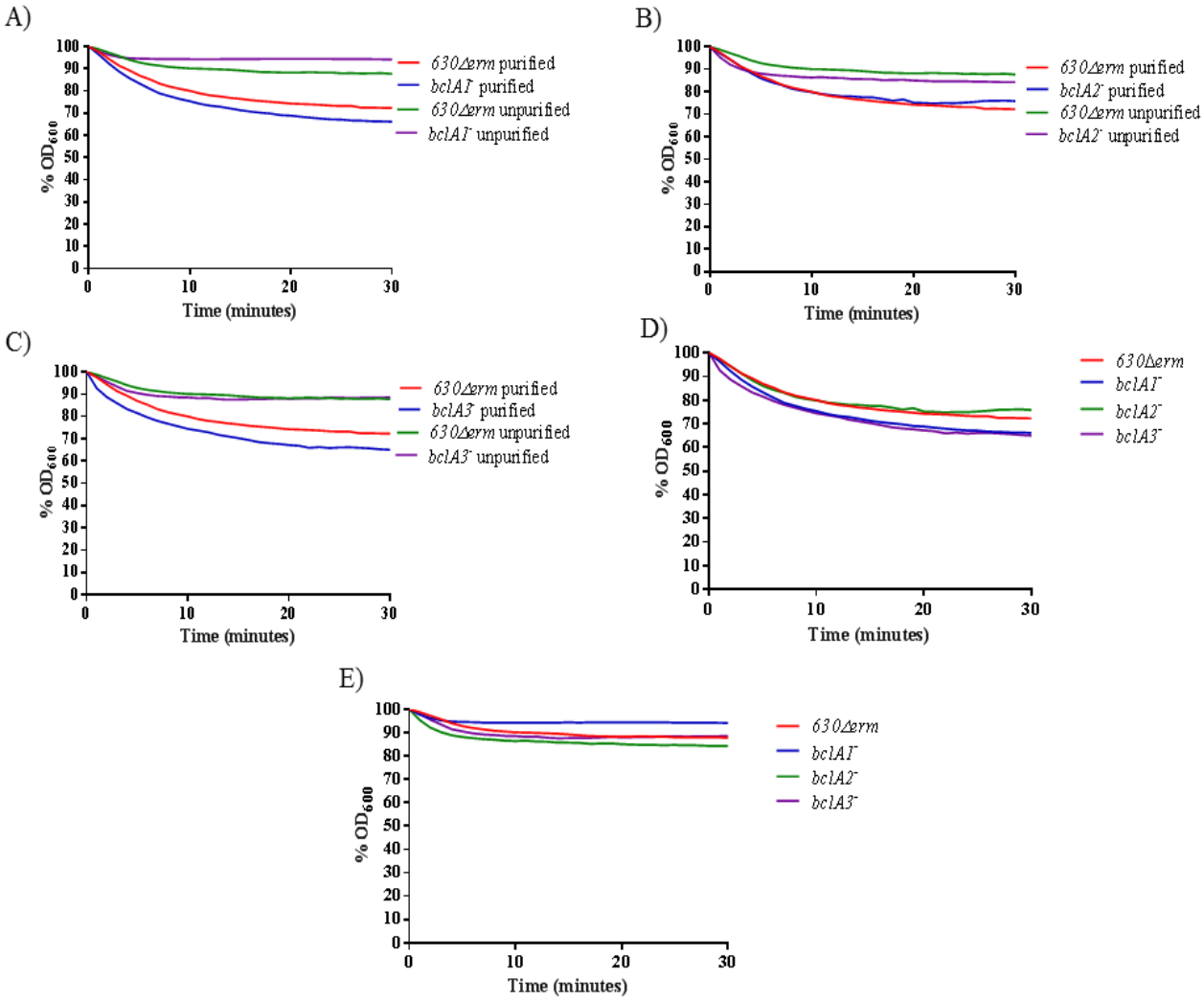


Figure 3.1. Germination of *bclA*⁻ spores in BHI media supplemented with 0.1% sodium taurocholate. A-C) Shows the rate of germination of HistoDenz purified and water washed unpurified *bclA*⁻ mutant spore suspensions compared with wild type 630Δ*erm* spores. D) Shows the rate of germination of HistoDenz purified spores. E) Shows the rate of germination of water washed unpurified spores. % fall in OD was determined as (recorded OD_{600} at time interval/initial OD_{600}) x 100. All samples had an initial OD of approximately 1.0.

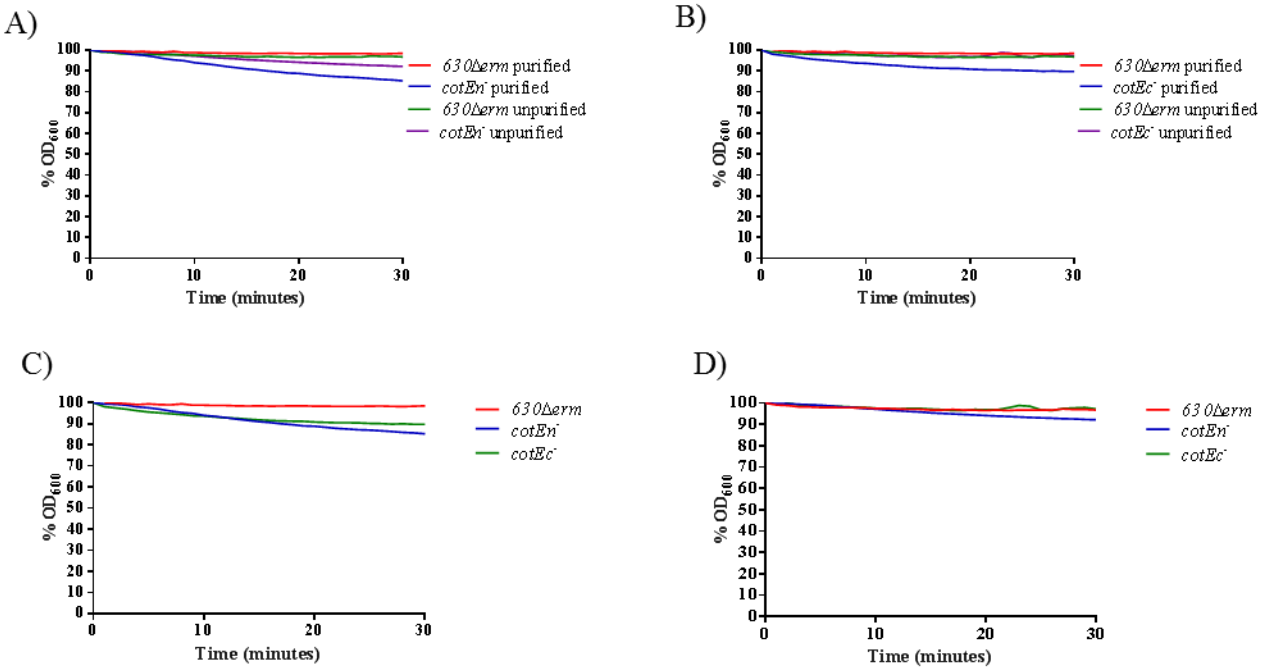


Figure 3.2. Germination of *cotE*⁻ spores in BHI media supplemented with 0.1% sodium taurocholate. A-B) Shows the rate of germination of HistoDenz purified and water washed unpurified *cotE*⁻ mutant spore suspensions compared with wild type 630Δ*erm* spores. C) Shows the rate of germination of HistoDenz purified spores. D) Shows the rate of germination of water washed unpurified spores. % fall in OD was determined as (recorded OD_{600} at time interval/initial OD_{600}) x 100. All samples had an initial OD of approximately 1.0.

3.2.2 Germination of *C. difficile* spores in cell culture media

Water washed mutant unpurified *bclA*$^{-}$, mutant CotE spores lacking either N- or C-terminal domain and wild type 630Δ*erm* spores were assessed for their ability to germinate in the presence of culture media used during the epithelial cell adhesion assays (Figure 3.3). Spores at an OD_{600} of ~0.8–1.0 (~1 x 10^{8}/ml) were suspended in 1ml of either DMEM or McCoy's 5A media. The initial OD_{600} was recorded and then measured at 1 min intervals over 120 min using a microplate reader. Germination correlates to a loss in OD_{600} as spores rehydrate and become phase dark. Results show that *C. difficile* spores were able to germinate in the presence of cell culture medium (Figure 3.3). Both *bclA*$^{-}$ mutant spores and *cotE*$^{-}$ mutant spores devoid of the N- or C- terminal domain germinated faster than 630Δ*erm* in both culture mediums (Figure 3.3) with greater spore germination occurring in DMEM (Figure 3.3A and 3.3C).

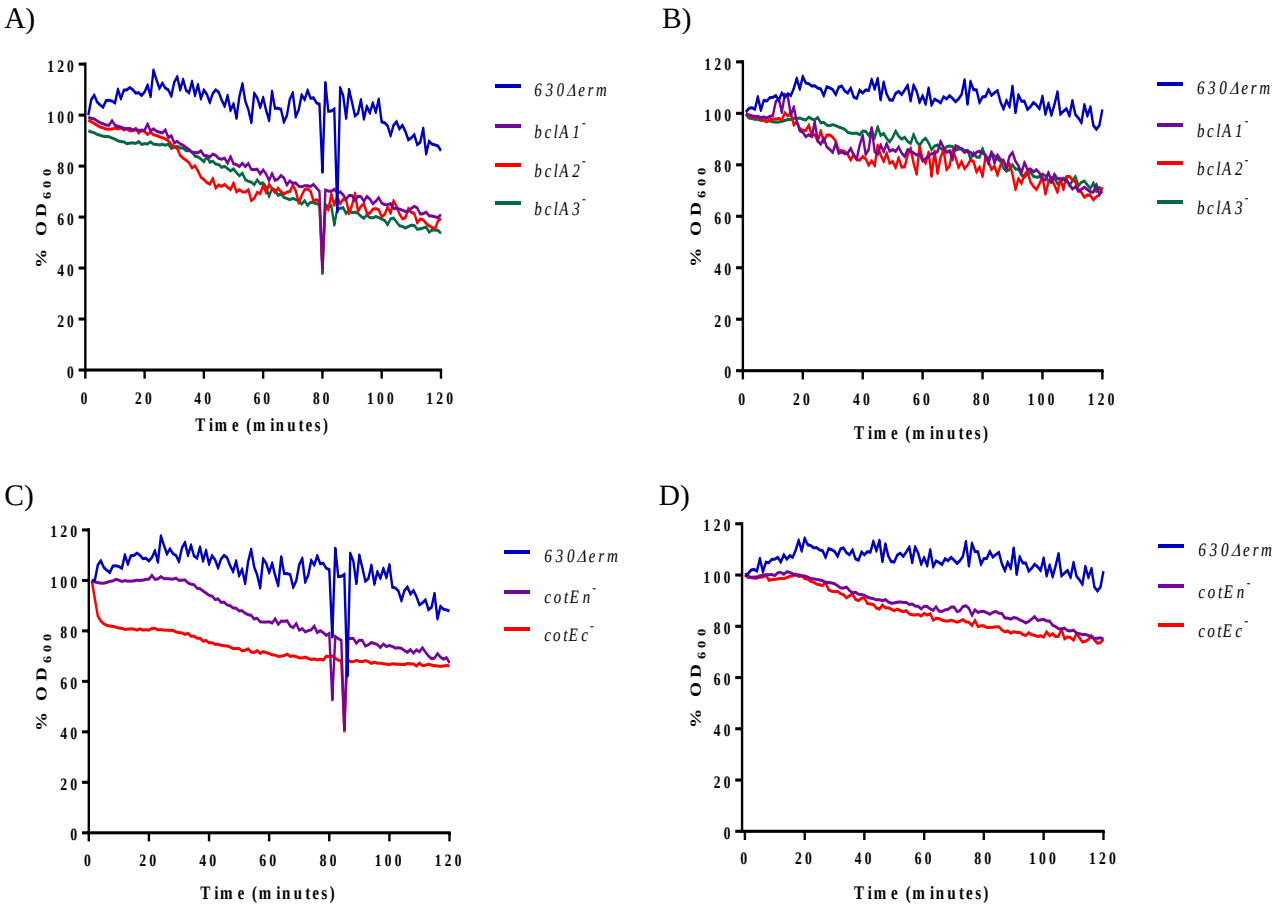


Figure 3.3. Rate of germination of unpurified water washed spores in the presence of cell culture media. A) Rate of germination of *bclA*- mutant spores in DMEM compared to wild type. B) Rate of germination of *bclA*- mutant spores in McCoy's 5A media compared to wild type. C) Rate of germination of c*otE*- mutant spores devoid of N- or C-terminal domain in DMEM compared to wild type. D) Rate of germination of *cotE*- mutant spores devoid of N- or C-terminal domain in McCoy's 5A media compared to wild type.

3.2.3 Hydrophobicity of *C. difficile* spores

The hydrophobicity of spores was assessed by measurement of OD of the aqueous layer after mixing with hexadecane. The stability of the exosporium is currently under much debate with several reports suggesting that this layer is fragile and easily lost (Permpoonpattana et al., 2011) (Permpoonpattana et al., 2013) whereas other reports suggest that it is a reasonably stable layer that is only removed by proteases and sonication treatments (Escobar-Cortes et al., 2013). The stability of the exosporium could be related to the type of purification method used. Hydrophobicity has been found to be a useful marker of *C. difficile* exosporium removal, with spores lacking most of the exosporium having lower hydrophobicity (Escobar-Cortes et al., 2013). Therefore the hydrophobicity of HistoDenz purified spores and water washed unpurified spores was compared (Figure 3.4 and 3.5). Results show that *bclA*$^{-}$, *cotEn*$^{-}$ and *630Δerm* wild type water washed unpurified spores are more hydrophobic than HistoDenz purified spores (Figure 3.4A and 3.5A). HistoDenz purified *bclA*$^{-}$ and HistoDenz purified and water washed unpurified *cotE*$^{-}$ mutant spores are significantly more hydrophobic than spores of wild type 630Δ*erm* (Figure 3.4B, 3.5B and 3.5C), whereas no significant difference in hydrophobicity was observed between unpurified water washed *bclA*$^{-}$ mutant spores and 630Δ*erm* wild type (Figure 3.4C).

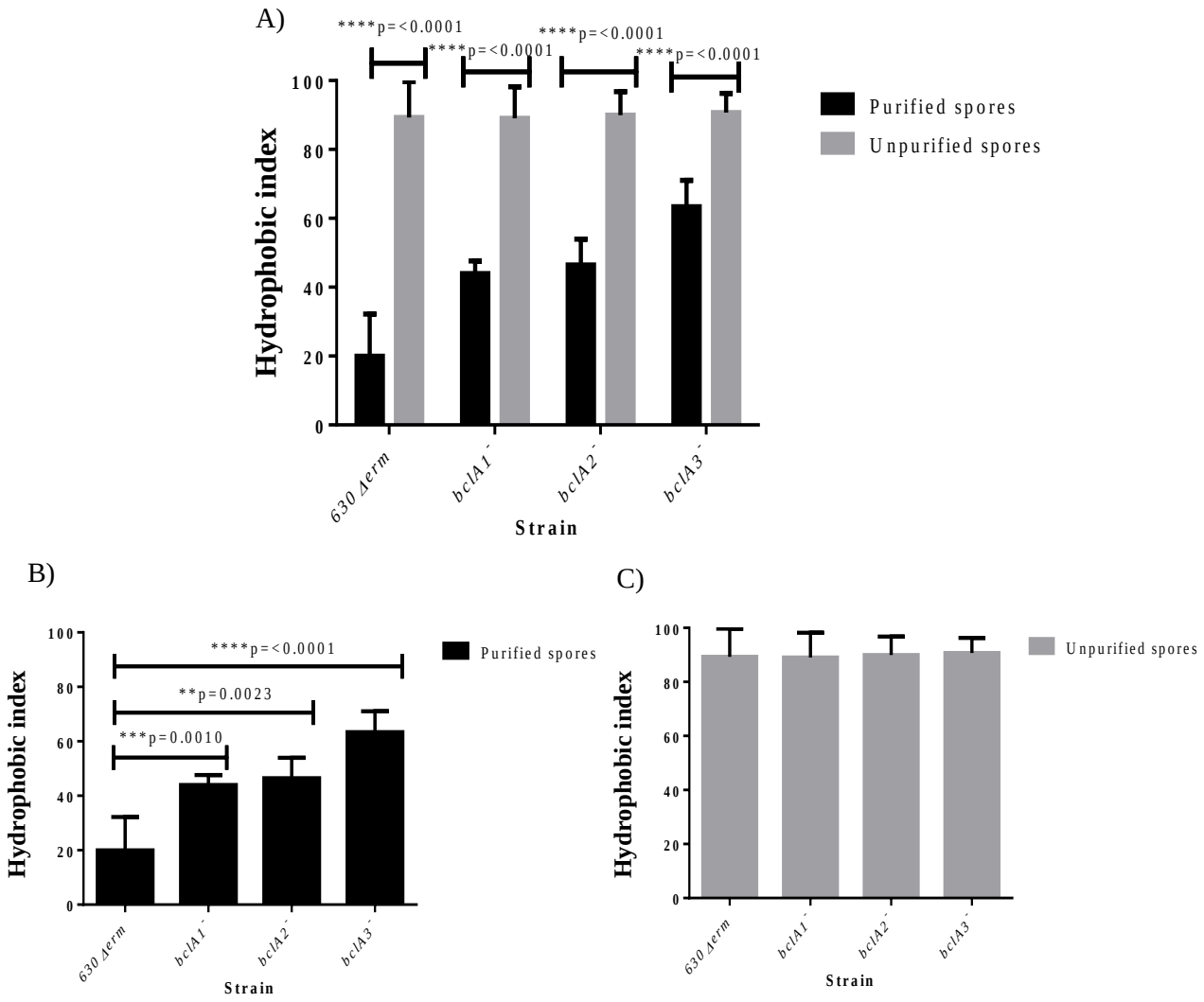


Figure 3.4. Hydrophobicity of HistoDenz purified and unpurified water washed *bclA*⁻ mutant spores. The SATH assay was used to calculate hydrophobicity of HistoDenz purified and unpurified water washed *bclA*⁻ mutant spores. Hydrophobicity was determined from the absorbance of the original spore suspension (A1) and the absorbance of the aqueous phase after incubation with hydrocarbon (A2) using the equation H=[(A1-A2)/A1]. A) Comparison of hydrophobicity of HistoDenz purified spores and unpurified water washed spores. B) Hydrophobicity of HistoDenz purified *bclA*⁻ mutant spores compared to 630Δ*erm* wild type. C) Hydrophobicity of unpurified water washed *bclA*⁻ mutant spores compared to 630Δ*erm* wild type.

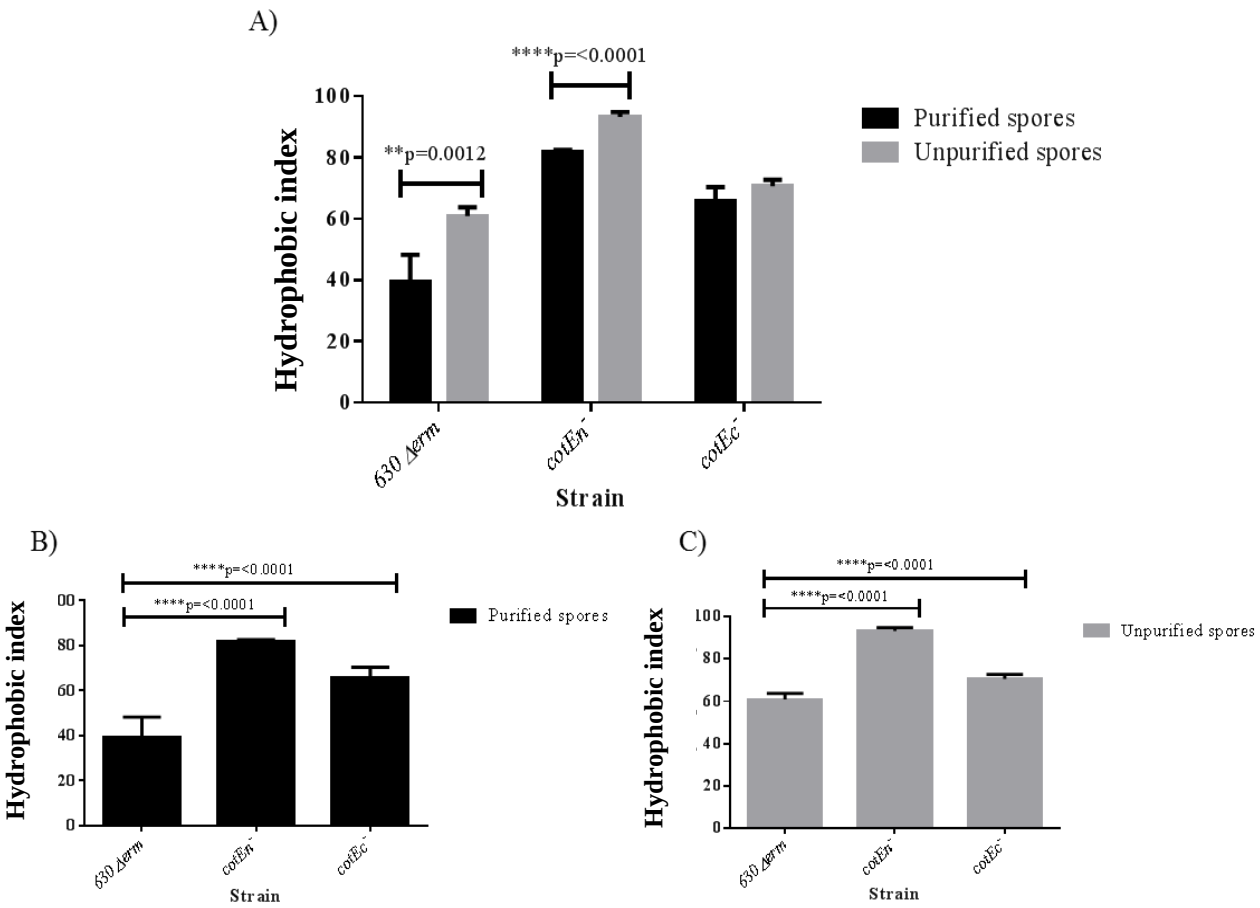


Figure 3.5. Hydrophobicity of HistoDenz purified and unpurified water washed *cotE*⁻ mutant spores. The SATH assay was used to calculate hydrophobicity of HistoDenz purified and unpurified water washed *cotE*⁻ mutant spores. Hydrophobicity was determined from the absorbance of the original spore suspension (A1) and the absorbance of the aqueous phase after incubation with hydrocarbon (A2) using the equation H=[(A1-A2)/A1]. A) Comparison of hydrophobicity of HistoDenz purified spores and unpurified water washed spores. B) Hydrophobicity of HistoDenz purified *cotE*⁻ mutant spores compared to 630Δ*erm* wild type. C) Hydrophobicity of unpurified water washed *cotE*⁻ mutant spores compared to 630Δ*erm* wild type.

3.2.4 Adherence of *C. difficile* spores to collagen I

HistoDenz purified and water washed unpurified *bclA*$^{-}$ mutant and mutant *cotE*$^{-}$ spores devoid of N- and C- terminal domain were assessed for their ability to adhere to ECM protein collagen I by ELISA (Figure 3.6 and 3.7). Spores were incubated with 96-well plates coated with collagen I for 2 h. Non-adherent bacteria were removed by washing and adherent spores were detected using mouse serum raised against *C. difficile* spores followed by detection with secondary antibody. Results show that both HistoDenz purified and water washed unpurified *bclA*$^{-}$ mutant spores had significantly lower adherence to collagen I compared to 630Δ*erm* (Figure 3.6A). Also significant differences were observed between the adherence of HistoDenz and water washed unpurified *cotEc*$^{-}$ spores and 630Δ*erm* (Figure 3.7B and 3.7C), with *cotEc*$^{-}$ spores adhering greater compared to 630Δ*erm* wild type (Figure 3.7A). Results could suggest that BclA plays a role in the adherence of spores to collagen I.

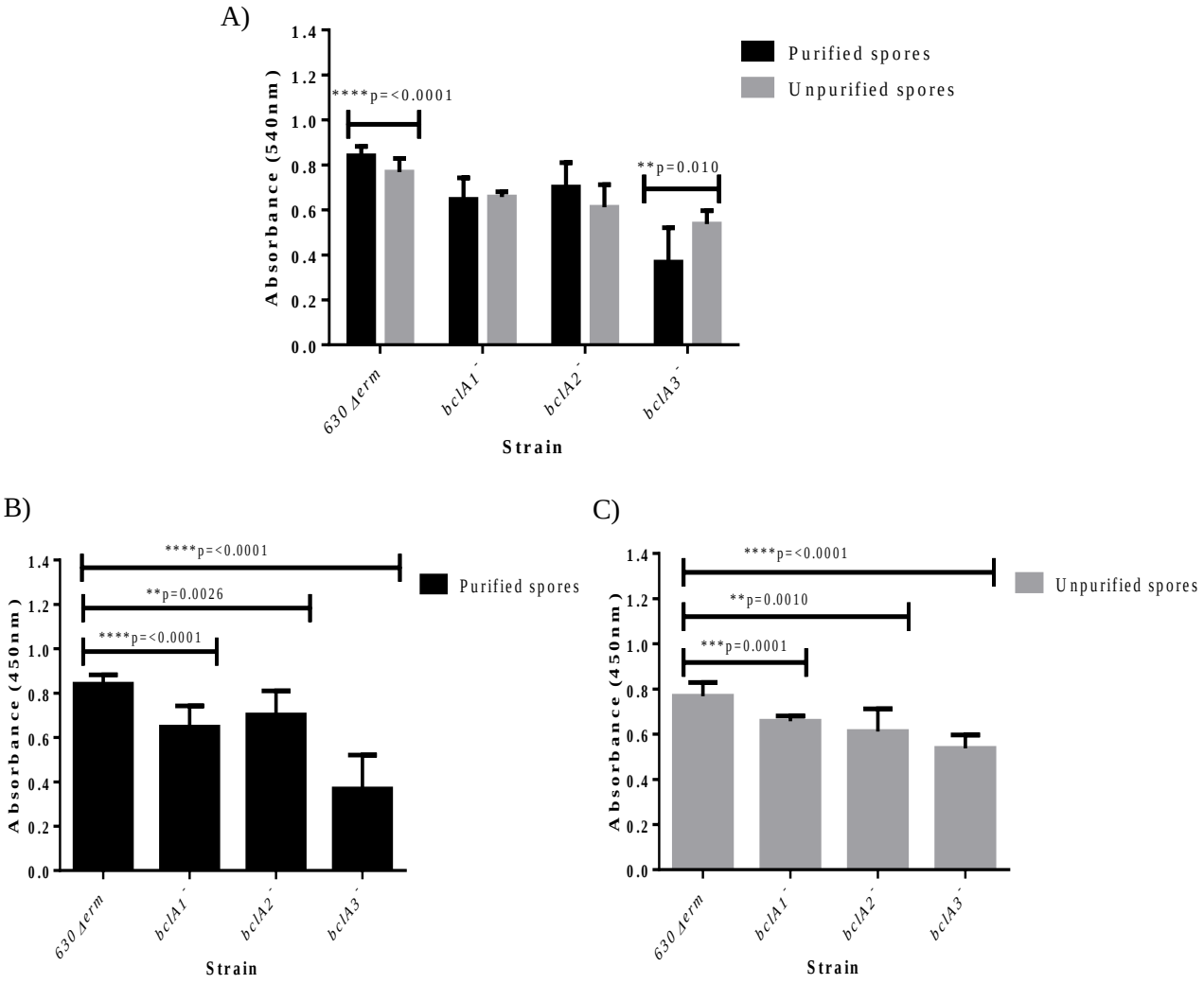


Figure 3.6. Adherence of HistoDenz purified and unpurified water washed *bclA*⁻ mutant spores to collagen I. HistoDenz purified and water washed unpurified mutant *bclA*⁻ *C. difficile* spores were incubated with human purified collagen type I for 2 h. Adherent bacteria were detected using mouse anti-serum against *C. difficile* spores. A) Compares adherence of both Histodenz purified and unpurified water washed *bclA*⁻ mutant *C. difficile* spores to collagen I. B) Adhesion of HistoDenz purified *bclA*⁻ mutant *C. difficile* spores to collagen I compared to wild type. C) Adhesion of unpurified water washed *bclA*⁻ mutant *C. difficile* spores to collagen I compared to wild type.

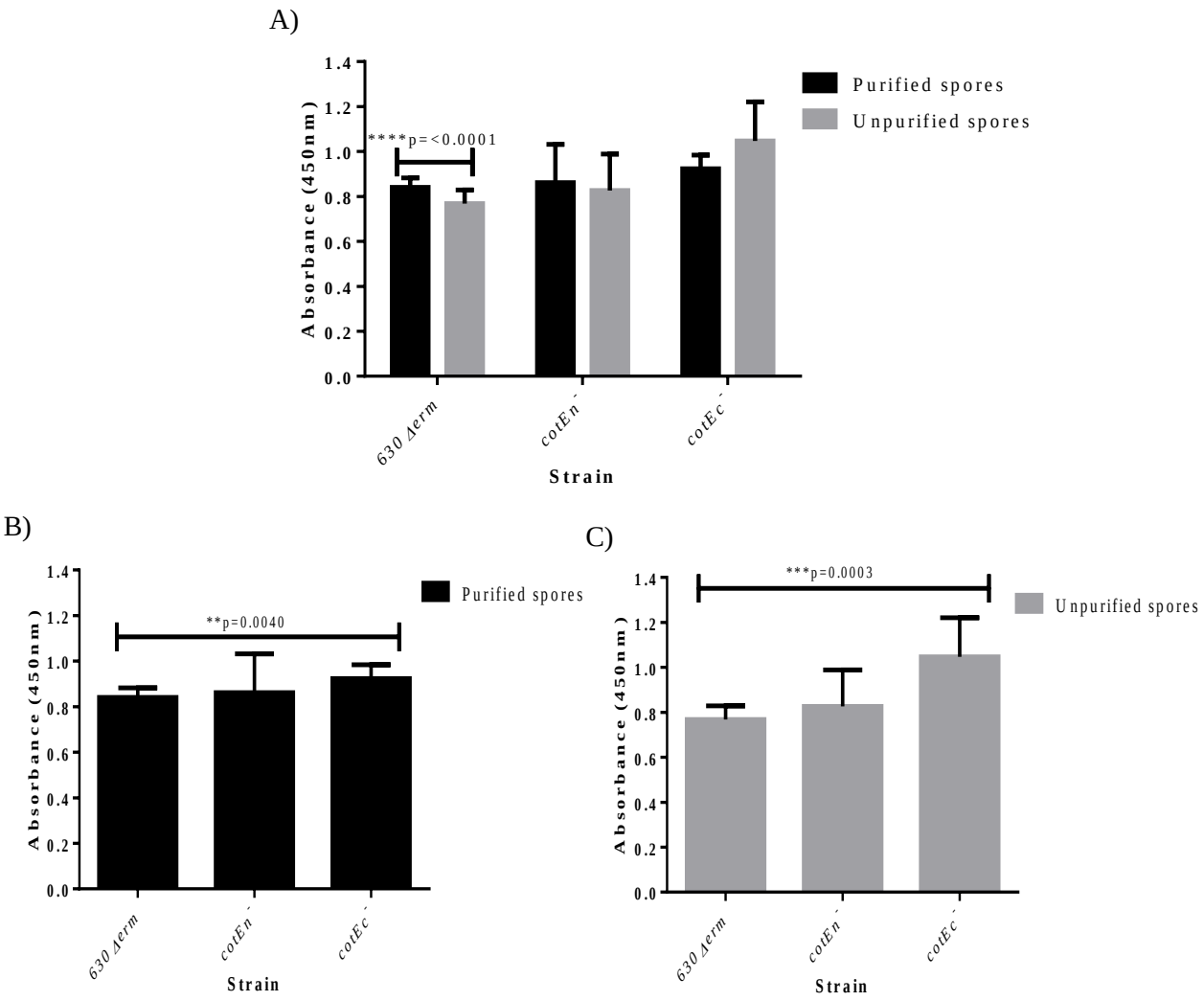


Figure 3.7. Adherence of HistoDenz purified and unpurified water washed c*otE*$^{-}$ mutant spores to collagen I. HistoDenz purified and water washed unpurified mutant c*otE*$^{-}$ *C. difficile* spores were incubated with human purified collagen type I for 2 h. Adherent bacteria were detected using mouse anti-serum against *C. difficile* spores. A) Compares adherence of both Histodenz purified and unpurified water washed c*otE*$^{-}$ mutant *C. difficile* spores to collagen I. B) Adhesion of HistoDenz purified *cotE*$^{-}$ mutant *C. difficile* spores to collagen I compared to wild type. C) Adhesion of unpurified water washed *cotE*$^{-}$ mutant *C. difficile* spores to collagen I compared to wild type.

3.2.5 Adherence of *C. difficile* spores to fibronectin

HistoDenz purified and water washed unpurified *bclA*$^{-}$ mutant and mutant *cotE*$^{-}$ spores devoid of N- and C- terminal domain were assessed for their ability to adhere to ECM protein fibronectin by ELISA (Figure 3.8 and 3.9). Spores were incubated with 96-well plates coated with fibronectin for 2 h. Non-adherent bacteria was removed by washing and adherent spores were detected using mouse serum raised against *C. difficile* spores followed by detection with secondary antibodies. Results showed that HistoDenz purified and water washed unpurified *bclA*$^{-}$ mutant spores had lower adherence to fibronectin compared to the wild type (Figure 3.8A). With significant differences observed for *bclA1*$^{-}$ and *bclA2*$^{-}$ HistoDenz purified spores (Figure 3.8B) and *bclA1*$^{-}$, *bclA2*$^{-}$ and *bclA3*$^{-}$ water washed unpurified spores (Figure 3.8C). *CotEc*$^{-}$ HistoDenz purified spores showed significant reduced adherence to fibronectin compared to 630Δ*erm* whereas *cotEn*$^{-}$ HistoDenz purified spores showed a significant increase in adherence to fibronectin compared to 630Δ*erm* (Figure 3.9B). No significant differences were observed between the adherence of water washed unpurified *cotE*$^{-}$ mutant spores compared to wild type (Figure 3.9C). Results could suggest that BclA plays a role in the adherence of spores to fibronectin.

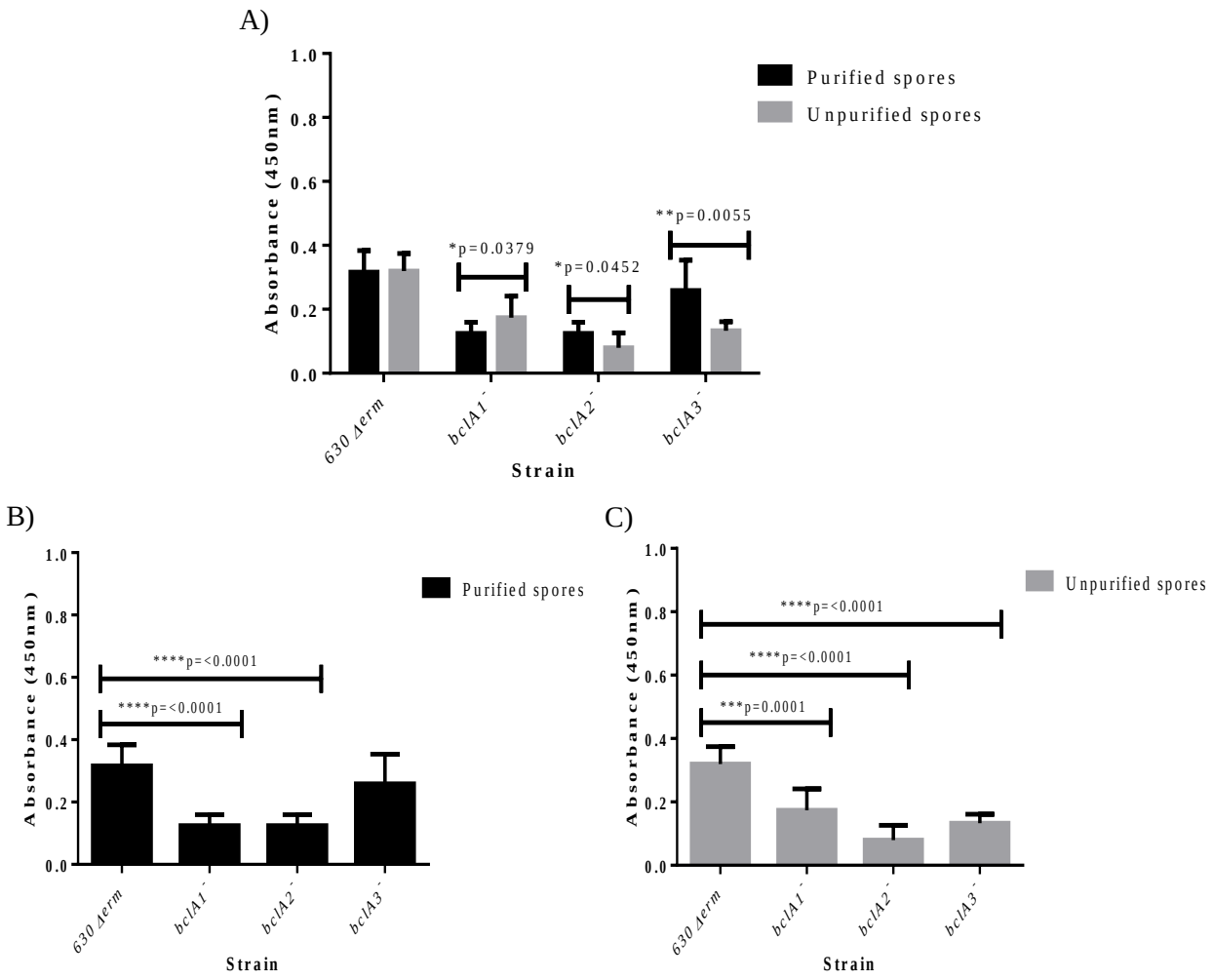


Figure 3.8. Adherence of HistoDenz purified and unpurified water washed *bclA*⁻ mutant spores to fibronectin. HistoDenz purified and water washed unpurified mutant *bclA*⁻ *C. difficile* spores were incubated with purified human plasma fibronectin for 2 h. Adherent bacteria were detected using mouse anti-serum against *C. difficile* spores. A) Compares adherence of both HistoDenz purified and unpurified water washed *bclA*⁻ mutant *C. difficile* spores to fibronectin B) Adhesion of HistoDenz purified *bclA*⁻ mutant *C. difficile* spores to fibronectin compared to wild type. C) Adhesion of unpurified water washed *bclA*⁻ mutant *C. difficile* spores to fibronectin compared to wild type.

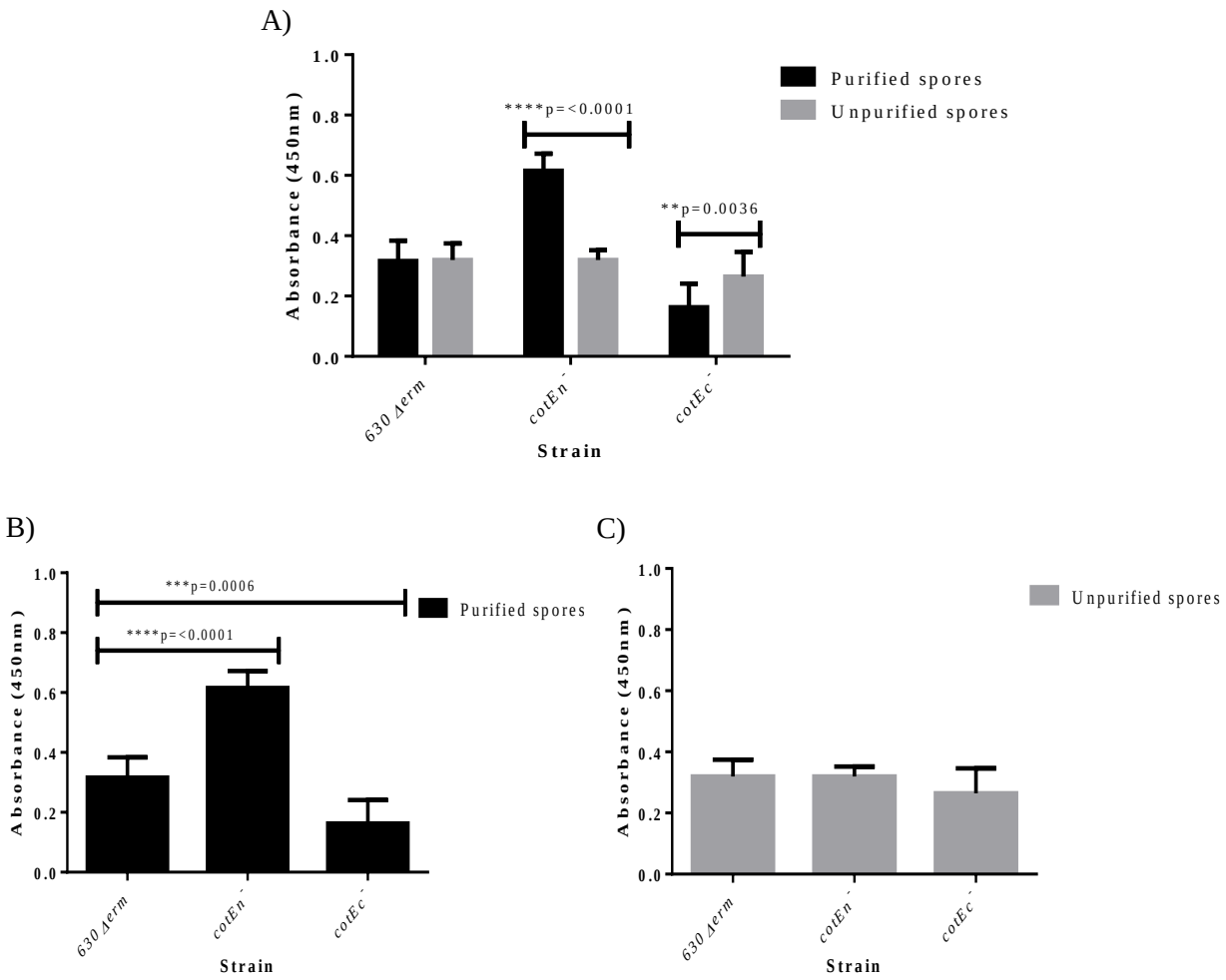


Figure 3.9. Adherence of HistoDenz purified and unpurified water washed *cotE*⁻ mutant spores to fibronectin. HistoDenz purified and water washed unpurified mutant *cotE*⁻ *C. difficile* spores were incubated with purified human plasma fibronectin for 2 h. Adherent bacteria were detected using mouse anti-serum against *C. difficile* spores. A) Compares adherence of both Histodenz purified and unpurified water washed *cotE*⁻ mutant *C. difficile* spores to fibronectin. B) Adhesion of HistoDenz purified *cotE*⁻ mutant *C. difficile* spores to fibronectin compared to wild type. C) Adhesion of unpurified water washed *cotE*⁻ mutant *C. difficile* spores to fibronectin compared to wild type.

3.2.6 Staining for mucin on HT29-MTX cells

To verify that HT29-MTX cells, a mucin producing cell line secretes mucus, Alcian blue staining of HT29 and HT29-MTX cell monolayers was carried out at days 7 and 14 post seeding. Alcian blue is a basic dye that has affinity for acidic tissue components such as mucin. Staining shows that acidic sulphated mucosubstances, hyaluronic acid and sialomucins are stained blue and nuclei are stained pink/red (Figure 3.10).

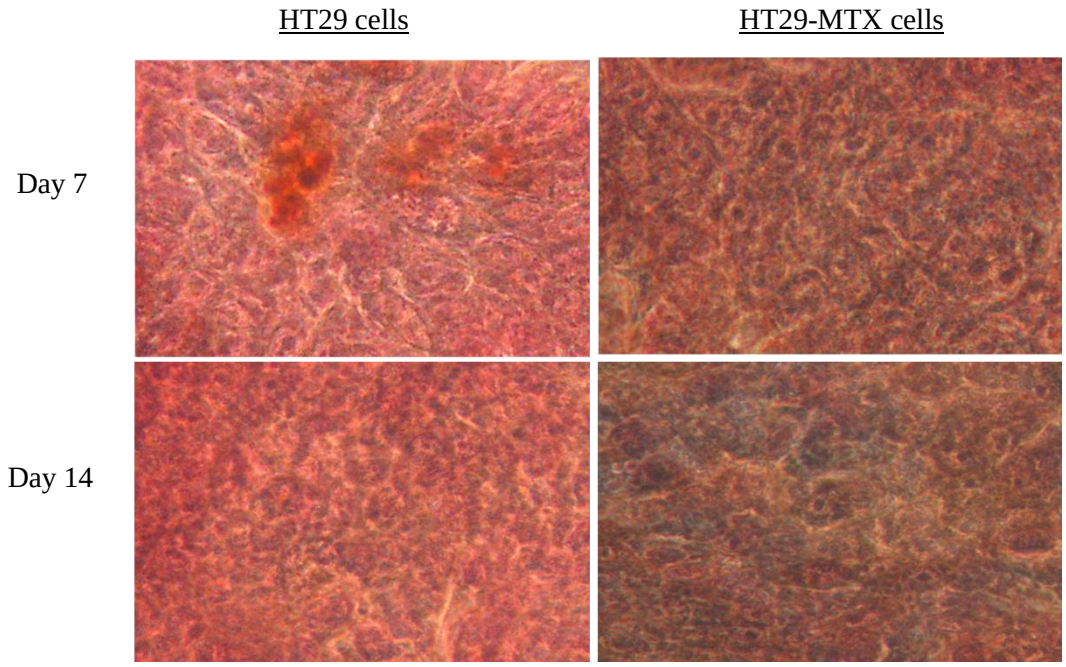


Figure 3.10. Alcian blue staining of HT29 and HT29-MTX cell monolayers days 7 and 14 post seeding. Acidic sulphated mucosubstances, hyaluronic acid and sialomucins are stained in blue by Alcian blue solution and nuclei are stained red using nuclear fast red solution.

3.2.7 Adherence of *C. difficile* spores to HT29 and HT29-MTX cells

BclA^{-} mutant and mutant *cotE*$^{-}$ spores devoid of N- and C- terminal domain and vegetative cells were assessed for their ability to adhere to 7 and 14 day old HT29 and HT29-MTX cell monolayers (Figure 3.11, 3.12. 3.13 and 3.14). Water washed unpurified spores were incubated for 2 h at a MOI 100:1 with 7 and 14 day old cell monolayers. Wells were washed five times with DPBS to remove non-adherent bacteria. To determine the number of spores that had adhered to monolayers cells were lysed, serially diluted and plated onto BHISS plates for CFU enumeration. For adhesion to 7-day old HT29 and HT29-MTX cell monolayers results show that *C. difficile* spores adherence was greater to cell monolayers compared to vegetative cells (Figure 3.11A and 3.12A). For 7-day old HT29 and HT29-MTX monolayers there is a significant difference in the adherence of 630Δ*erm* spores with an increased adherence of spores to HT29-MTX cell monolayers compared to HT29 (Figure 3.11A). *BclA3*$^{-}$ spores show increased adherence to 7-day old HT29 cell monolayers compared to the wild type (Figure 3.11B) and a decrease in adherence to 7-day old HT29-MTX cell monolayers is observed for *bclA1*$^{-}$ and *bclA2*$^{-}$ spores compared to 630Δ*erm* spores (Figure 3.11C).

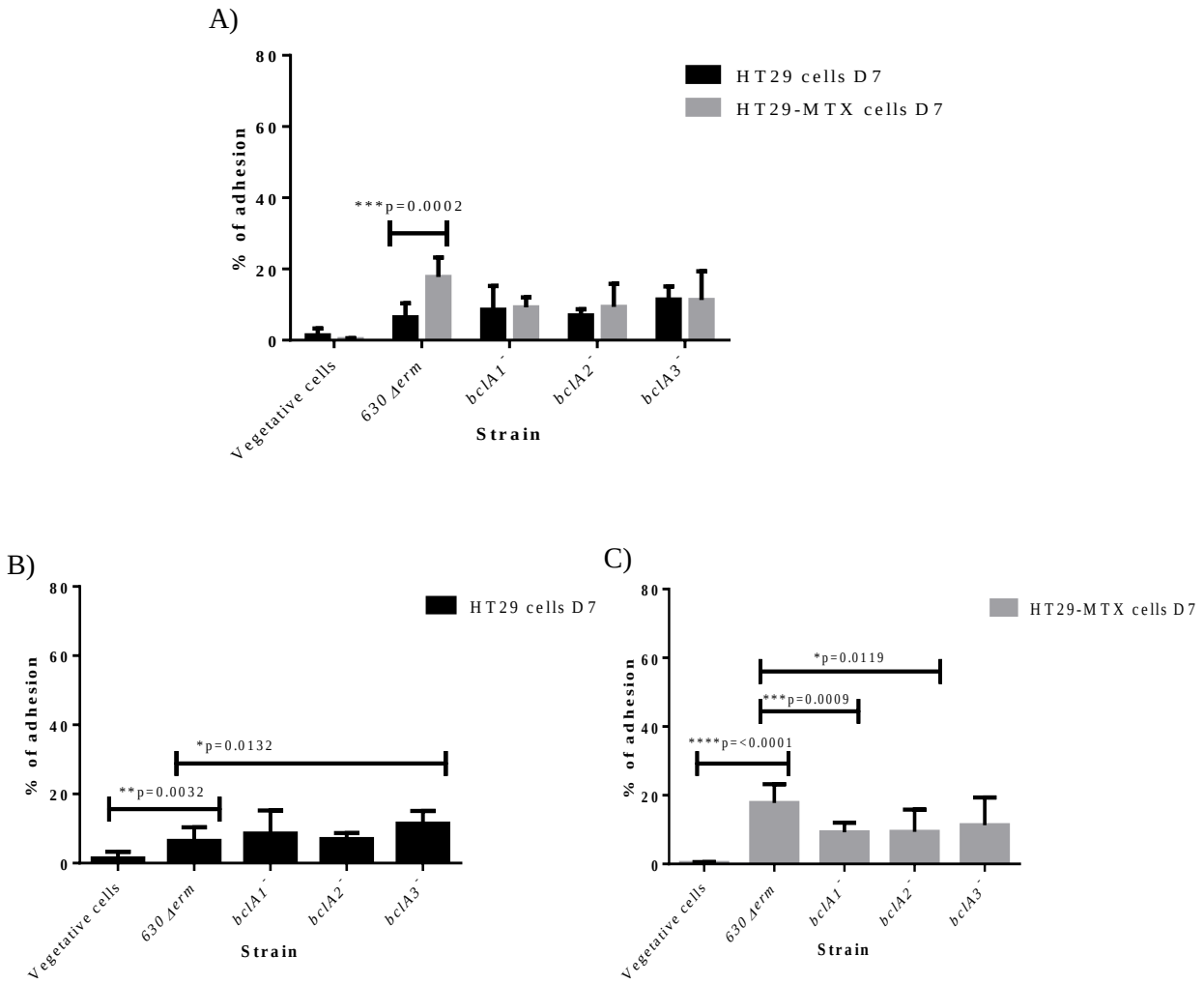


Figure 3.11. Adherence of unpurified water washed 630Δ*erm* and *bclA*⁻ mutant spores to 7-day old HT29 and HT29-MTX cell monolayers. Cell monolayers were infected at MOI 100:1 with *C. difficile* spores or vegetative cells for 2 h, monolayers were washed to remove non-adherent bacteria. To determine number of spores or vegetative cells adhering to monolayers, cells were lysed and samples were serially diluted and plated onto BHISS plates for CFU enumeration. Number of CFU was determined and the percentage of adherence was calculated using the following formula (final CFU/initial CFU) x 100. A) Compares the adherence of vegetative cells, wild type and *bclA*⁻ mutant spores to 7-day old HT29 and HT29-MTX cell monolayers. B) Adherence of vegetative cells and *bclA*⁻ mutant spores to 7-day old HT29 cell monolayers compared to the wild type. C) Adherence of vegetative cells and *bclA*⁻ mutant spores to 7-day old HT29-MTX cell monolayers compared to the wild type.

For adhesion to 14-day old HT29 and HT29-MTX cell monolayers, results show that there was a significant difference observed between adherence of 630Δ*erm* and *bclA*$^{-}$ mutant spores to HT29 and HT29-MTX cell monolayers with greater adherence observed to HT29-MTX cell monolayers (Figure 3.12A). The adherence of spores to HT29-MTX cell monolayers also increases from day 7 to day 14, this could be due to the increase of mucus present on HT29-MTX cell monolayers as days post seeding increases. Results also showed that *bclA2*$^{-}$ spores showed significantly less adherence to HT29-MTX cell monolayers compared to the wild type (Figure 3.12C). Although there is no significant difference, *bclA1*$^{-}$ and *bclA3*$^{-}$ spores show an increased adherence to 14-day old HT29 and HT29-MTX cell monolayers (Figure 3.12B and 3.12C).

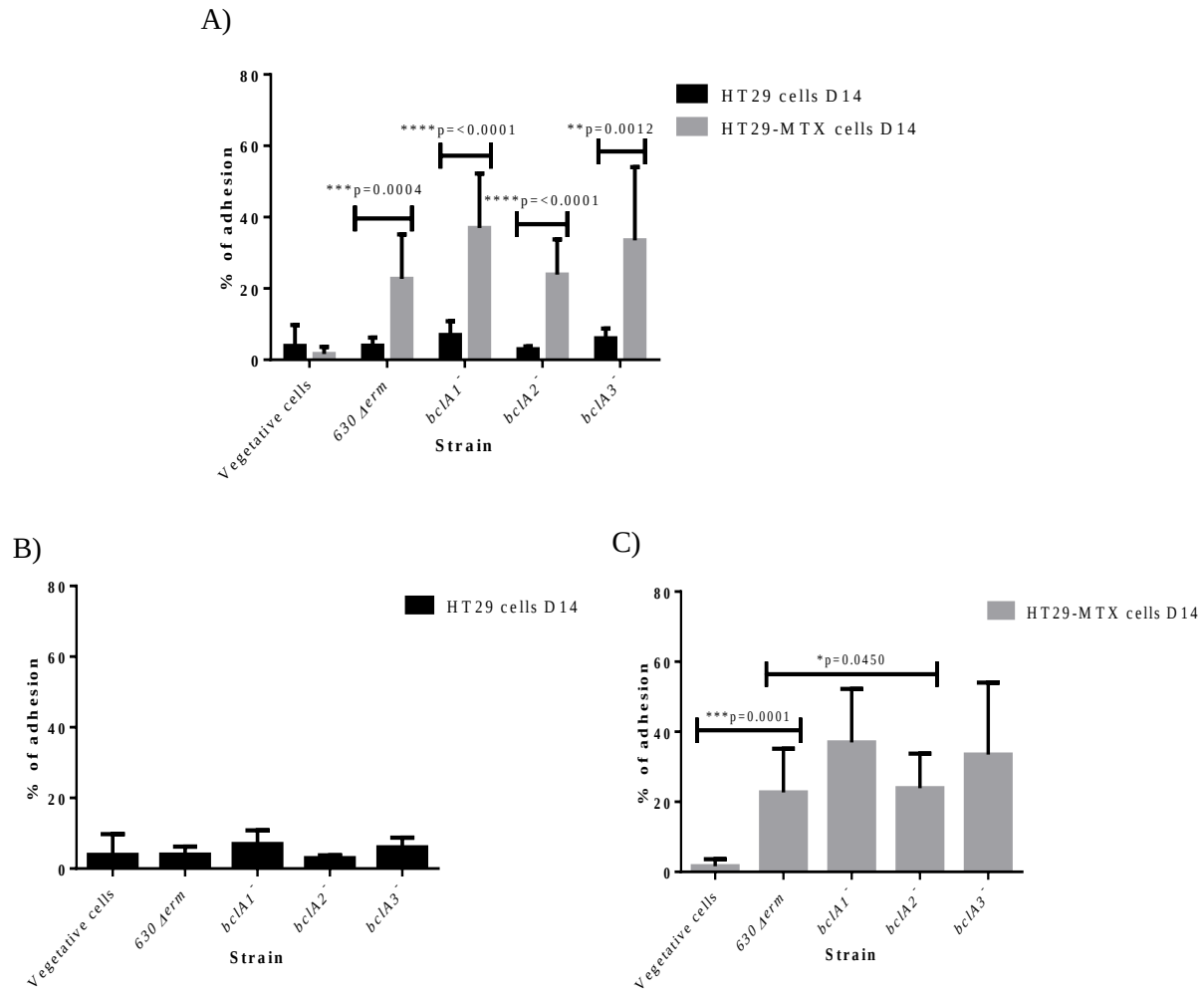


Figure 3.12. Adherence of unpurified water washed 630Δ*erm* and *bclA*⁻ mutant spores to 14-day old HT29 and HT29-MTX cell monolayers. Cell monolayers were infected at MOI 100:1 with *C. difficile* spores or vegetative cells for 2 h, monolayers were washed to remove non-adherent bacteria. To determine number of spores or vegetative cells adhering to monolayers, cells were lysed and samples were serially diluted and plated onto BHISS plates for CFU enumeration. Number of CFU was determined and the percentage of adherence was calculated using the following formula (final CFU/initial CFU) x 100. A) Compares the adherence of vegetative cells, wild type and *bclA*⁻ mutant spores to 14-day old HT29 and HT29-MTX cell monolayers. B) Adherence of vegetative cells and *bclA*⁻ mutant spores to 14-day old HT29 cell monolayers compared to the wild type. C) Adherence of vegetative cells and *bclA*⁻ mutant spores to 14-day old HT29-MTX cell monolayers compared to the wild type.

For adhesion to 7-day old HT29 and HT29-MTX cell monolayers results showed that there was a significant difference observed between adherence of *cotE*⁻ mutant spores to HT29 and HT29-MTX cell monolayers with greater adherence observed to HT29-MTX cell monolayers (Figure 3.13A). 630Δ*erm* spores adhere greater to both HT29 and HT29-MTX cell monolayers compared to mutant *cotE*⁻ spores devoid of N- and C- terminal domain (Figure 3.13B and 3.13C).

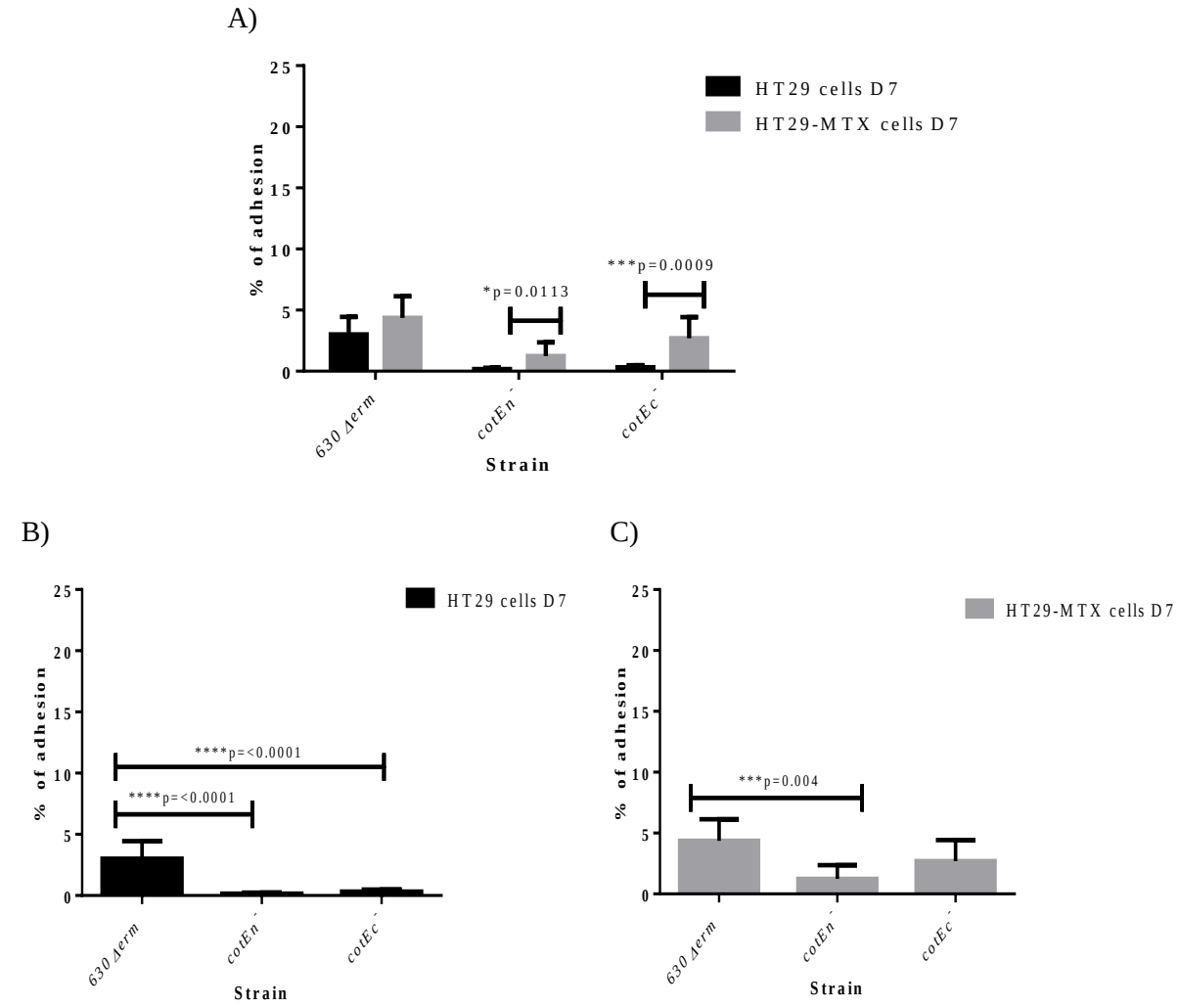


Figure 3.132. Adherence of unpurified water washed 630Δ*erm* and *cotE*⁻ mutant spores to 7-day old HT29 and HT29-MTX cell monolayers. Cell monolayers were infected at MOI 100:1 with *C. difficile* spores for 2 h, monolayers were washed to remove non-adherent bacteria. To determine number of spores adhering to monolayers, cells were lysed and samples were serially diluted and plated onto BHISS plates for CFU enumeration. Number of CFU was determined and the percentage of adherence was calculated using the following formula (final CFU/initial CFU) x 100. A) Compares the adherence of wild type and *cotE*⁻ mutant spores to 7-day old HT29 and HT29-MTX cell monolayers. B) Adherence of *cotE*⁻ mutant spores to 7-day old HT29 cell monolayers compared to the wild type. C) Adherence of *cotE*⁻ mutant spores to 7-day old HT29-MTX cell monolayers compared to the wild type.

For adhesion to 14-day old HT29 and HT29-MTX cell monolayers results show that there is a significant difference observed between adherence of 630Δ*erm* and *cotE*$^{-}$ mutant spores to HT29 and HT29-MTX cell monolayers with greater adherence observed to HT29-MTX cell monolayers (Figure 3.14A). 630Δ*erm* spores significantly adhere greater to both HT29 and HT29-MTX cell monolayers compared to mutant *cotE*$^{-}$ spores devoid of N- and C- terminal domain (Figure 3.14B and 3.14C). Results from adhesion assays to cell monolayers at day 7 and day 14 are suggestive that CotE may have a role to play in the adherence of *C. difficile* spores to epithelial cells and mucus.

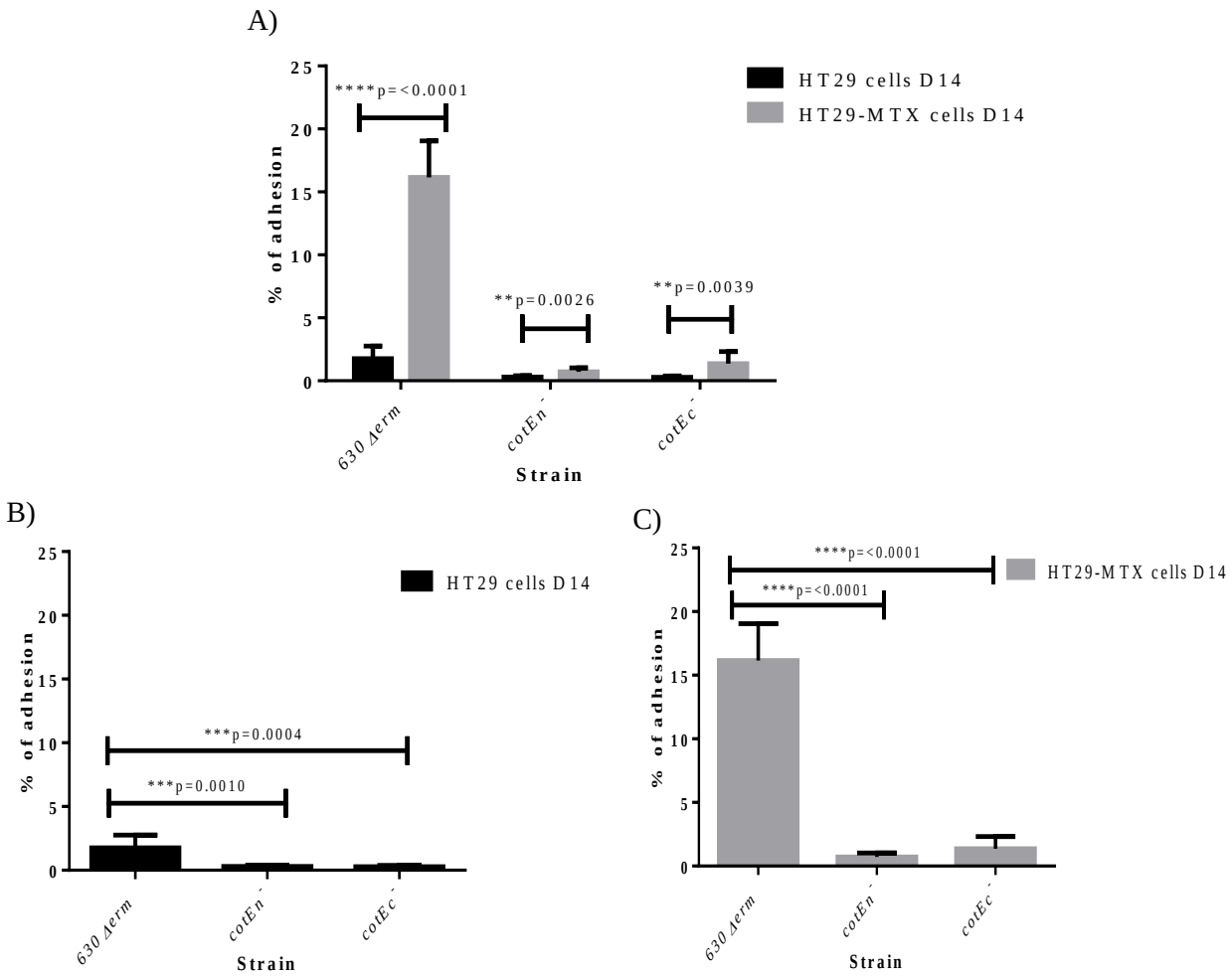


Figure 3.14. Adherence of unpurified water washed 630Δ*erm* and *cotE*⁻ mutant spores to 14-day old HT29 and HT29-MTX cell monolayers. Cell monolayers were infected at MOI 100:1 with *C. difficile* spores for 2 h, monolayers were washed to remove non-adherent bacteria. To determine number of spores adhering to monolayers, cells were lysed and samples were serially diluted and plated onto BHISS plates for CFU enumeration. Number of CFU was determined and the percentage of adherence was calculated using the following formula (final CFU/initial CFU) x 100. A) Compares the adherence of wild type and *cotE*⁻ mutant spores to 14-day old HT29 and HT29-MTX cell monolayers. B) Adherence of *cotE*⁻ mutant spores to 14-day old HT29 cell monolayers compared to the wild type. C) Adherence of *cotE*⁻ mutant spores to 14-day old HT29-MTX cell monolayers compared to the wild type.

As *C. difficile* spores were shown to germinate in culture media, samples were heat treated at 60°C for 20 min to kill any vegetative cells before plating out onto BHISS plates to determine the population of dormant spores (Figure 3.15, 3.16, 3.17 and 3.18). No significant difference was observed between the adherence of *bclA*$^{-}$ mutant spores to HT29 and HT29-MTX 7-day old cell monolayers (Figure 3.15A). There was also no significant difference observed between the adherence of *bclA*$^{-}$ mutants and 630Δ*erm* spores (Figure 3.15B and 3.15C).

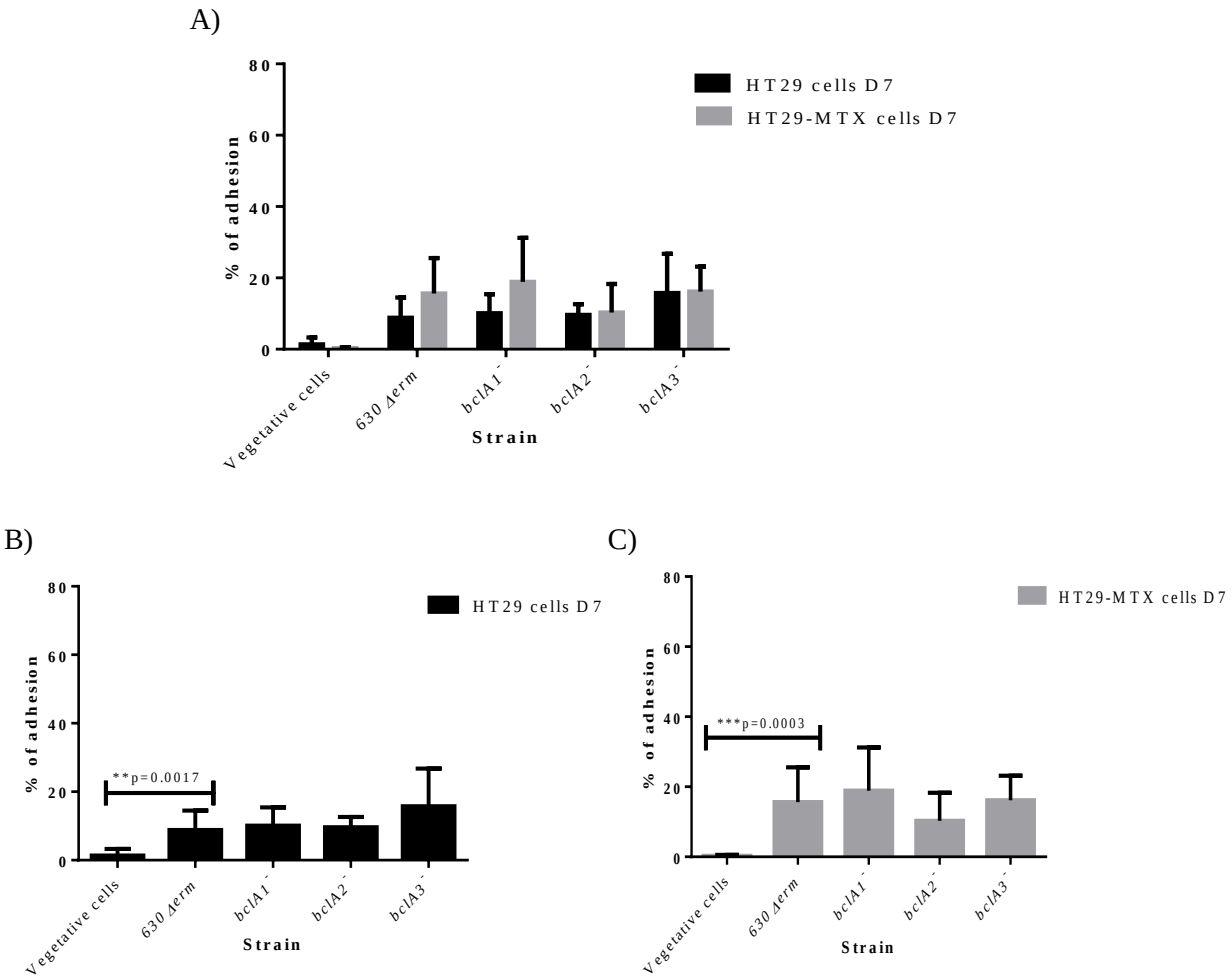


Figure 3.15. Adherence of unpurified water washed 630Δ*erm* and *bclA*⁻ mutant spores to 7-day old HT29 and HT29-MTX cell monolayers, heat resistant counts. Cell monolayers were infected at MOI 100:1 with *C. difficile* spores or vegetative cells for 2 h, monolayers were washed to remove non-adherent bacteria. To determine number of spores or vegetative cells adhering to monolayers, cells were lysed and samples were heat treated at 60ºC for 20 min and serially diluted and plated onto BHISS plates for CFU enumeration. Number of CFU was determined and the percentage of adherence was calculated using the following formula (final CFU/initial CFU) x 100. A) Compares the adherence of vegetative cells, heat resistant wild type and *bclA*⁻ mutant spores to 7-day old HT29 and HT29-MTX cell monolayers. B) Adherence of vegetative cells and heat resistant *bclA*⁻ mutant spores to 7-day old HT29 cell monolayers compared to the wild type. C) Adherence of vegetative cells and heat resistant *bclA*⁻ mutant spores to 7-day old HT29-MTX cell monolayers compared to the wild type.

For adhesion to 14-day old HT29 and HT29-MTX cell monolayers results show that there was a significant difference observed between adherence of 630Δ*erm* and *bclA*$^{-}$ mutant spores to HT29 and HT29-MTX cell monolayers with greater adherence observed to HT29-MTX cell monolayers (Figure 3.16). There was also no significant difference observed between the adherence of *bclA*$^{-}$ mutants and 630Δ*erm* spores. However *bclA1*$^{-}$ spores show greater adherence compared to the wild type and *bclA2*$^{-}$ spores showed reduced adherence compared to the wild type to both 7 and 14 day old HT29 and HT29-MTX cell monolayers (Figure 3.16B and 3.16C), a similar trend was observed for the total viable count without heat treatment.

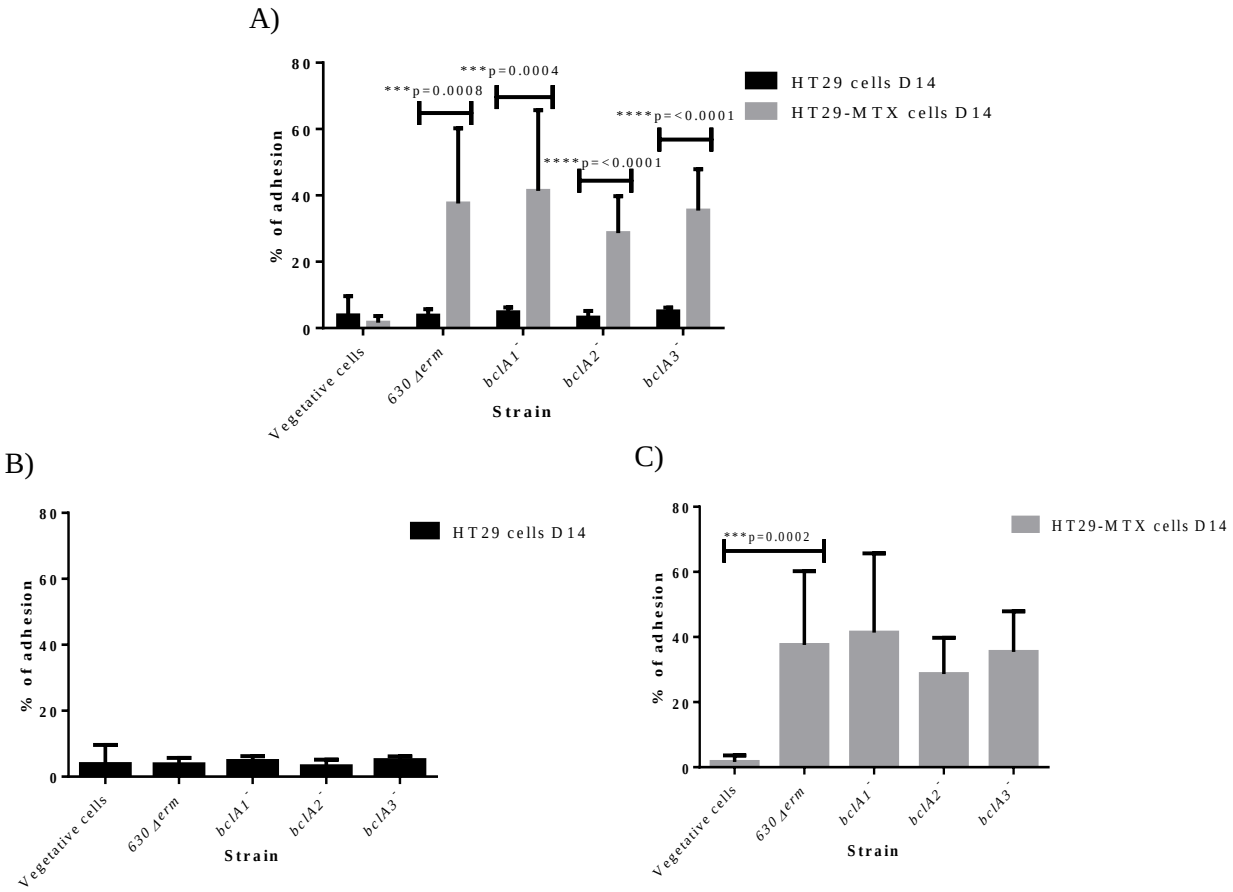


Figure 3.16. Adherence of unpurified water washed 630Δ*erm* and *bclA*$^{-}$ mutant spores to 14-day old HT29 and HT29-MTX cell monolayers, heat resistant counts. Cell monolayers were infected at MOI 100:1 with *C. difficile* spores or vegetative cells for 2 h, monolayers were washed to remove non-adherent bacteria. To determine number of spores or vegetative cells adhering to monolayers, cells were lysed and samples were heat treated at 60ºC for 20 min and serially diluted and plated onto BHISS plates for CFU enumeration. Number of CFU was determined and the percentage of adherence was calculated using the following formula (final CFU/initial CFU) x 100. A) Compares the adherence of vegetative cells, heat resistant wild type and *bclA*$^{-}$ mutant spores to 14-day old HT29 and HT29-MTX cell monolayers. B) Adherence of vegetative cells and heat resistant *bclA*$^{-}$ mutant spores to 14-day old HT29 cell monolayers compared to the wild type. C) Adherence of vegetative cells and heat resistant *bclA*$^{-}$ mutant spores to 14-day old HT29-MTX cell monolayers compared to the wild type.

For adhesion to 7-day old HT29 and HT29-MTX cell monolayers results show that there was a significant difference observed between adherence of *cotE*$^{-}$ mutant spores to HT29 and HT29-MTX cell monolayers with greater adherence observed to HT29-MTX cell monolayers (Figure 3.17A). 630Δ*erm* spores adhered greater to both HT29 and HT29-MTX cell monolayers compared to mutant c*otE*$^{-}$ spores devoid of the N- and C- terminal domains (Figure 3.17B and 3.17C).

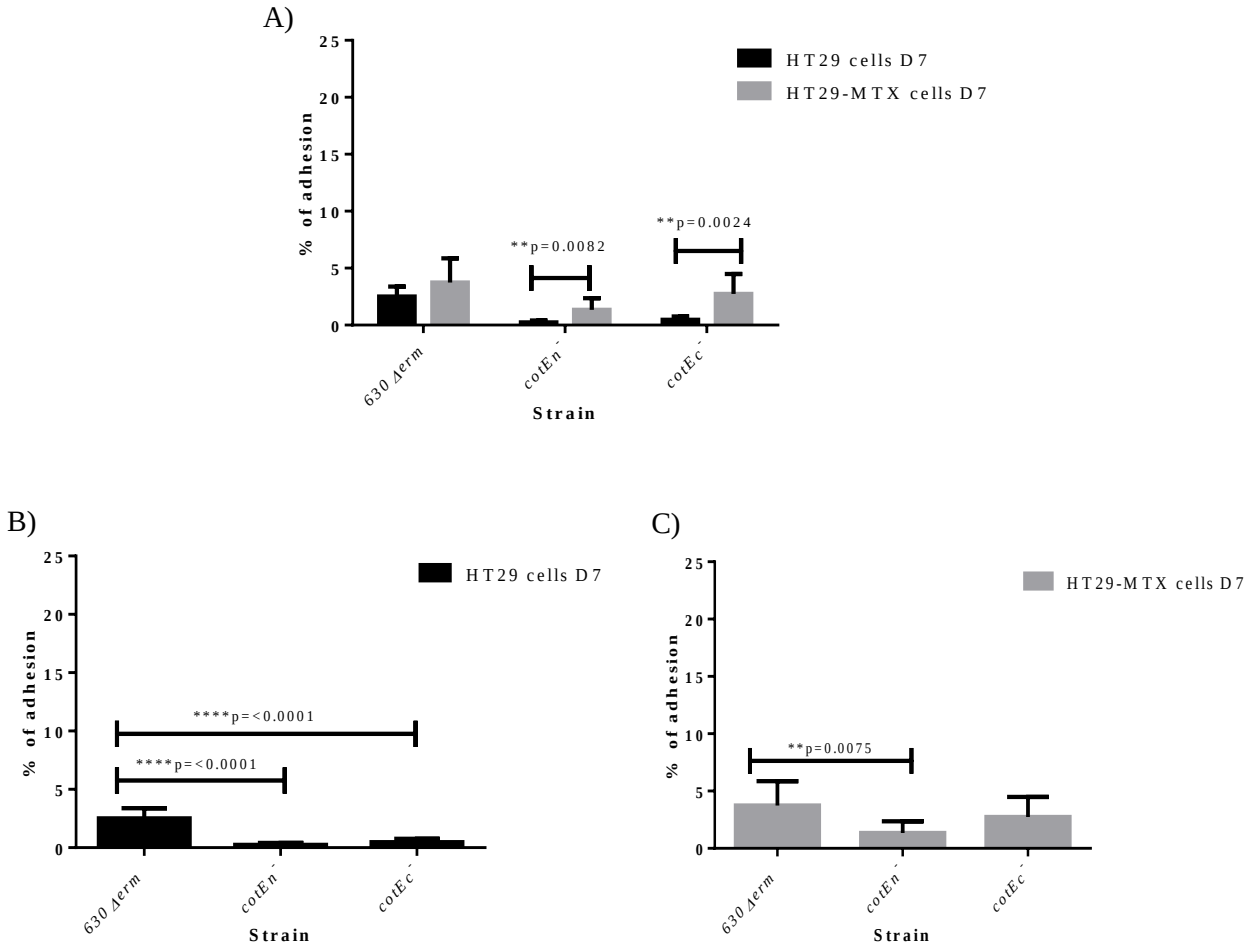


Figure 3.17. Adherence of unpurified water washed 630Δ*erm* and *cotE*$^{-}$ mutant spores to 7-day old HT29 and HT29-MTX cell monolayers, heat resistant counts. Cell monolayers were infected at MOI 100:1 with *C. difficile* spores for 2 h, monolayers were washed to remove non-adherent bacteria. To determine number of spores adhering to monolayers, cells were lysed and samples were heat treated 60ºC for 20 min and serially diluted and plated onto BHISS plates for CFU enumeration. Number of CFU was determined and the percentage of adherence was calculated using the following formula (final CFU/initial CFU) x 100. A) Compares the adherence of heat treated wild type and *cotE*$^{-}$ mutant spores to 7-day old HT29 and HT29-MTX cell monolayers. B) Adherence of heat treated *cotE*$^{-}$ mutant spores to 7-day old HT29 cell monolayers compared to the wild type. C) Adherence of heat treated *cotE*$^{-}$ mutant spores to 7-day old HT29-MTX cell monolayers compared to the wild type.

For adhesion to 14-day old HT29 and HT29-MTX cell monolayers results show that there was a significant difference observed between adherence of 630Δ*erm* and *cotE*$^{-}$ mutant spores to HT29 and HT29-MTX cell monolayers with greater adherence observed to HT29-MTX cell monolayers (Figure 3.18A). 630Δ*erm* spores significantly adhered greater to both HT29 and HT29-MTX cell monolayers compared to mutant *cotE*$^{-}$ spores devoid of the N- and C- terminal domain (Figure 3.18B and 3.18C). These results follow the same trend observed for total viable count without heat treatment.

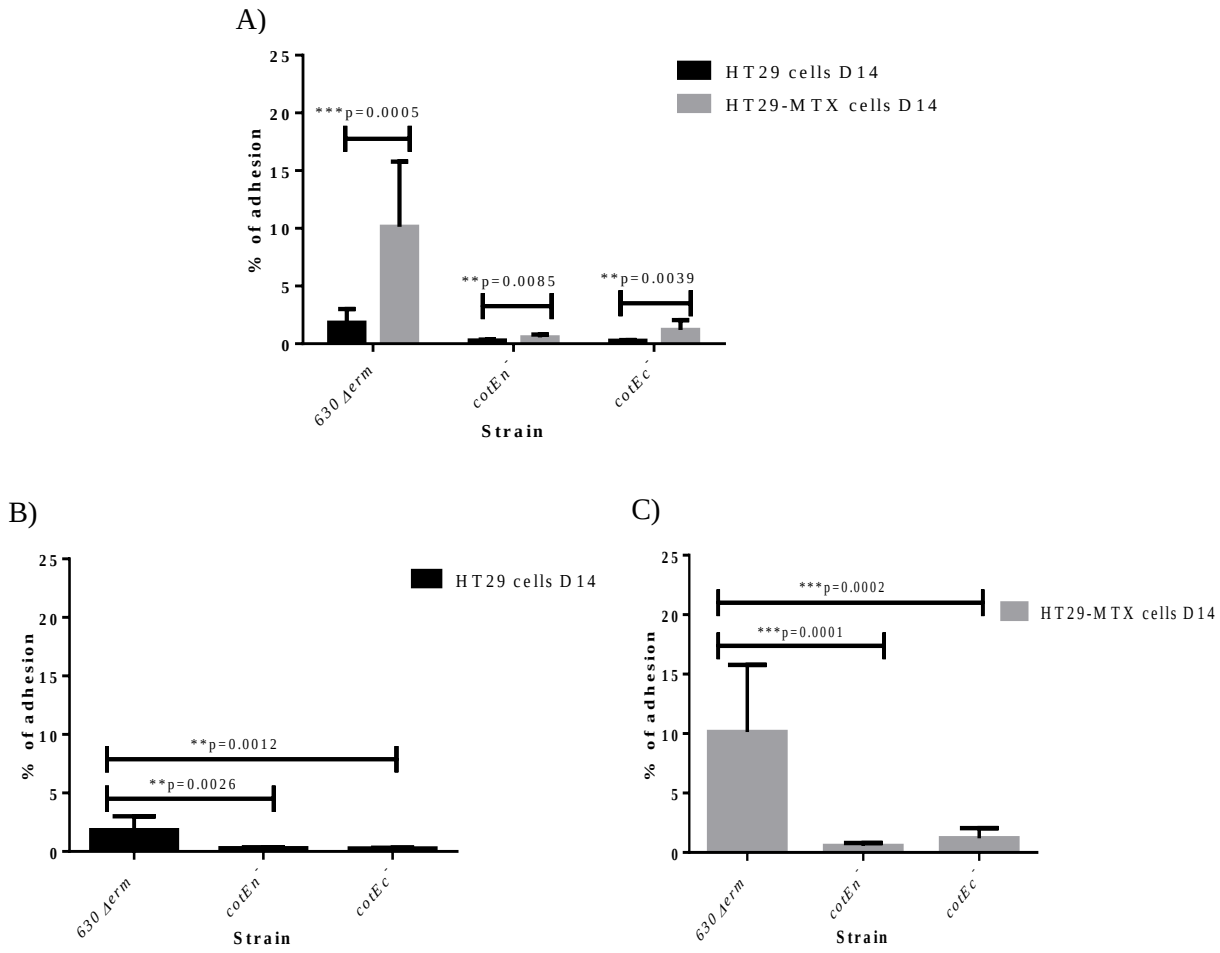


Figure 3.18. Adherence of unpurified water washed 630Δ*erm* and c*otE* - mutant spores to 14-day old HT29 and HT29-MTX cell monolayers, heat resistant counts. Cell monolayers were infected at MOI 100:1 with *C. difficile* spores for 2 h, monolayers were washed to remove non-adherent bacteria. To determine number of spores adhering to monolayers, cells were lysed and samples were heat treated 60ºC for 20 min and serially diluted and plated onto BHISS plates for CFU enumeration. Number of CFU was determined and the percentage of adherence was calculated using the following formula (final CFU/initial CFU) x 100. A) Compares the adherence of heat treated wild type and *cotE*- mutant spores to 14-day old HT29 and HT29-MTX cell monolayers. B) Adherence of heat treated *cotE*- mutant spores to 14-day old HT29 cell monolayers compared to the wild type. C) Adherence of heat treated *cotE*- mutant spores to 14-day old HT29-MTX cell monolayers compared to the wild type.

3.2.8 Immunofluorescence of *C. difficile* spore adherence to HT29 and HT29-MTX cells

To further confirm the adherence of mutant *bclA*$^{-}$, mutant *cotE*$^{-}$ spores devoid of the N- and C- terminal domain and 630Δ*erm* spores to HT29 and HT29-MTX cell monolayers. *C. difficile* spores were incubated for 2 h with 7 and 14 day old cell monolayers. Wells were washed five times with DPBS to remove non-adherent bacteria. Samples were fixed, permeabilized and adherent spores were labelled with mouse antiserum against *C. difficile* for 1 h. Spores were subsequently labelled with anti-mouse IgG-FITC antibody for 1 h. Cell monolayers were washed and cell nuclei of HT29 and HT29-MTX cells were stained with DAPI. Samples were mounted and examined using EVOS FL digital microscope at magnification 20x. Results show adherence of spores to HT29 and HT29-MTX cell monolayers at both days 7 and 14 (Figure 3.19). 7 and 14 day old HT29-MTX cell monolayers appeared to have the greatest adherence of *C. difficile* spores compared to 7 and 14 day old HT29 cell monolayers, with 14-day old HT29-MTX cell monolayers showing the greatest adherence for *bclA1*$^{-}$ and *bclA2*$^{-}$ spores. There appears to be reduced adherence of *cotE*$^{-}$ mutant spores compared to 630Δ*erm* spores to 14-day old HT29-MTX cell monolayers.

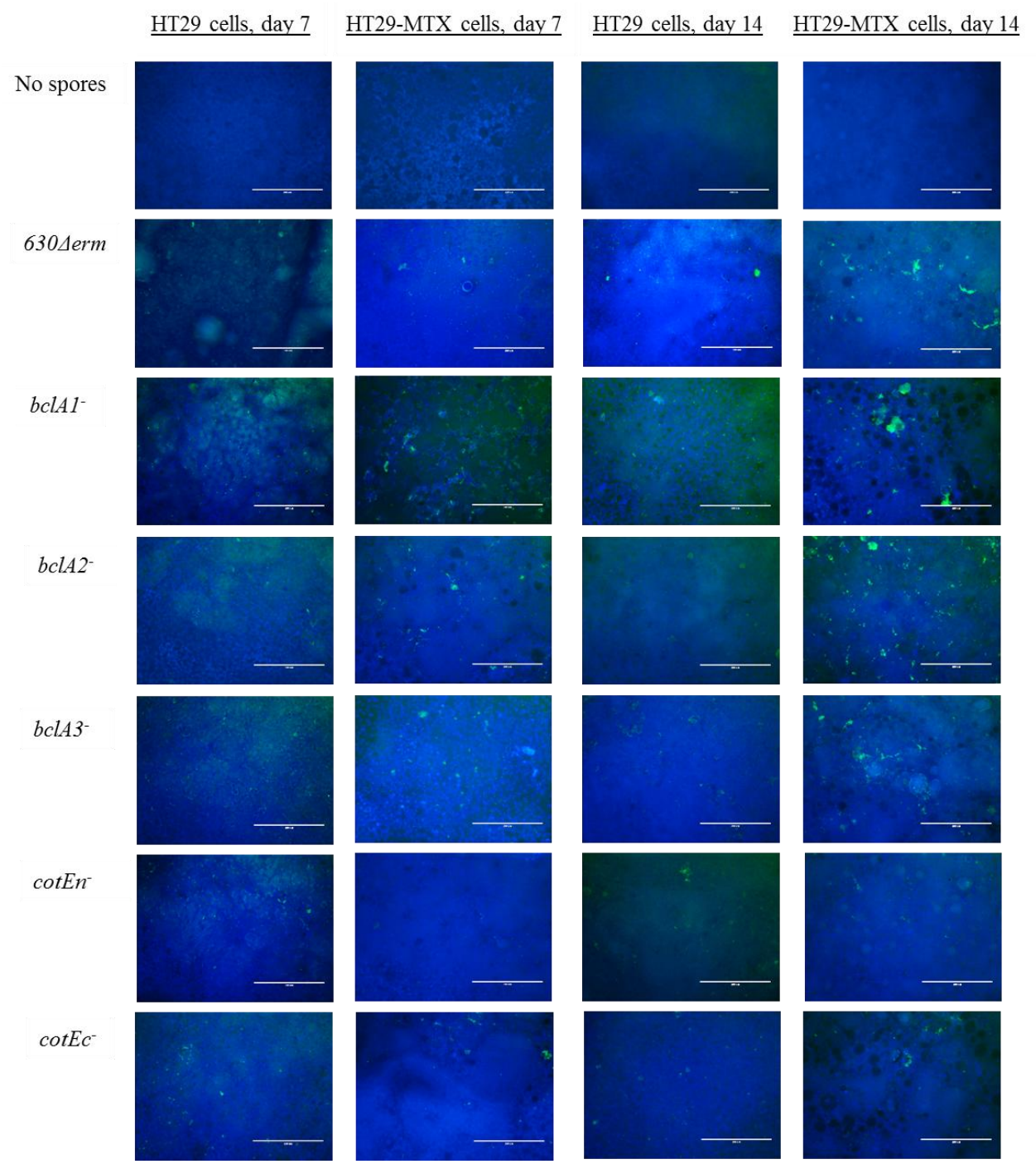


Figure 3.19. Adherence of *bclA*$^{-}$, *cotE*$^{-}$ and 630Δ*erm* spores to 7 and 14 day old HT29 and HT29-MTX monolayers. Monolayers were incubated with spores at a MOI of 100:1 for 2 h. Spores were labelled with mouse antiserum against *C. difficile* and anti-mouse IgG-FITC antibody (green). Cell nuclei was stained with DAPI (blue). Samples examined at 20x magnification using EVOS FL digital microscope. Scale bars = 20μm

3.3 Summary of results

Results suggest that the exosporium of *C. difficile* spores may have a role in spore hydrophobicity and act as a protective shield from germinants. Results showed that HistoDenz purified *C. difficile* spores had reduced hydrophobicity compared to water washed unpurified *C. difficile* spores (Figure 3.4 and 3.5). These results therefore suggest that during HistoDenz spore purification the exosporium may be removed.

The exosporium may also provide resistance to germinants. *C. difficile* spore germination was fastest in HistoDenz purified spore suspensions compared to unpurified water washed spore suspensions (Figure 3.1 and 3.2). This suggests that during spore purification the exosporium is lost or damaged allowing for greater access of germinants to receptors located in the inner membrane.

BclA1^{-} mutant spores were shown to have increased adherence to epithelial cell monolayers compared to the wild type (Figure 3.12, 3.15 and 3.16). In contrast results show that *bclA*$^{-}$ mutant spores had significantly lower adherence to collagen I and fibronectin (Figure 3.6 and 3.8), suggesting BclA might play a role in the adherence of spores to ECM components of the gut.

CotE mutant *C. difficile* spores were shown to have reduced adherence to epithelial cell monolayers compared to the wild type (Figure 3.13, 3.14, 3.17 and 3.18), suggesting a possible role of CotE protein in adherence to the intestinal epithelium and mucus.

Knowledge regarding which proteins of the *C. difficile* spore are involved in the interaction of the spore with the host could lead to the development of strategies that might inhibit attachment of spores to the host and therefore block the infection cycle.

Chapter 4

Interaction of *C. difficile* exosporial proteins and macrophages

4.1 Introduction

4.1.1 *C. difficile* and the innate immune response

During the onset of CDI, *C. difficile* begins a sporulation cycle in the colon which leads to persistence of spores in the colonic tract. Studies have shown that spores have the ability to adhere to epithelial cell lines (Paredes-Sabja et al., 2012). These persistent spores then germinate, colonize and produce recurrent CDI episodes. Following colonization of *C. difficile* to epithelial cells of the colon, *C. difficile* vegetative cells produce the enterotoxin TcdA and the cytotoxin TcdB. These toxins are responsible for the abundant tissue damage, epithelial barrier disruption and fluid accumulation during the disease. Both toxins lead to a huge inflammatory response from host epithelial cells, inducing the production of pro-inflammatory cytokines and chemokines which in turn leads to the recruitment of additional immune cells such as neutrophils and macrophages to the site of infection (Cowardin et al., 2014). This suggests that there is an interaction between *C. difficile* and the innate immune system. It has recently been shown that TLR4 and nucleotide binding oligomerization domain (NOD1) recognize *C. difficile* vegetative cells and mediate protection against CDI (Hasegawa et al., 2011) (Ryan et al., 2011).

The innate immune system provides the first line of defence against the presence of pathogenic microbes and plays a crucial role in affecting the adaptive immune response. The innate immune response is influential during CDI as multiple innate signalling pathways have been shown to play a role is disease susceptibility. Pattern Recognition Receptors (PRRs) present on host cells recognize conserved microbial molecules known as Pathogen Associated Molecular Patterns (PAMPs) which initiate immune responses. Stimulation of these PRRs by pathogens leads to the induction of several immune molecules, including antimicrobial peptides, cytokines, chemokines and adhesion molecules.

TLRs have been shown to recognize *C. difficile* PAMPS and contribute to the initiation of the host inflammatory response. Specifically the TLR adaptor protein Myeloid Differentiation Primary Response 88 (MyD88) has been shown to be involved in host defence. MyD88 mediated signalling is necessary for the production of chemokine CXC motif ligand 1 (CXCL1) which is responsible for the recruitment of neutrophils to colonic lamina propria. Mice lacking this molecule and therefore the majority of TLR signalling show decreased survival during CDI (Lawley et al., 2009) (Ryan et al., 2011). NOD1, an intracellular Nod-like receptor (NLR) is another PRR which recognizes *C. difficile,* it is known to recognize diaminopimelic acid derived from peptidoglycan (PG). Though NOD1 does not signal via MyD88, deletion of this receptor impairs production of CXCL1 and therefore decreases neutrophil recruitment resulting in more severe disease (Hasegawa et al., 2011).

4.1.2 Toll like receptors in innate immunity

Toll family of receptors play a key role in the initiation of cellular innate responses. TLRs are transmembrane molecules linking the extracellular compartment where contact with and recognition of microbial pathogens occurs and the intracellular compartment where signalling cascades leading to cellular responses are initiated. They are a type I transmembrane protein and consist of an ectodomain which contains leucine-rich repeats that mediate the recognition of PAMPs, a transmembrane region and cytosolic Toll-IL-1 receptor (TIR) domains that activate downstream signalling pathways. Ten functional TLRs for humans and twelve functional TLRs for mice have been identified. Each TLR detects distinct PAMPs derived from viruses, bacteria, mycobacteria, fungi and parasites. These include lipoproteins (recognized by TLR1, 2 and 6), double stranded RNA (TLR3), lipopolysaccharide (LPS) (TLR4), flagellin (TLR5), single stranded RNA (TLR7 and 8) and DNA (TLR9). Upon recognition of PAMPs, TLRs recruit adaptor molecules which contain a TIR domain and initiate downstream signalling events that lead to the secretion of inflammatory cytokines, chemokines and antimicrobial peptides. These responses in turn lead to the recruitment of neutrophils, activation of macrophages resulting in the direct killing of infected pathogens. Intact microbial pathogens are usually composed of a number of PAMPs, which activate multiple PRRs, also different PRRs may recognize the same PAMP. Bacteria consist of various PAMPs that are detected by TLRs. Bacterial cell wall components are normally recognized by cell surface TLRs whereas nucleic acids are recognized by intracellular TLRs. LPS from Gram-negative bacteria is recognized by TLR4. TLR2 recognizes PAMPs of both Gram-positive and Gram-negative bacteria and detects lipoproteins and peptidoglycans which are present in Gram-positive and Gram-

negative bacteria and lipoteichoic acid (LTA) from Gram-positive bacteria (Kawai et al., 2011).

An example of a target molecule recognised by TLR2 is LPS, the principal endotoxin of Gram-negative bacteria. CD14 present on macrophages acts as a high affinity receptor for LPS. The binding of LPS leads to activation of macrophages and the initiation of an inflammatory response. Interactions between LPS and CD14 have been shown to be important in the innate immune response to Gram-negative bacterial infections. However, there have been recent *in vitro* experiments that show that CD14 is also able to bind to components of Gram-positive bacterial cell wall such as lipopolysaccharide binding protein (LBP) and LTA (Olivia et al., 2009).

Little is known about the clearance of *C. difficile* from the gut. Investigating mechanisms of clearance is of crucial importance in understanding CDI, because many strains of this bacterium are not efficiently cleared from the gut. When this occurs, patients may be susceptible to recurrent infection or may develop colitis, which in severe cases can lead to sepsis and death (Madan et al., 2012). Clearance of pathogens during infection involves immune cells such as neutrophils and macrophages migrating to the site of infection and using mechanisms such as phagocytosis to clear the pathogen. Macrophages arrive early to the site of infection and begin production of pro-inflammatory cytokines and chemokines. Secreted chemokines recruit other cell types to the site of infection, in some cases initiating an adaptive immune response. Macrophages also phagocytose pathogens, which reduce bacterial load and contribute overall clearance (Collins et al., 2014). It has been shown that surface layer proteins (SLPs) from *C. difficile* activate key mechanisms in macrophages that are important in bacterial clearance. Exposing murine

macrophage J774.1 cell lines to *C. difficile* SLPs induce the macrophage to produce pro-inflammatory cytokines and chemokines, to increase expression of PRRs and other cell surface markers TLR2, TLR4 and their associated co-receptor CD14 leading to an increase in phagocytosis and migration activity (Collins et al., 2014). This suggests that SLPs are important in the recognition of *C. difficile* and in the induction of immune responses to the bacterium leading to clearance of this pathogen by macrophages during infection.

4.1.3 Macrophages

Macrophages are situated throughout the body tissues where they ingest and process foreign materials, dead cells and debris and also recruit additional macrophages in response to inflammatory signals. Mononuclear phagocytic system is a system that consists of bone marrow derived cells that have different morphologies and are mainly responsible for phagocytosis, cytokine secretion and antigen presentation. Macrophage precursors are released into circulation as monocytes and within a few days are found throughout tissues in the body. When monocytes migrate from circulation and extravasate through the endothelium they differentiate into macrophages or dendritic cells (DCs). Therefore the main role of monocytes is to continually restore the number of tissue resident macrophages and DCs. Professional phagocytic cells include monocytes, DCs, macrophages, neutrophils and mast cells, they differ from non-professional phagocytes depending on how effective they are at phagocytosis. A key feature that distinguishes professional and non-professional phagocytes is that professional phagocytes express a variety of receptors on their surfaces that detect signals which are not present on healthy tissues. Also professional phagocytes express PRRs such as TLRs. Macrophages are divided into

subpopulations based on where they are located in the body and their functional phenotype. The gut harbours the largest population of macrophages in the body which have distinct phenotypes and functions but work together to maintain tolerance to the gut flora and food. Classically activated macrophages (M1 macrophages) which are a macrophage subset that is activated by TLR ligands and IFN-γ. M1 macrophages express pro-inflammatory cytokines and mediate defence of the host from a variety of bacteria, protozoa and viruses. Alternatively, activated macrophages (M2 macrophages) are a macrophage subset that are stimulated by IL-4 or IL-13. When stimulated macrophages take on an environment-dependent phenotype that either promote or inhibit host antimicrobial defence, anti-tumour immunity and inflammatory responses. At a steady state, tissue macrophages have intrinsic anti-inflammatory functions, for example colonic macrophages are surrounded by IL-10 and dampen any inflammatory response to the gut flora and their products. Disruption of normal quantities of IL-10 or IL-10 signalling in immune cells leads to massive inflammation in the gut. Mature macrophages are strategically located throughout the body and have an important immune surveillance function. They look for signs of tissue damage and invading organisms and are ready to stimulate lymphocytes and other immune cells to respond when danger signals are phagocytosed and/or detected by cell surface receptors. In addition to fighting infections, resident tissue macrophages are involved in maintaining healthy tissues by removing dead and dying cells and toxic materials. Tissue macrophages also supress inflammation mediated by inflammatory monocytes, in doing so ensuring that tissue homeostasis is re-established following infection or injury. Macrophages are selective over what they phagocytose so that healthy cells of the body are not mistakenly removed. During and following phagocytosis, PRRs recognize signals

associated with invading pathogens, foreign substances and dead and dying cells. Some PRRs function in pathogen binding and phagocytosis whereas signalling PRRs sense microbial products and aberrant self on the cell surface or in the cytoplasm of cells and activate transcriptional mechanisms that lead to phagocytosis, cellular activation and release of cytokines, chemokines and growth factors. Macrophages also express a number of secreted molecules including complement and Fc receptors that bind opsonin molecules which activate the complement cascade, enhancing process of phagocytosis by tagging the pathogen surface (Murray et al., 2011).

4.1.4 Killing mechanisms used by macrophages

The killing mechanisms used by phagocytes to destroy bacteria can be organized into two groups: oxygen dependent and oxygen independent mechanisms.

Oxygen dependent mechanisms involve reactive oxygen species (ROS) which are crucial components of the antimicrobial repertoire of macrophages. Macrophages recognize and engulf bacteria into phagosomes which subsequently acidy. These phagosomes mature into phagolysosomes upon vesicle mediated delivery of various antimicrobial effectors such as proteases, antimicrobial peptides and lysozyme. The multi-subunit NADPH-dependent phagocytic oxidase is assembled on the phagolysosome membrane and pumps electrons into the phagolysosome compartment to reduce oxygen to superoxide anion. It is the phagolysosome where ROS are produced. Most bacteria are rapidly killed and degraded in the phagolysosome, making it difficult to determine the mechanism of death of bacteria by macrophages however it is the thought that phagocytic ROS kill bacteria by damaging DNA (Slauch et al., 2011).

Oxygen independent mechanisms which can aid the destruction of microorganisms in the phagolysosome are pH, the pH of the phagolysosome can be as low as pH4 and this alone can inhibit the growth of many types of bacteria. This low pH also enhances the activity of lysozyme, glycosylases, phospholipases and nucleases present in the phagolysosome that degrade various parts of the microbe (Baldwin, C., 2002).

4.1.5 Phagocytosis

For its survival the host depends on an elaborate internal defence system to protect it against persistent invasion by pathogenic microorganisms. This system is composed primarily of cellular and humoral components of the circulatory system which includes professional phagocytes. Professional phagocytes include polymorphonuclear granulocytes (PMNs), monocytes and macrophages which destroy harmful microorganisms by the process of phagocytosis. Phagocytosis is defined as the receptor mediated engulfment of large (>0.5μm) particles into plasma membrane derived vacuoles called phagosomes (Botelho et al., 2011). Phagocytosis is triggered when specialised receptors engage cognate ligands on the target particle. Following separation from the plasma membrane, the phagosome undergoes a maturation process sequentially fusing with endosomes and lysosomes finally becoming phagolysosomes which are highly acidic and hydrolase rich organelles that degrade the internalized particles.

Once phagocytosed the fate of the bacterial spore will vary depending on their specific virulence traits that will either enable them to escape or regulate the host innate immune system. For example depending on the germination ability of

Clostridium perfringens (*C. perfringens*) spores their fate is significantly different. Spore isolates that were more prone to germinate inside macrophages were killed by macrophages however isolates that germinated poorly were able to survive and remain dormant inside macrophages (Paredes-Sabja et al., 2011). Studies demonstrated *B. anthracis* wild type spores were efficiently phagocytosed and upon germination inside the phagolysosome they were efficiently killed by macrophages. However *B. anthracis* germination deficient spores were able to survive longer periods of time than germination proficient wild type spores indicating that spore survival inside macrophages is dependent on the ability of spores to remain dormant (Kang et al., 2005).

The ability of *C. difficile* spores to germinate is dependent upon the presence of co-germinants taurocholate and glycine which are not present in the phagolysosome environment. Therefore, the fate of *C. difficile* spores during macrophage infection could possibly be different to that observed for spores of *C. perfringens* and *B. anthracis*.

4.1.6 Experimental objectives

The objective of this chapter was to investigate the interaction of *C. difficile* exosporial proteins and macrophages. Little is known about the clearance of *C. difficile* from the gut and investigating mechanisms of clearance is of crucial importance in understanding CDI because if bacteria are not efficiently cleared from the gut patients may be susceptible to recurrent infection. Using ClosTron mutants of *bclA* genes I have evaluated the interactions of *C. difficile* exosporial proteins with murine macrophage cell line J774.1. Understanding interactions of *C. difficile* spores

with cells of the innate immune system may explain mechanisms of spore persistence and survival in the host.

4.2 Results

4.2.1 Phagocytosis of *C. difficile* spores

To assess the ability of murine macrophage cell line J774.1 to internalize spores by phagocytosis, cell monolayers were infected at MOI 10:1 with *bclA*$^{-}$ mutant and 630Δ*erm* wild type spores for 30 min. Monolayers were washed to remove any spores that had not been internalized. To determine the number of spores that had been phagocytosed, cells were lysed and samples were serially diluted and plated onto BHISS plates for CFU enumeration. This experimental method is similar to that carried out for the adhesion of spores to epithelial cell lines, however it is expected that J774.1 cells will phagocytose spores as this is the main function of macrophages whereas HT29 cells do not phagocytose spores and instead function as a barrier to microorganisms from colonizing and establishing infection in the host (Janeway, 2001) (Alberts, 2002). This therefore allows the use of epithelial cell lines for spore adhesion assays and macrophage cell lines for phagocytosis assays. Results show that after 30 min of incubation of *C. difficile* spores with macrophages approximately 40% of 630Δ*erm* spores had been internalized whereas there was a reduction of spore phagocytosis of *bclA*$^{-}$ mutant spores by macrophages, approximately 30% of *bclA*$^{-}$ mutant spores were internalized by macrophages (Figure 4.1). There is a significant difference between the number spores phagocytosed by 630Δ*erm* spores and *bclA*$^{-}$ mutant spores, with a decrease in phagocytosis of *bclA*$^{-}$ mutant spores. Therefore results could be suggestive that BclA could have a role to play in the phagocytosis of *C. difficile* spores by macrophages.

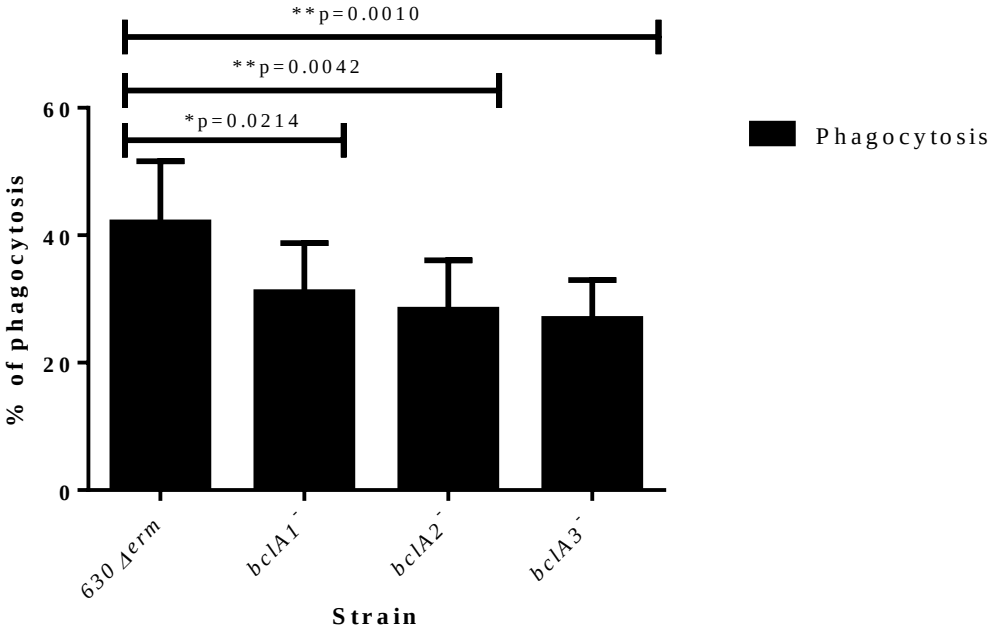


Figure 4.1. Phagocytosis of *bclA*$^{-}$ mutant spores by J774.1 cells. *BclA*$^{-}$ mutant and wild type spores were incubated with J774.1 cells for 30 min. Monolayers were washed to remove any spores that had not been internalized. To assess the number of spore's phagocytosed, cells were lysed and plated onto BHISS plates for CFU enumeration. % of phagocytosis was calculated using the following formula: (CFU count / Initial number of spores added) x 100

4.2.2 Survival of *C. difficile* spores in macrophages

As *C. difficile* spores were efficiently recognized and internalized by J774.1 cells, the survival of spores inside J774.1 cells was evaluated. After 30 min incubation of spores with J774.1 cells, spores that had not been internalized were removed by washing and spore survival was evaluated at 12 and 24 h post infection. To determine the number of spore survival, cells were lysed and samples were serially diluted and plated onto BHISS plates for CFU enumeration. Results show there was no significant difference between the number of spores surviving at 12 and 24 h post infection with the exception of *bclA2*$^{-}$ spores, where there was a significant decrease in the number of spores surviving at 24 h compared to 12 h (Figure 4.2A). There is a significant increase in the number of *bclA*$^{-}$ mutant spores surviving, approximately 60-80% in macrophages compared to 630Δ*erm* spores where spore survival is approximately 50% (Figure 4.2B and 4.2C). Results show that *bclA*$^{-}$ mutant spores are better suited to survive within macrophages (Figure 4.2) suggesting that BclA is required for the phagocytosis and destruction of spores by macrophages.

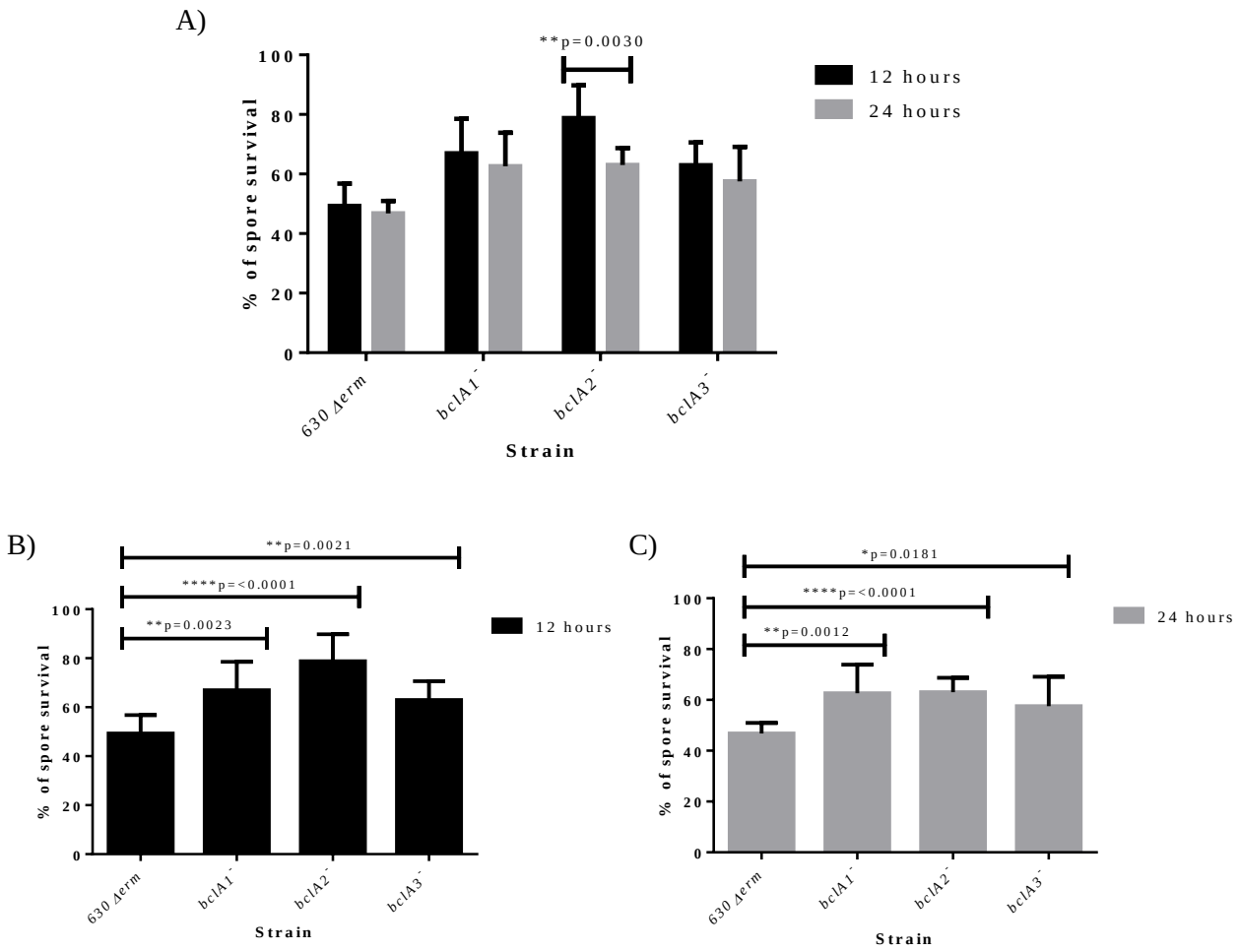


Figure 4.2. Survival of *bclA*⁻ mutant *C. difficile* spores during infection of J774.1 macrophages. J774.1 cells were infected with *bclA*⁻ mutant and 630Δ*erm* spores at a MOI 10:1 for 30 min, spores that had not been internalized were washed away. Spore survival was evaluated at 12 and 24 h. To determine number of spores surviving, cells were lysed and samples were serially diluted and plated onto BHISS plates for CFU enumeration. Number of spores surviving was calculated using the following formula (CFU count / Number of spores phagocytosed) x 100. A) Compares spore survival of *bclA*⁻ mutants and 630Δ*erm* at 12 and 24 h post infection. B) Compares the spore survival of *bclA*⁻ mutants and 630Δ*erm* at 12 h post infection. C) Compares the spore survival of *bclA*⁻ mutants and 630Δ*erm* 24 h post infection.

4.2.3 Cytotoxic effects of *C. difficile* spores on macrophages

As results show that spores are able to survive within macrophages there could be a possibility that *C. difficile* spores could be cytotoxic to J774.1 cells. To assess the cytotoxic effects of *bclA*$^{-}$ mutant and 630Δ*erm* spores on J774.1 cells a MTT cell viability assay was carried out. J774.1 cell monolayers were infected at MOI 10:1 and 100:1 for 30 min. Monolayers were washed to remove any spores that had not been internalized and cell viability was assessed at 24 h post infection. Results show that *C. difficile* spores are cytotoxic to J774.1 cells with a greater significant cytotoxic effect at MOI 100:1 for 630Δ*erm*, *bclA1*$^{-}$ and *bclA3*$^{-}$ spores compared to MOI 10:1 (Figure 4.3A). At MOI 10:1, *bclA1*$^{-}$, *bclA2*$^{-}$ and *bclA3*$^{-}$ spores are significantly more cytotoxic compared to J774.1 cell control (Figure 4.3B) and *bclA2*$^{-}$ and *bclA3*$^{-}$ spores are significantly more cytotoxic than 630Δ*erm* (Figure 4.3D). At MOI 100:1, 630Δ*erm*, *bclA1*$^{-}$, *bclA2*$^{-}$ and *bclA3*$^{-}$ spores are significantly more cytotoxic compared to J774.1 cell control (Figure 4.3C) and *bclA3*$^{-}$ spores are significantly more cytotoxic than 630Δ*erm* (Figure 4.3E). Therefore results indicate that *C. difficile* spores are cytotoxic to J774.1 cells with *bclA3*$^{-}$ spores exhibiting the greatest cytotoxic effect.

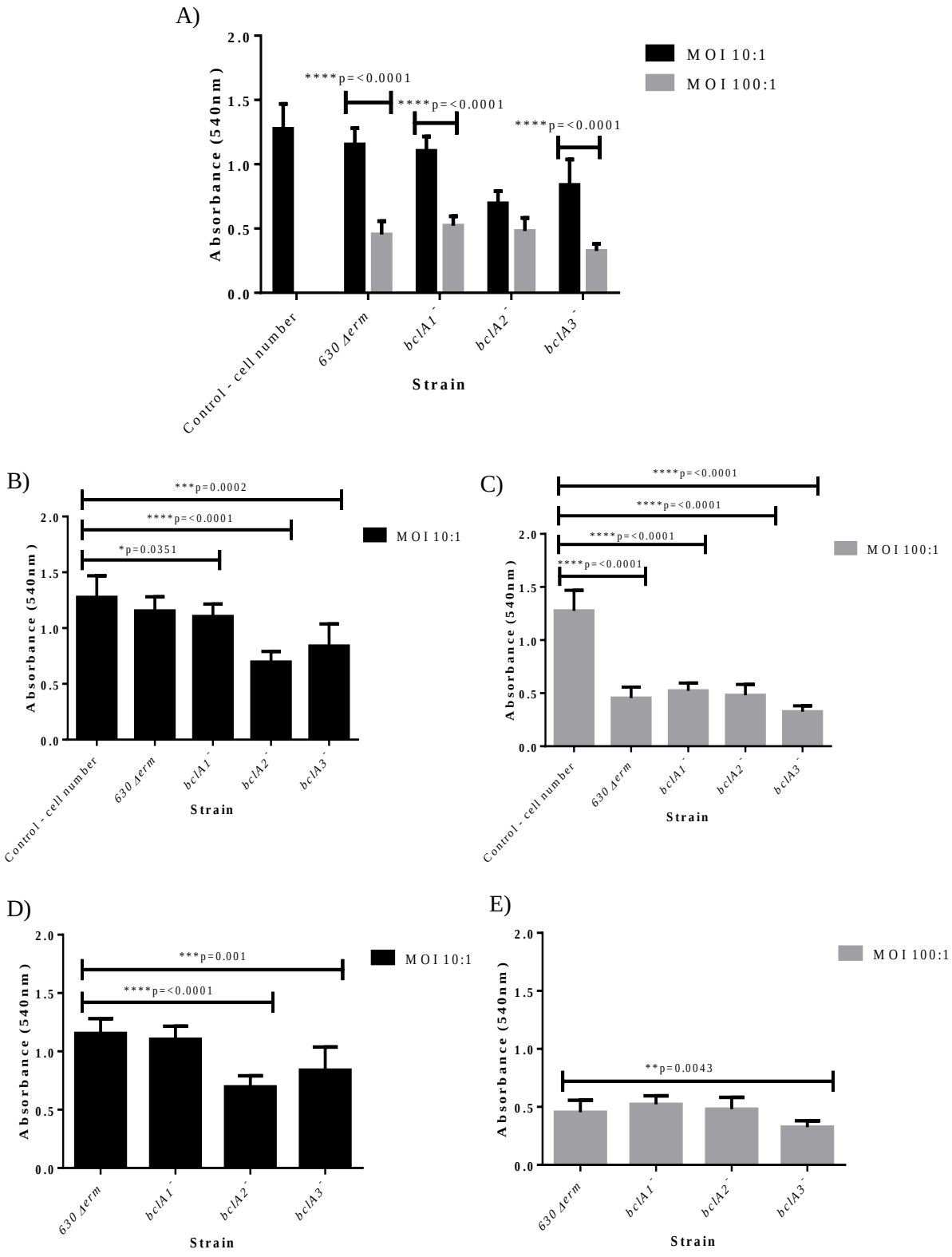


Figure 4.3. Cytotoxic effects of *bclA*⁻ mutant *C. difficile* spores on J774.1 cells. J774.1 cells were infected with 630Δ*erm* and *bclA*⁻ mutant *C. difficile* spores at a MOI 10:1 and 100:1 for 30 min and J774.1 cell viability was assessed at 24 h post infection using an MTT assay. A) Compares viability of J774.1 cells after infection with 630Δ*erm* and *bclA*⁻ mutants at MOI 10:1 and 100:1. B) Compares viability of J774.1 cells at MOI 10:1 to control cell number. C) Compares viability of J774.1 cells at MOI 100:1 to control cell number. D) Compares viability of J774.1 cells at MOI 10:1 to 630Δ*erm* spores. E) Compares viability of J774.1 cells at MOI 100:1 to 630Δ*erm* spores.

4.2.4 Fate of *C. difficile* spores inside macrophages

It has been suggested that the ability of *C. difficile* spores to survive inside macrophages depends on their germination capabilities inside the environment of the macrophage. To investigate the fate of *C. difficile* spores during macrophage infection, J774.1 cells were infected with 630Δ*erm* and *bclA*$^{-}$ mutant spores for 30 min at MOI 10:1. After 30 min incubation of spores with J774.1 cells, spores that had not been internalized were removed by washing. The fate of spores inside macrophages were determined at 30 min, 12 h and 24 h by lysing cells, serially diluting samples and plating onto BHISS plates for CFU enumeration. Germination of spores within macrophages was assessed by bacterial counts without heat treatment which corresponds to the total spore population (dormant and germinated spores) and viability after heat treatment (60ºC, 20 min), was a measure of the population of dormant spores. Results show that there was no significance difference in the CFU between bacterial counts without heat treatment and after heat treatment suggesting that *C. difficile* spores did not germinate inside macrophages and spores remained dormant (Figure 4.4).

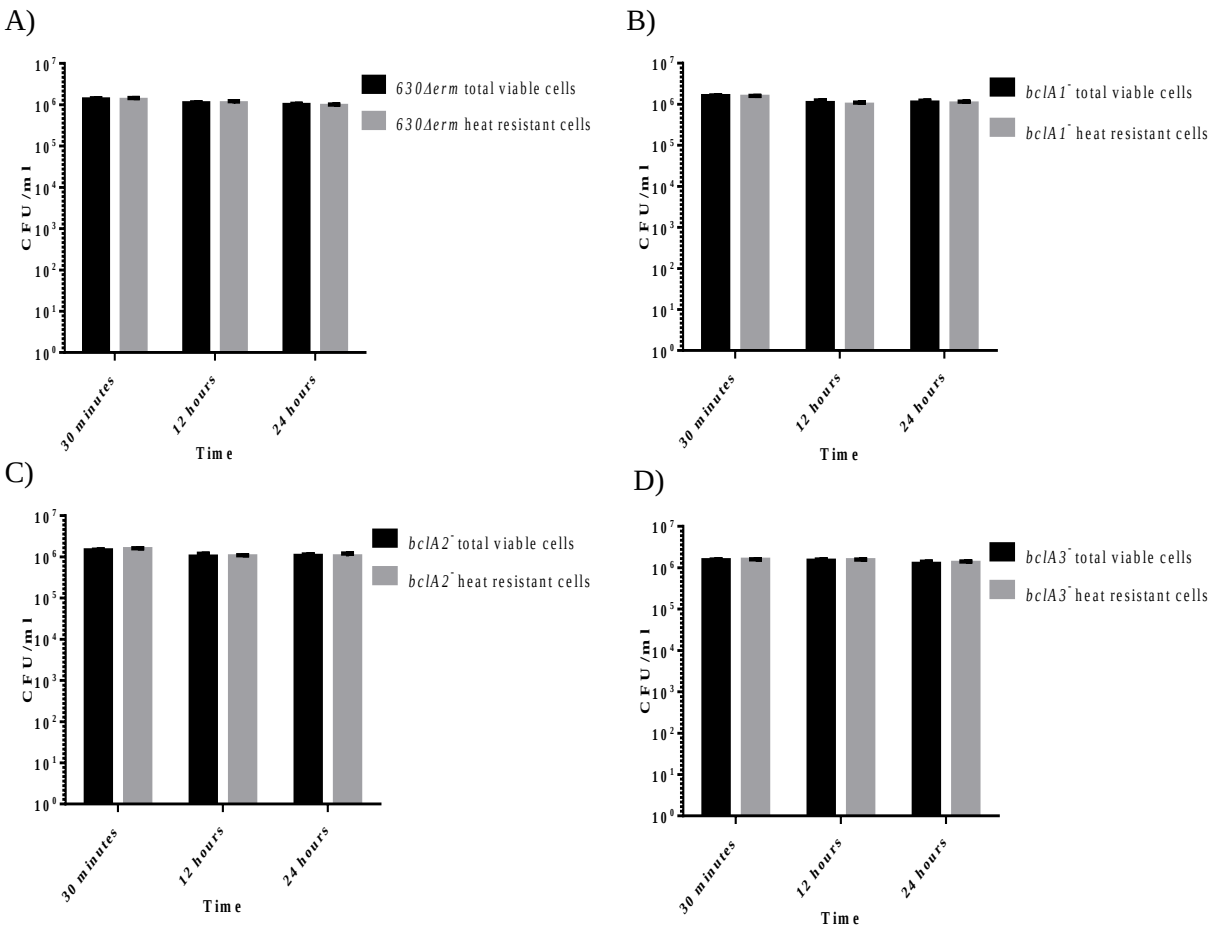


Figure 4.4. Germination of 630Δ*erm* and *bclA*⁻ mutant spores inside macrophages. J774.1 cells were infected with *bclA*⁻ mutant and 630Δ*erm* spores at a MOI 10:1 for 30 min, spores that had not been internalized were washed away. Spore germination was evaluated at 30 min, 12 and 24 h. Germination of spores within macrophages was assessed by bacterial counts with and without heat treatment, samples were serially diluted and plated onto BHISS plates for CFU enumeration. A) Germination of 630Δ*erm* spores. B) Germination of *bclA1*⁻ spores. C) Germination of *bclA2*⁻ spores. D) Germination of *bclA3*⁻ spores.

4.2.5 Interaction of *C. difficile* spores and J774.1 cells

To investigate the interaction between *C. difficile* spores and macrophages, J774.1 cells were labelled with a biotinylated cross-linking agent, solubilizing the surface proteins and screening those which bound to wild type 630Δ*erm* spores. Biotin labelled spore bound proteins derived from lysed J774.1 cells were resolved on 18% SDS-PAGE gel (Figure 4.5A) and transferred to PVDF membrane. Proteins were detected by western blot using anti-streptavidin HRP. A protein of approximately 16 kDa was detected (Figure 4.5B).

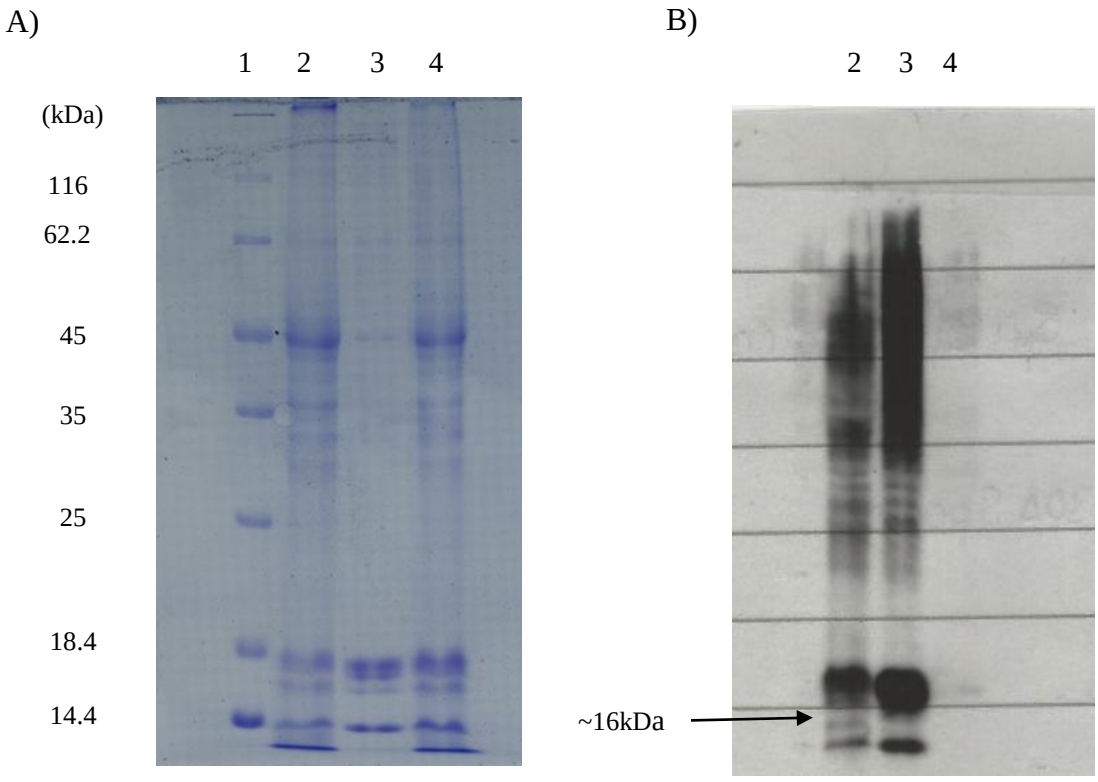


Figure 4.5. Interaction of 630Δ*erm C. difficile* spores with J774.1 cells. Cells were labelled with biotinylation cross-linking agent and incubated with *C. difficile* 630Δ*erm* spores at MOI 25:1 for 45 min. Spore bound proteins centrifuged and pellet resuspended in lysis buffer. Spore bound proteins released with sample buffer and analysed by western blot with HRP-conjugated streptavidin. A) Coomasie blue staining of biotinylated J774.1 cells and 630Δ*erm* spores, lane 1- ladder, 2- biotinylated J774.1 cells and 630Δ*erm* spores, 3- biotinylated J774.1 cells and 4- J774.1 cells and 630Δ*erm* spores. B) Western blot detected using anti-streptavidin HRP revealing a band of approximately 16 kDa.

To identify the protein of approximately 16 kDa, SDS-PAGE gels were silver stained (Figure 4.6) and the band of interest was excised and sent for mass spectrometric analysis. Mass spectrometric analysis revealed the protein to be histone H4. One of the 5 main histone proteins involved in the structure of chromatin.

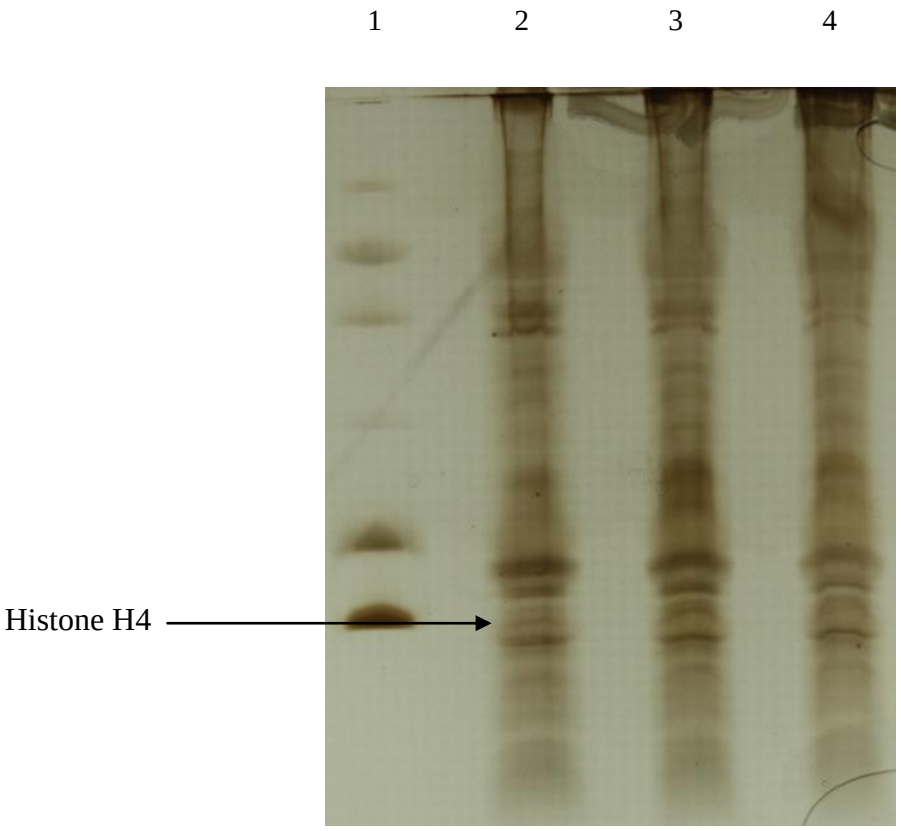


Figure 4.6. Silver stained 18% SDS-PAGE gel of biotinylated J774.1 cells and 630Δ*erm* spores. SDS-PAGE gel was stained using ProteoSilver Plus Silver Stain Kit according to the manufacturer's instructions. Arrow points to band that was excised and sent for mass spectrometric analysis. Lane 1 – ladder, 2, 3 and 4 - biotinylated J774.1 cells and 630Δ*erm* spores.

4.3 Summary of results

Results show a decrease in phagocytosis of *bclA*$^{-}$ mutant *C. difficile* spores compared to the wild type (Figure 4.1), suggesting that BclA could have a role to play in the efficient phagocytosis of *C. difficile* spores by macrophages.

C. difficile spores were shown to be able to survive inside macrophages 12 and 24 h post infection (Figure 4.2). Spores were shown not to germinate inside macrophages (Figure 4.4) and this could be due to their ability to remain dormant inside macrophages.

C. difficile spores were shown to be cytotoxic to murine macrophage cell line J774.1 at MOI 10:1 and 100:1, with greater cytotoxicity observed at MOI 100:1 (Figure 4.3).

Results have shown that *C. difficile* spores are recognized by macrophages however the nature of phagocytic receptors mediating *C. difficile* spore recognition by J774.1 cells has not yet been determined. Further investigation is required to discover precise protein-protein interactions of *C. difficile* spores and macrophages.

Chapter 5

Discussion

5.1 Role of adhesion in the pathogenesis of CDI

The outcome of any infection is the end product of a complex set of interactions between the host and the pathogen (Borriello., 1998). Most bacterial infections are initiated by the adherence of microorganisms to host tissues, this process involves the interaction of specific bacterial surface structures called adhesins with host components (Joh et al., 1999). Adhesion to host tissues is important for full expression of virulence factors for many pathogens (Borriello., 1998). In addition to toxins other factors are likely to be important during the colonisation process, after germination the adherence of *C. difficile* to mucus is considered to be the first step for the settlement of the pathogen in the gut. Once the gut is occupied, *C. difficile* is thought to penetrate the mucosa and interact with epithelial cells and components of the ECM of the gut releasing its virulence factors. This interaction between bacteria and epithelial cells at the host surface requires the involvement of surface displayed adhesins and eukaryotic cellular targets. However, the overall strategy by which *C. difficile* colonises the host is unclear and not much is known about the role the *C. difficile* spore has to play in the interaction with ECM components or epithelial cells of the gut. *C. difficile* vegetative cells have been shown to adhere to specific components of the ECM such as fibrinogen, laminin, fibronectin, collagen I, III and IV (Cerquetti et al., 2002). Several *C. difficile* cell surface proteins involved in host-pathogen interactions have been characterised including S-layer proteins (Calabi et

al., 2002), the flagellar components FliC and FliD (Tasteyre et al., 2001), the Cwp66 adhesin (Waligora et al., 2001), the fibronectin binding protein, Fbp68 (Hennequin et al., 2003) and collagen binding protein A, CbpA (Tulli et al., 2013). Complexity of CDI symptoms suggest that other non-toxin virulence factors and traits could be involved in the pathogenesis of *C. difficile*. It is widely accepted that that the *C. difficile* spores are the persistent and infectious morphotype as well as the channel of transmission of CDI. During the course of CDI an increase of *C. difficile* spores has been observed in the stools (Deneve et al., 2009) indicating that *C. difficile* sporulates inside the host leading to persistence of *C. difficile* spores in the intestinal tract. CDI also has a relatively high relapse rate approximately 20% (Pepin et al., 2005) suggesting that *C. difficile* might have spores with unique properties to adhere to hosts intestinal epithelial cells leading to persistence of the spore. Before CDI develops it could be suggested that the spore may have a role to play, by attaching to the gut mucosa. As adhesion has a critical role in the infection process, bacterial adherence to host tissues represents a potential target for the development of agents that can block adherence and therefore help in combating infectious disease.

There are many factors that can contribute to the adherence of spores to the host such as hydrophobicity and the presence of surface structures such as appendages and the outer spore layer known as the exosporium (Joshi et al., 2012). Results show that HistoDenz purified *C. difficile* spores had reduced hydrophobicity compared to water washed unpurified *C. difficile* spores (Figure 3.4 and 3.5). Hydrophobicity is a useful marker of *C. difficile* exosporium removal, with spores lacking most of the exosporium having lower hydrophobicity (Escobar-Cortés et al., 2013). These results therefore suggest that during HistoDenz spore purification the exosporium may be removed. The suggestion of a link between spore hydrophobicity and the presence of

an exosporium was first suggested by Takubo et al. who showed that *B. megaterium* spores with a defective or absent exosporium showed reduced affinity for hexadecane (Takubo et al., 1988). Also spores of *B. subtilis*, *B. licheniformis* and *B. macerans* do not have a distinct exosporium and are less hydrophobic than exosporium containing *B. cereus*, *B. brevis* and *B. thuringiensis* spores (Koshikawa et al., 1989). It has been shown that comparing spores from two different isolates of *C. difficile* that isolates containing an exosporium were more hydrophobic than isolates not containing an exosporium. Also spores containing an exosporium showed an increase adherence to 7-day old HT29 cells compared to spores without. It has been previously been observed that spores of *C. difficile* and *C. sporogenes* attach to the apical microvilli of human epithelial cells by use of an exosporium-like structure. This suggests that the hydrophobic nature of the spore is due to the presence of an exosporium and this structure also has a role in aiding the adherence of spores to epithelial cells (Joshi et al., 2012). A connection has also been exhibited between spore hydrophobicity and the presence of an exosporium leading to the ability of *B. cereus* to adhere to Caco-2 epithelial cells (Andersson et al., 1998). Also a similar correlation between spore hydrophobicity and adherence of *C. difficile* spores to Caco-2 cells has also been reported. It was shown that sonication of *C. difficile* spores altered the ultrastructure of the exosporium, which resulted in a significant reduction in spore hydrophobicity and adherence to Caco-2 cells (Pardes-Sabja et al., 2012). Bacteria and other microorganisms have evolved ways to use hydrophobic effect to adhere to surfaces and there is reason to believe that hydrophobic interactions participate in the initial adhesion of pathogens to tissues, leading to colonization, invasion or tissue destruction (Doyle., 2000). Very few microbes have entirely hydrophobic or charged surfaces however the spore coat is a

structure which contributes to hydrophobicity of *C. difficile* spores. It has been suggested that the increased hydrophobicity of bacterial spores compared to vegetative cells is due to the relative abundance of protein in the outer coats and the exosporium compared to the peptidoglycan on Gram-positive vegetative cell surfaces (Wiencek et al., 1990).

The exosporium not only provides a hydrophobic surface that enhances the spore's adhesive properties but it also may provide resistance to chemical and enzymatic treatments (Henriques et al., 2007). *C. difficile* spore germination was fastest in HistoDenz purified spore suspensions compared to unpurified water washed spore suspensions (Figure 3.1 and 3.2) this suggests that during spore purification the exosporium is lost or damaged allowing for greater access of germinants to receptors located in the inner membrane. This suggests that the exosporium has a role in protecting the spore from germinants. *BclA1*$^{-}$ and *bclA2*$^{-}$ mutant *C. difficile* spores were shown to have noticeable defects within the spore coat (Phetcharaburanin et al., 2014). Aberrations in the spore coat were observed in *bclA1*$^{-}$ and *bclA2*$^{-}$ mutants compared to 630Δ*erm* wild type strain. Sheets of coat like material were found present in the medium as well as angular projections of material at the spore surface, suggesting that both these proteins are major exosporial proteins that could have a role in stabilizing the exosporium to the spore coat. Germination rates of HistoDenz purified *bclA1*$^{-}$ mutant spores (Figure 3.1D) and water washed unpurified *bclA2*$^{-}$ mutant spores (Figure 3.1E) were faster compared to 630Δ*erm* wild type spores, this could be due to aberrations of the spore coat allowing for access of germinants to receptors located in the inner membrane of the spore. Increased germination rates of *bclA* mutant spores was also observed in *B. anthracis* and is presumably a result of a

defective exosporium, allowing access of geminants to receptors located in the inner most spore membranes (Brahmbhatt et al., 2007).

There is much debate about the stability of the exosporium of the *C. difficile* spore, images of this outer loose-fitting sac like structure vary from electron-dense outer surface (Lawley et al., 2009) to diffuse layers that are easily removed from the spore coat by purification methods (Permpoonpattana et al., 2013). It is thought that the exosporium of *C. difficile* is quite fragile and it is thought that the exosporial layer can be easily removed under conditions used to prepare spores in the laboratory, only spores that have been washed with water and not purified using HistoDenz were assessed for their binding ability to epithelial cell lines, HT29 and HT29-MTX (Figure 3.11, 3.12, 3.13 and 3.14). Using *C. difficile* spores which have not undergone HistoDenz purification will allow for spores to be in a more physiologically relevant form allowing for a more accurate representation of the action of the spore during pathogenesis of CDI.

Before CDI develops it is assumed the spore has a role to play by attaching to the gut mucosa. Little is known about which aspects of the spores are involved in this process. Therefore *C. difficile* spores devoid of the N- and C- termini of CotE protein (*cotEn*$^{-}$ and *cotEc*$^{-}$) and *C. difficile* spores devoid of BclA proteins (*bclA1*$^{-}$, *bclA2*$^{-}$ and *bclA3*$^{-}$) and isogenic wild type strain 630Δ*erm* have been assessed for their binding efficiency to monolayers of intestinal epithelial cell lines HT29 and HT29-MTX and to the main structural components of the ECM of the gut, collagen and fibronectin.

Adherence of 630Δ*erm C. difficile* spores compared to vegetative cells to HT29 and HT29-MTX cell monolayers show that *C. difficile* spores have increased adherence

to cell monolayers compared to vegetative cells (Figure 3.11 and 3.12). It has been reported previously that approximately 1% of *C. difficile* vegetative cells adhere to Caco-2 cells (Dingle et al., 2010) which is much lower compared to approximately 5-40% adherence of 630Δ*erm* spores. This further supports the theory that the *C. difficile* spore uses mechanisms of adherence as a way of persisting in the host. Results also show an increase in adhesion of *C. difficile* spores to HT29-MTX cell monolayers compared to HT29 cell monolayers (Figure 3.11A, 3.12A, 3.13A and 3.14A), with an increase in adhesion of spores to HT29-MTX cell monolayers at 14-days post seeding compared to 7-days post seeding. The increase in adhesion of spores to HT29-MTX cell monolayers compared to HT29 cells is most likely due to the increase in the amount of mucus produced by HT29-MTX cells at late confluency (Kerneis et al., 1994). Which is also evidenced by the Alcian blue staining of HT29 and HT29-MTX cell monolayers at 7 and 14 days post seeding (Figure 3.10). Results show a significant decrease in the adhesion of *bclA1*$^{-}$ and *bclA2*$^{-}$ spores to 7-day old HT29-MTX cell monolayers compared to the wild type (Figure 3.11C) suggesting that BclA could have a role in the adherence of *C. difficile* spores to mucus secreting epithelial cells. BclA1 has been shown to be involved in the initial stages of colonization and infection. In mice and hamster models of infection spores which were devoid of BclA1 were up to 2-logs less infective than isogenic wild type spores and hamsters also showed an increased time to death. This work suggests that BclA1 could be involved in the initial stages of host colonization and this even could be mediated by the spore. Also spores of R20291, the 'hypervirulent' strain of *C. difficile* which carries a truncated BclA1 protein was less infective in a mouse model of infection than 630 suggesting a relationship of animal susceptibility to the presence of an intact BclA1 protein in *C. difficile* spores.

Therefore spores of strains carrying full length BclA1 protein such as 630 were more infectious than those carrying a truncated or missing BclA1 protein (Phetcharaburanin et al., 2014).

Although no significant difference is observed between the adherence of *bclA1*$^{-}$ and *bclA3*$^{-}$ spores compared to 630Δ*erm* spores to 7-day old HT29 cell monolayers and 14-day old HT29 and HT29-MTX cell monolayers a similar trend of adherence is observed for these strains. *BclA1*$^{-}$ and *bclA3*$^{-}$ spores show increased adherence to these cell monolayers compared to the wild type (Figure 3.11B and 3.15). A similar result has also been observed, where most of the removal of the BclA1 protein on *C. difficile* spores led to an increase in adhesion of spores to Caco-2 cell monolayers (Pizarro-Guajardo et al., 2014). It is possible that BclA protein may have a similar role to that of BclA in *B. anthracis* where BclA has been shown to promote tropism towards macrophages and decrease the adherence to lung epithelium cells (Bozue et al., 2007). Which in turn leads to the interaction of BclA with components of complement which mediates internalization into epithelium or professional phagocytes (Xue et al., 2011) (Gu et al., 2013). When examining the adherence of heat resistant spores no significant difference is observed between the adherence of *bclA*$^{-}$ mutant spores and the wild type, however a similar trend is observed of an increase in adherence of *bclA1*$^{-}$ mutant spores to HT29 and HT29-MTX cell monolayers at 7 and 14 days post seeding compared to 630Δ*erm* spores (Figure 3.15 and 3.16). Therefore it can be speculated that the role BclA has in *C. difficile* is similar to that of BclA in *B. anthracis*. That BclA has a role in pathogenesis by inhibiting binding of spores to epithelial cells during the initial stages of infection and therefore spores are more readily directed towards macrophages (Bouze et al., 2007).

cotEn^{-} and *cotEc*$^{-}$ mutant spores showed reduced adherence to 7 and 14 day old monolayers of HT29 and HT29-MTX cell monolayers compared to 630Δ*erm* wild type strain (Figure 3.13, 3.14, 3.17 and 3.18) suggesting a possible role of CotE protein in adherence to the epithelium and mucus. CotE is a novel bifunctional protein with peroxiredoxin (amino-terminal) and chitinase (carboxyl-terminal) activity. Peroxiredoxin activities are potential antioxidants which would reduce cellular toxicity of hydrogen peroxide by conversion to oxygen and water. Previous studies in *B. subtilis* sporulation showed that hydrogen peroxide may play a role in spore coat synthesis and could act as a substrate for the oxidative cross linking of spore coat monomer (Henriques et al., 1998). CotE as a 1-Cys peroxiredoxin is expected to have a similar function and participate in the cross linking of tyrosine rich spore coat proteins (Permpoonpattana et al., 2011). Chitinases are expected to be involved in the breakdown of fungi and other biological matter whether in the soil or in the intestine. It is thought that chitinase activity may be released during spore germination enabling a potential source of nutrients as *C. difficile* cell emerges from its coats. It has been shown that chitinase activity decreased as spores matured however increased during spore germination and sonication which are events that would rupture spore coat layers (Permpoonpattana et al., 2011).

There could be other functions related to the peroxiredoxin and chitinase displayed on CotE. There is a potential link between peroxiredoxin, chitinase and inflammation. Peroxiredoxin-1 secreted from tumour cells were shown to induce pro-inflammatory cytokines in macrophages through interactions with TLR4 to promote chronic inflammation (Riddell et al., 2010). In the case of chitinase, some inflammatory conditions of the GI tract such as IBD and UC lead to the induction of host cell chitinases which enhance the adhesion and invasion of pathogenic bacteria

to colonic epithelial cells (CECs) and subsequently enhance the exacerbation of intestinal inflammation (Kawada et al., 2007). As some symptoms of CDAD are similar to that of IBD and UC it can be suggested that chitinase may play a direct role in infection. It is possible that chitinase could have a role to play in the degradation of mucus by targeting GluNAc which is one of the four main mucin oligosaccharides present in mucus (Linden et al., 2008) and is also the main component of chitin. This would allow for the vegetative cell to penetrate and secrete its toxins causing the abundant tissue damage, epithelial barrier disruption and fluid accumulation seen during CDI.

Results show that both HistoDenz purified and water washed unpurified *bclA*$^{-}$ mutant spores had significantly lower adherence to collagen I and fibronectin compared to 630Δ*erm* (Figure 3.6 and 3.8). Results suggested that BclA could have a role to play in the adherence of spores to ECM components of the gut. It is possible that BclA could act as a cell surface adhesin which specifically interacts with ECM components known as MSCRAMMs. To be classified as a MSCRAMM, the molecule of interest must be localized to the microbial cell surface and the microbial component must recognize a macromolecular ligand which can be found within the ECM such as collagen and fibronectin. It is possible that a single MSCRAMM can bind to several ECM ligands (Patti et al., 1994). Therefore BclA could act as a MSCRAMM for both collagen and fibronectin leading to persistence of the spore in the host by binding to major ECM components of the gut.

Fully understanding which aspects of the spore are involved in the interaction of the spore with the host could lead to the development of strategies that could inhibit attachment of spores to the host and therefore block infection cycle.

5.2 Interaction of *C. difficile* spores with macrophages

Macrophages are the first line of defence in the innate immune system against bacterial infections. Previous studies on the earliest stages of *C. difficile* have been focused on the interactions between *C. difficile* vegetative cells and macrophages. However there is a lack of knowledge on the earliest stages of macrophage infection by spores of *C. difficile*. Results from this work show that *C. difficile* spores do interact with macrophages. Results suggest that BclA could have a role to play in the efficient phagocytosis of *C. difficile* spores by macrophages. That spores are able to survive within macrophages and this could be due to their ability to remain dormant. *C. difficile* spores have also been shown to be cytotoxic to murine macrophage cell line J774.1 cells. Further investigation is required to discover precise protein-protein interactions of *C. difficile* spores and macrophages.

Following colonization of *C. difficile* to epithelial cells of the colon, *C. difficile* vegetative cells produce the enterotoxin TcdA and the cytotoxin TcdB. These toxins are responsible for the abundant tissue damage, epithelial barrier disruption and fluid accumulation during the disease. Both toxins lead to a huge inflammatory response from host epithelial cells, inducing the production of pro-inflammatory cytokines and chemokines which in turn leads to the recruitment of additional immune cells such as neutrophils and macrophages to the site of infection (Cowardin et al., 2014).

PRRs are responsible for sensing the presence of microorganisms, they do this by recognizing structures conserved among microbial species called PAMPs. Currently four different types of PRR families have been identified that are expressed by macrophages. These families include transmembrane proteins such as TLRs and C-type lectin receptors (CLRs) and cytoplasmic proteins such as the Retinoic acid-

inducible gene (RIG)-1-like receptors (RLRs) and NLRs (Takeuchi et al., 2010). Recognition of PAMPs on microorganisms by PRRs on professional phagocytes leads to phagocytosis of the microorganism. Results show that *bclA*$^{-}$ mutant spores were less efficiently phagocytosed compared to wild type strain (Figure 4.1). It is possible that BclA acts as a PAMP which is recognized by PRRs of macrophages leading to phagocytosis of the spore. The outermost layer of *B. anthracis* spores are also composed of an exosporium that is formed by the collagen-like glycoprotein BclA. As BclA forms the outermost component of the spore it was hypothesized that it would play a role in spore host interactions. It was shown that CD14 expressed on macrophages is involved in the binding and uptake of *B. anthracis*, specifically CD14 binds to the rhamnose residues in the oligosaccharides of BclA and induces an inside-out signalling pathway involving TLR2 and PI3K that leads to enhanced Mac-1 dependent spore internalization. Therefore BclA plays a central role in the targeting of *B. anthracis* spores to macrophages (Oliva et al., 2008) (Oliva et al., 2009).

BclA on *B. anthracis* has also been shown to activate the classical complement pathway (CCP) leading to efficient phagocytosis of spores. In the presence of complement BclA interacts with C1q which is the first subcomponent of the C1 complex of the classical pathway of complement activation (Kishore et al., 2000). This interaction mediates spore entry into epithelial cells via integrin $\alpha_2\beta_1$ (Xue et al., 2010). Interaction of BclA and C1q leads to the activation of CCP resulting in deposition of C3 fragments on the spore surface. C3 is further cleaved into iC3b, recognition of iC3b by CR3 on phagocytic cells results in phagocytosis of *B. anthracis* spores. When BclA is absent there is significantly less C1q and C3

fragments on the spore surface and consequently less efficient spore entry into host cells compared with that of wild type spores (Gu et al., 2012).

Once phagocytosed the fate of bacterial spores will vary depending on their specific virulence traits that will enable them to either escape or to modulate the host innate immune system. Results show that *C. difficile* spores are able to survive within macrophages and this could be due to their ability to remain dormant and not germinate (Figure 4.4). The fate of *C. perfringens* spores depends on their germination ability, isolates with germination proficient spores were efficiently inactivated during macrophage infection, while those that germinated poorly were able to survive for extended periods of time inside macrophages (Paredes-Sabja et al., 2012). Dormant *B. subtilis* spores are able to survive inside macrophages and upon germination they lose their resistance properties, becoming killed by macrophages antimicrobial compounds (Ceragioli et al., 2009). Studies have also demonstrated that *B. anthracis* wild type spores are efficiently phagocytosed and upon germination inside the phagolysosome they were efficiently killed by macrophages (Guidi-Rontani et al., 1999) (Guidi-Rontani et al., 2001). However *B. anthracis* germination deficient spores were able to survive longer periods of time that germination capable wild type spores, indicating that spore survival inside macrophages is dependent on the ability of spores to remain dormant (Kang et al., 2005). Only *B. anthracis* vegetative cells that can escape the hostile intracellular environment of the phagosome and replicate in the cytoplasm of macrophages are able to survive and cause infection to be spread (Dixon et al., 2000). The fate of *C. difficile* were shown to be significantly different than that of *C. perfringens* and *B. anthracis*. *C. difficile* spores germinate in the presence of co-germinants taurocholate and glycine however cholates are not part of the phagolysosomes environment

therefore results show that *C. difficile* spores did not germinate inside macrophages. A possible reason for their survival could be the ability of *C. difficile* to remain dormant.

The exosporium of *B. anthracis* also appears to have a role to play in protecting the bacterium from antibacterial activity of macrophages. The exosporium contains homologs to enzymes such as superoxide dismutase, alkyl hydroperoxide reductase, catalase and DNA repair enzyme endonuclease IV which are known to evade the toxic events initiated by ROS and hydrogen peroxide produced as a result of the oxidative burst of macrophages (Kang et al., 2005). *C. difficile* also contains enzymes such as superoxide dismutase, catalase (CotCB and CotD) and peroxiredoxin ($CotE_n$). Superoxide dismutase catalyses the reduction of superoxide anions to hydrogen peroxide and catalase and peroxiredoxin act as antioxidants which would reduce cellular toxicity of hydrogen peroxide by conversion to water and oxygen. Therefore these enzymes present on the *C. difficile* spore could have a critical role against the toxic effects of ROS produced in the phagolysosome of macrophages during oxidative burst. *Pseudomonas aeruginosa* (*P. aeruginosa*) has been shown to counteract oxidative stress using antioxidant enzymes peroxiredoxin. Peroxiredoxin acts a virulence factor that contributes to *P. aeruginosa* arsenal against host defences (Kaihami et al., 2014).

Also results show that *bclA*$^-$ mutant spores showed greater spore survival in macrophages at both 12 and 24 h post infection compared to wild type spores (Figure 4.2) suggesting that BclA is required for the efficient phagocytosis of spores and also for the destruction of *C. difficile* spores when inside the environment of the macrophage. There is a possibility that BclA is recognized by a component present

in macrophages that leads to its destruction, when BclA is not present, the macrophage is unable to detect the spore and in turn is unable to clear the pathogen.

It has been demonstrated that some bacteria are able to survive within macrophages via several different methods, i) prevention of lysosome-phagosome fusion e.g. *Legionella pneumophila* (*L. pneumophila*) (Barker et al., 1994) therefore maintaining a protected niche inside the host cell. ii) Escape into the cytoplasm before phagosome-lysosome fusion occurs e.g. *Listeria monocytogenes* (*L. monocytogenes*) (Finlay et al., 1997). iii) Resistance to antimicrobial response e.g. *Salmonella typhimurium* (*S. typhimurium*) (Slauch et al., 1997) by expressing several gene products that enhance intracellular survival by neutralizing lysosomal killing mechanisms such as delaying and reducing phagolysosome acidification. It could be possible that BclA mutant spores could be using these evasion techniques to survive within macrophages better than the wild type.

C. difficile spores were shown to produce cytotoxic effects to J774.1 cells at MOI 10:1 and 100:1, with greater cytotoxicity observed at MOI 100:1 (Figure 4.3). It has been demonstrated that *C. difficile* spores are phagocytosed by J774.1 cells and are able to survive inside macrophages. Once phagocytosed the fate of spores is dependent on their ability to escape the phagosome for example *B. anthracis* spores localize to the late phagolysosome whereas *C. perfringens* vegetative cells escape from the phagosome by degrading the phagosome membrane. Paredes-Sabja et al., recently showed that *C. difficile* 630 spores directly interacted with the phagosomes membrane of Raw 264.7 cells. They demonstrated that *C. difficile* was capable of inducing permanent damage to Raw 264.7 cells leading to disruption of plasma membrane and loss of viability of Raw 264.7 cells. *C. difficile* spores were shown to

directly interact with the phagosomes membrane of Raw 264.7 cells and presumably induce membrane disruption and cell death. However, the precise nature of this interaction is not known and it is hypothesized this interaction likely involves interactions between spore surface proteins and the phagosomes membrane and/or membrane embedded proteins (Paredes-Sabja et al., 2012). These interactions have been shown to occur between *C. perfringens* vegetative cells and the phagosome of J774.1 cells, where bacteria were in the process of being phagocytosed but were able to break down the phagosome membrane before it could completely engulf the bacteria (O'Brien et al., 2000). And also with hair like nap of *B. anthracis* spores and the phagosomes membrane (Guidi-Rotani et al., 2001).

Results have shown that *C. difficile* spores are recognized by macrophages however the nature of phagocytic receptors mediating *C. difficile* spore recognition by J774.1 cells has not yet been determined. Biotinylation spore pull down assays were carried out using J774.1 cells and 630Δ*erm* spores by labelling J774.1 cells with biotin cross-linking agent and solubilizing the surface proteins and screening for those that had bound to 630Δ*erm* spores. Protein with apparent molecular mass of 16 KDa was detected (Figure 4.5). Mass spectrometric analysis identified the protein as histone H4, a protein which is a major component of chromatin and not a phagocytic receptor. Therefore further investigation into the nature of phagocytic receptors mediating *C. difficile* spore recognition by J774.1 cells is needed.

5.3 Conclusions

During the course of CDI, *C. difficile* undergoes sporulation and releases spores to the colonic environment. The elevated relapse rates of CDI suggest that *C. difficile* spores has mechanisms to efficiently persist in the host colonic environment however little is known about the role of proteins in the outermost layer of the spore has to play in persistence. Results from this work suggest BclA could have a role to play in the adherence of spores to ECM components of the gut. It is possible that BclA could act as a cell surface adhesin which specifically interacts with ECM components known as MSCRAMMs.

CotE protein could have a role in the adherence of *C. difficile* spores to the intestinal epithelium and mucus. It is possible that chitinase could have a role to play in the degradation of mucus by targeting GluNAc which is one of the four main mucin oligosaccharides present in mucus and is also the main component of chitin. This would allow for the vegetative cell to penetrate and secrete its toxins causing the abundant tissue damage, epithelial barrier disruption and fluid accumulation seen during CDI.

BclA1 mutant spores show increased adherence to epithelial cells compared to the wild type strain. It is possible that BclA protein may have a similar role to that of BclA in *B. anthracis* where BclA has been shown to promote tropism towards macrophages and decrease the adherence to lung epithelium cells. Which in turn leads to the interaction of BclA with components of complement which mediates internalization into epithelium or professional phagocytes.

Interaction of *C. difficile* spores with macrophages suggest that BclA could have a role to play in the efficient phagocytosis of *C. difficile* spores by macrophages. Results also show that spores are able to survive within macrophages and this could be due to their ability to remain dormant. *C. difficile* spores have also been shown to be cytotoxic to murine macrophage cell line J774.1 cells.

Persistence of *C. difficile* in the host might be mediated through the adherence of spores to the host's intestinal epithelium and surviving attacks of phagocytic cells. Investigating mechanisms of persistence of the spore in the host is of great importance in understanding the pathogenesis of CDI and will lead to effective diagnosis, treatment and prevention of the infection.

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