Clostridium difficile infection; establishment, prevention and the role of the microbiota

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Declaration of Authorship

I declare that this work was carried out in accordance with the Regulations of the University of London. I declare that this submission is my own work, and to the best of my knowledge does not represent the works of others, published or unpublished, except where duly acknowledged in the text. No part of this thesis has been submitted for a higher degree at another university or institution.

Signed: Date: 09/09/2019

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Abstract

Clostridium difficile is a leading cause of nosocomial, antibiotic-associated, diarrhoea in industrialised countries. *C. difficile* spores are dormant and resistant structures responsible for the colonisation and persistence of *C. difficile* infection (CDI) within patients as well as the transmission between them. This thesis examines firstly, the role of a *C. difficile* spore coat protein, CotE, in the establishment of CDI, and secondly, the role of allochthonous *Bacillus* within the GI tract in suppressing CDI. CotE is a protein displayed on the *C. difficile* spore surface which carries two functional elements, an N-terminal peroxiredoxin and a C-terminal chitinase domain. Using isogenic mutants, it was demonstrated *in vitro* and *ex vivo* that CotE enables binding of spores to mucus by direct interaction with mucin and contributes to its degradation. In animal models of CDI, it was shown that when CotE was absent, both colonisation and virulence were markedly reduced. It is demonstrated here that the attachment of spores to the intestine is essential in the development of CDI. The developments of infection within the host GI tract requires germination of the spore, followed by outgrowth of the vegetative cell; a process which requires a multitude of factors to favour *C. difficile* proliferation. In a series of experiments, it was shown that environmentally acquired *Bacillus* help to suppress CDI by the production of lipopeptides *in vivo* with activity against *C. difficile* and that the use of antibiotics abrogates this phenomenon. Using hamster and mouse models, it was demonstrated that the administration of these *Bacillus* provides complete colonisation resistance against CDI in clindamycin treated animals. Reversed-phase high-performance liquid chromatography, dynamic light scattering and MALDI-TOF analysis identified the active molecules to be a micellar complex of lipopeptides comprised of chlorotetaine, iturins, fengycins and surfactins. Through this work, it is demonstrated that the attachment of spores to the intestine is essential in the development of CDI and suggests a direct role for the spore in the establishment and promotion of disease.

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Abbreviations

ACN - acetonitrile

AcOOH - acetic acid

$AmSO_4$ - ammonium sulphate

BHI - brain heart infusion

BSA - bovine serum albumin

CA - community acquired

CD - *Clostridium difficile*

CDAD - *Clostridium difficile*-associated diarrhoea

CDI - *Clostridium difficile* infection

cDNA - complementary deoxyribonucleic acid

CDT - *Clostridium difficile* binary toxin

CDTA - C-terminus domain of Toxin A

CFU - colony-forming unit(s)

CWP - cell wall protein

Da - Daltons

DLS - dynamic light scattering

DMEM - Dulbecco's Modified Eagle Medium

DNA - deoxyribonucleic acid

EDTA - ethylenediaminetetraacetic acid

EIA - enzyme immunoassay

ELISA - enzyme linked immunosorbent assay

EM - electron microscopy

Erm - erythromycin

EtOH - ethanol

FBS - foetal bovine serum

FCS - foetal calf serum

GalNAc - N-acetylgalactosamine

GCW - germ-cell wall

GI - gastrointestinal

GlcNAc - N-acetylglucosamine

GM - genetically modified

HMW - high molecular weight

HPA - Health Protection Agency

HPLC - high-performance liquid chromatography

HRP - horse radish peroxidase

IBD - inflammatory bowel disease

i.g. - intragastric

Ig - immunoglobulin

IgA - immunoglobulin isotype A

IgG - immunoglobulin isotype G (major systemic Ig)

IL- interleukin

i.p. - intraperitoneal

IPTG - isopropyl β-D-thiogalactopyranoside

kDa - kilodaltons

LB - Luria Bertani

LCTs - large clostridial toxins

LMW - low molecular weight

LTA - lipoteichoic acid

MALDI - matrix-assisted laser desorption/ionisation

MALDI-TOF - matrix-assisted laser desorption/ionisation – time of flight

MeOH - methanol

MIP - macrophage inflammatory protein

MOI - multiplicity of infection

mwt - molecular weight

OD - optical density

ORF - open reading frame

PaLoc - pathogenicity locus

PBS - phosphate buffered saline

PCR - polymerase chain reaction

PEG - polyethylene glycol

PG - peptidoglycan

PMC – pseudomembranous colitis

ppt. - precipitate

PS - polysaccharide

PSI - polysaccharide I of *C. difficile*

PSII - polysaccharide II of *C. difficile*

RHUL - Royal Holloway University of London

RNA - ribonucleic acid

RP-HPLC - reversed phase high-performance liquid chromatography

rpm - revolutions per minute

RT - room temperature

RT-PCR - reverse transcriptase PCR

SASP - small acid-soluble spore protein

SATH - spore adhesion to hydrocarbon

SD - standard deviation

SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEC - size-exclusion chromatography

SEM - scanning electron microscopy

S-layer - surface layer

Slp - S layer protein

SMC - sporulation medium supplemented with L-cysteine

TA - teichoic acid

Tcd - Clostridial toxin

TEM - transmission electron microscopy

TFA – trifluoroacetic acid

TGY - tryptic-glucose-yeast extract medium

Th-1 - T helper lymphocyte type 1

TI – Terminal ileum

TLR4 - Toll-like receptor 4

TMB - tetramethyl benzidine

TNF - tumour necrosis factor

UDP - uridine diphosphate

UV - ultraviolet

v/v - volume per volume

w/v - weight per volume

CHAPTER 1

Introduction

1.1 Identification of *C. difficile* as a pathogen

The Clostridia are believed to have emerged as a taxonomic Class approximately 2.9 billion years ago, prior to the 'Great Oxygenation Event' which resulted in the induction of free oxygen into the atmosphere (Battistuzzi *et al.*, 2004). Clostridia, of the phylum Firmicutes, are a Gram positive, polyphyletic Class with the ability to form highly resistant endospores. Consistent with an origin prior to the Great Oxygenation Event, the Clostridia Class are obligate anaerobes. Most Clostridia are saprophytic organisms and can be found throughout the environment, most commonly in soil. There appear several human pathogens within Clostridia; the toxins produced by some being amongst the deadliest known to mankind. *Clostridium tetani* and *Clostridium botulinum* both produce potent neurotoxins which can lead to severe autonomic nervous system dysfunction and death (Thwaites, 2017). *Clostridium perfringens*, *Clostridium septicum*, *Clostridium sordellii*, *Clostridium histolyticum*, and *Clostridium novyi* can all cause gangrenous soft tissue infections (Stevens *et al.*, 2012). *C. perfingens* is associated with necrotising soft tissue infections, foodborne gastroenteritis and endometritis. Several species of Clostridia, including *Clostridium saccharaobutylicum*, *Clostridium acetobutylicum* and *Clostridium beijerinckii* are capable of converting carbohydrates into solvents and are therefore of interest in the potential development of biofuels (Galadima *et al.*, 2015). Non-toxigenic or attenuated strains of Clostridia are currently being investigated as drug delivery vehicles for potential use in the treatment of cancer; *Clostridium novyi* has been shown to confer a survival advantage in canine soft tissue carcinoma via induction of anti-tumour responses following local spore administration of an attenuated strain (Heap *et al.*, 2014; Roberts *et al.*, 2014; Theys and Lambin, 2015). Clostridial spores are being assessed as selective delivery vehicles of high therapeutic doses of anti-cancer

agents to tumours (Minton, 2003). This design system relies on the ability of intravenously injected spores to infiltrate, germinate and survive within the hypoxic environment of solid tumours. It has been demonstrated that engineered *Clostridium sporogenes* strains expressing a prodrug-converting enzyme can convert a non-toxic prodrug to a cytotoxic drug specifically at tumour sites (Heap *et al.*, 2014).

Clostridium difficile was first isolated from the stool of new-born infants by Hall and O'Toole in 1935 and was initially named *Bacillus difficile*, due to the difficulty in culturing the bacteria (Hall and O'Toole, 1935). In 2016 *Clostridium difficile* was reclassified and assigned to a new genus following restiction of the genus to *Clostridium butyricum* and other related species in 2015 (Lawson *et al.*, 2016). *C. difficile* was originally assigned to the *Clostridium* genus due to its broad phenotypical similarties with other members. Recently performed phenotypic, chemotaxonomic and phylogenetic analyses found *C. difficile* to share the most similarities with species within the Peptostreptococcaceae family. Commercial and medical concerns regarding the change in name prompted the creation of the genus *Clostridioides* and the species *Clostridioides difficile*.

Initial investigators found *C. difficile* to be toxic in guinea pigs and rabbits, but its presence in neonates led them to believe that the pathogen was a normal species of a healthy human intestinal flora. The asymptomatic carriage of *C. difficile* by neonates, and their clearance after approximately 12 months is now widely acknowledged by researchers (Al-Jumaili *et al.*, 1984; Lees *et al.*, 2016; Kuiper *et al.*, 2017). However, the explanation for the lack of *C. difficile* toxicity in infants remains unknown (Jangi and Lamont, 2010).

Although cases of pseudomembranous colitis (PMC) symptoms began to rise in the 1950's due to the increased use of antibiotics (with rates as high as 27% in postoperative patients), *C. difficile* was believed to not be associated with the condition and rather *Staphylococcus aureus* was presumed to be the causative agent (Bartlett, 2008). This hypothesis was undermined in a study by Tedesco *et al.*

(1974), who reported high levels of PMC in patients being treated with clindamycin. Of the 200 patients receiving clindamycin, 42 (21%) developed PMC and despite the ease of isolating *S. aureus,* stool samples were found to be negative for the bacteria. This article spawned interest in discovering the cause, the pathophysiology and modes of treatment for "clindamycin colitis".

Following this, a succession of studies started to illuminate a clear link between these cases and *C. difficile.* Most important among these studies included work by Bartlett *et al.* (1977) and Chang *et al.* (1978) who demonstrated that *C. difficile* causes PMC in hamsters. This work was supported by controlled analyses in hospitals such as University Hospital in Ann Arbor, Michigan (Keighley *et al.*, 1978) and The General Hospital in Birmingham (Lusk *et al.*, 1977).

Although *C. difficile* had been recognised as the causative agent of *C. difficile* infection (CDI), funding was limited and remained so throughout the 80's and 90's despite the public outcry following an outbreak in 1991/2 of CDI in three hospitals in Manchester, UK causing the deaths of 17 patients (Cartmill *et al.*, 1994). It was not until the 21st Century that *C. difficile* was recognised as a 'hospital superbug' following a number of high-profile outbreaks coupled with extensive media coverage. An outbreak at Stoke Mandeville hospital in Buckinghamshire, UK, in 2005 led to a public enquiry following complaints made by staff of poor senior management, contributing to the conditions which facilitated the outbreak (Katikireddi, 2005). This outbreak was proceeded by another, at a hospital in Maidstone, UK, leading to 90 fatalities between 2004 and 2006. The subsequent increase in research funding has helped bring us to the present day, where our basic understanding of *C. difficile* and its implications on gastrointestinal health has risen exponentially, albeit with many questions remaining un-answered.

1.2 *C. difficile* infection

1.2.1 The disease

C. difficile is now considered to be one of the foremost nosocomial pathogens in humans. It has been responsible for numerous hospital outbreaks with symptoms ranging from diarrhoea to pseudomembranous colitis and death (Rodriguez *et al.*, 2016). CDI is acquired following antibiotic treatment which leads to perturbation of the gut microbiota (which is protective against *C. difficile* colonisation and growth) allowing outgrowth and infection by antibiotic resistant strains of *C. difficile.* Although those most at risk of CDI often have a history of antibiotic treatment, prolonged periods of hospitalisation, and are over 65 years of age, recent studies have described increased incidences of the infection spreading further into the community as well as increases in severity and occurrences of nosocomial CDI within North America and Europe (Kuijper *et al.*, 2006; Rodriguez *et al.*, 2014; Borali *et al.*, 2015). This increase has been principally attributed to the emergence of 'hypervirulent' ribotypes, notably ribotype 027 and 078 (Cookson, 2007). Strains within these ribotypes, when compared to endemic strains, typically demonstrate increased microbial resistance, particularly to quinolone antibiotics, increased infectiousness and an increased symptomatic disease rate. Since the identification of these strains in 2005, ribotype 027 has been reported in most European countries, forty US states and every province in Canada (Pepin *et al.*, 2004; Loo *et al.*, 2005; Hubert *et al.*, 2007; Kuijper *et al.*, 2008; Rupnik *et al.*, 2009). Meanwhile, *C. difficile* ribotype 078 has increased in prevalence from 3 to 13% in several European countries (Goorhuis *et al.*, 2008; Rupnik *et al.*, 2008). However, it should be noted that although the increasing rates of CDI can largely be attributed to hypervirulent strains, other endemic strains have continued to be associated with outbreaks (Borgmann *et al.*, 2008).

Every antibiotic has the potential to place a patient at risk of CDI, however several antibiotics pose a significantly higher risk than others. The use of penicillins, clindamycin and cephalosporins can place a patient at significant risk for acquiring CDI whilst the emergence of hypervirulent strains with resistance against

fluoroquinolones means that this antibiotic is also high risk (Owens *et al.*, 2008). It has been demonstrated that 25 to 30% of hospital patients are asymptomatic carriers of *C. difficile* (Shim *et al.*, 1998). Asymptomatic carriage is thought to be a consequence of the host's high anti-toxin IgG response against *C. difficile* Toxin A (following exposure to *C. difficile*) resulting in decreased morbidity (Kyne *et al.*, 2001). Asymptomatic carriage then is thought to play a role in transmission of *C. difficile* in many unexplained cases (Eyre *et al.*, 2013).

Non-toxigenic strains of *C. difficile* have also been observed colonising patients at a prevalence of between 0.4 and 6.9% (Boone *et al*., 2012). Studies in hamsters have demonstrated that non-toxigenic *C. difficile* can have a protective effect against toxigenic infection and trials are underway to find evidence for a therapeutic or preventative role (Natarajan *et al.*, 2013). A definitive mechanism for this protective effect is yet to be found, but it has been suggested that non-toxigenic strains compete with toxigenic strains for colonisation thereby attenuating the severity of infection (Nagaro *et al.*, 2013; Gerding *et al.*, 2018). This is because binding of *C. difficile* vegetative cells to colonic mucus producing cells is thought to play a role in colonisation, and it is possible that prior exposure of host cells to non-toxigenic *C. difficile* S-layer protein increases colonisation resistance by significantly reducing attachment of the subsequent toxigenic strain (Merrigan *et al*., 2013). Nutrient acquisition and competition for a niche in the gastrointestinal tract has also been suggested (Natarajan *et al*., 2013). Studies involving infecting hamsters with a non-toxigenic strain of *C. difficile* (CD1342) have perhaps provided evidence for an active role played by the spore itself during the infection process, where cell damage in the caecum and an inflammatory response were observed (Buckley *et al*., 2013).

Interestingly, among community acquired infections there has been a recent increase in the number of individuals who acquire CDI without any prior antibiotic use (Khanna *et al.*, 2012). It has also been observed that new-borns (humans and piglets) and infants can be colonised by *C. difficile* without any prior antibiotic use (Jangi and Lamont, 2010; Rousseau *et al.*, 2012; Moono *et al.*, 2016). These studies

would suggest that antibiotics are not necessarily a prerequisite to *C. difficile* germination and outgrowth.

In the previous two decades *C. difficile* has had a substantial economic and clinical worldwide impact (Hung *et al.*, 2015; Nanwa *et al.*, 2015). A recent review analysing 45 independent cost-of-illness studies described that the cost of hospitalised CDI patients ranges from $8,911 to $30,049 per patient in the USA (Nanwa *et al.*, 2015). CDI is the most frequently reported healthcare associated infection in the USA with the number of incidences ranging from 2.8 to 9.3 per 10,000 patient days (Evans and Safdar, 2015). In 2011, it was estimated that there were 453,000 cases of CDI in US hospitals and long-term care facilities resulting in 29,000 deaths (Lessa *et al.*, 2015). Although CDI is considered to be primarily a healthcare related disease, incidences of community acquired infections have increased and are now estimated to be in the range of 1.3 to 2.7 per 10,000 patient days (Evans and Safdar, 2015).

In Europe, the annual economic cost of CDI is approximately €3 billion, a figure expected to rise due to the increasing proportion of elderly people in European countries (Kuijper *et al.*, 2006). In Saxony, Germany, the incidence rate of CDI has risen from between 1.7 and 3.8 cases per 100,000 people in 2002 to 14.8 cases per 100,000 people in 2006 (Burckhardt *et al.*, 2008). While in Spain, the number of hospital discharges over the age of 65 years diagnosed with CDI tripled between 1997 and 2005 (Soler *et al.*, 2008).

CDI is most frequently associated with nosocomial diarrhoea in Europe and Northern America; however, this is partly due to the scarcity of data available in developing countries. In a study describing the rates of CDI in travellers, the highest number of cases were reported in low- and middle-income countries (Neuberger *et al.*, 2013). CDI was reported to often be acquired in the community by younger patients and was associated with antimicrobial use, particularly fluoroquinolones (Neuberger *et al.*, 2013).

An epidemiological review of CDI in Asia suggests that the prevalence of infection is similar to Europe and the US but with a predominance of toxin A-negative/toxin B-positive strains (Collins *et al.*, 2013). In stark contrast to Europe, ribotypes 027 and 078 have rarely been reported in Asia (Collins *et al.*, 2013). As is the case in Asia, there is little data of CDI prevalence in South America. In a study assessing the epidemiology of *C. difficile*-associated diarrhoea in a Peruvian hospital, 12.9 incidences per 1000 patients were reported. The high prevalence (for comparison, in a six-month prospective surveillance study covering nine Canadian provinces an overall CDI rate of 4.6 cases per 1,000 patients was reported) was attributed by the author to in-hospital transmission and the need for implementing more stringent hygiene programmes in low-resource settings was emphasised (Garcia *et al.*, 2007; Gravel *et al.*, 2009).

Of additional concern in developing countries is the comorbidity of HIV and CDI. A study in India discovered that *C. difficile* was the most commonly identified bacterial pathogen in adults with HIV, with a prevalence of 18% (Jha *et al.*, 2012). A similar trend was observed in Nigeria where HIV positive inpatients and outpatients were found to be positive for *C. difficile* in 43% and 14% of cases respectively (Onwueme *et al.*, 2011). These studies demonstrate the necessity in controlling and regulating access to antibiotics in low- and middle-income countries in addition to improving early diagnosis of intestinal pathogens, particularly among those infected with HIV.

1.2.2 Presentation of the disease

Once CDI has developed it can present in several ways (Rupnik *et al.*, 2009). The classical presentation of mild, symptomatic CDI presents with diarrhoea, which can range in severity from mild to moderate and often resolves itself after the discontinuation of antibiotic treatment (Rao and Higgins, 2016). The most common clinical manifestation is non-pseudomembranous colitis, with symptoms being inflammation of the colon, fever, abdominal pain and severe diarrhoea. PMC is diagnosed if upon endoscopical examination pseudomembranous-like structures

are observed (appearing as raised yellow plaques) on the colorectal mucosa (Cohen *et al.*, 2010). Severe CDI often shows systemic signs of infection including leucocytosis, fever and acute kidney injury (Cohen *et al.*, 2010). Complicated CDI presents with hypotension, shock, ileus and/or toxic megacolon (Cohen *et al.*, 2010). Approximately 3% of CDI cases are diagnosed as complicated (Kachrimanidou and Malisiovas, 2011). Toxic megacolon can result in complication; fever, colonic perforations, systemic inflammatory response and occasionally death (Kachrimanidou and Malisiovas, 2011). Cases of severe and complicated CDI cause significant morbidity and treatment may require a colectomy. Even with treatment, a median of 19% of patients will die within 30 days of initial diagnosis (Abou Chakra *et al.*, 2014).

Following recovery from CDI, recurrence within two months is common and occurs in approximately 25% of individuals (Abou Chakra *et al.*, 2014). Ordinarily, a second CDI episode occurring 8 weeks or more after resolution of a previous infection are considered to be a new infection but a study utilising molecular typing demonstrated that of these 65% were in fact recurrences (Kamboj *et al.*, 2011). The incidence of recurrent CDI is significantly higher in older adults and in those with irritable bowel syndrome (IBS) (Goodhand *et al.*, 2011; Abou Chakra *et al.*, 2014). In those who experience a first recurrence, 30-45% will have a second recurrence, and of those 45-60% will experience a third with ≤5% of those suffering from chronic, seemingly endless cycles of recurrence (Gough *et al.*, 2011; Rao and Higgins, 2016). Among those who do not recur, up to 35% may experience functional bowel disorder in the first two weeks following resolution of CDI (Piche *et al.*, 2007). Although a transient disorder, approximately 4% of patients will go on to develop IBS lasting more than three months from initial CDI diagnosis (Piche *et al.*, 2007).

1.2.3 Establishment of infection

CDI occurs following antibiotic perturbation of the microbiota residing within the gut. Recent studies are beginning to demonstrate, using 16S ribosomal RNA sequencing, that the perturbation inflicted upon the natural gut flora by antibiotic

treatment is more extensive than was previously realised, and contributes to the markedly disrupted and decreased diversity thought to play an integral role in recurrent CDI (Chang *et al.*, 2008; Dethlefsen *et al.*, 2008).

The antibiotic resistance of *C. difficile* enables the bacterium to colonize and outgrow within the host during antibiotic treatment (Figure 1.1). Studies using hamster models have shown that different antibiotics cause susceptibility to CDI for varying lengths of time after cessation of treatment. Colonisation resistance is restored rapidly after treatment with cephalosporins while clindamycin has a prolonged period of susceptibility (Merrigan *et al.*, 2003; Rupnik, Wilcox and Gerding, 2009). The recovery of the gut microbiota and consequently host colonisation resistance, is therefore necessary to prevent CDI.

As an obligate anaerobe, *C. difficile* relies on its spore form as the chosen method of transmission and dissemination. After ingestion by a host, the process by which *C. difficile* spores germinate, outgrow and cause disease involves complex interactions with a number of bile salts (Karen and John, 2011; Shen, 2015). In early experiments performed in hamsters, *C. difficile* spores were shown to germinate rapidly (~1 hour) within the small intestine in response to the bile salt sodium taurocholate (Wilson *et al.*, 1985).

This finding was supported by the later work of Sorg and Sonenshein (2008), where it was demonstrated that spores germinate in response to cholate derivatives in combination with amino acid co-germinants. Meanwhile, chenodeoxycholate, another primary bile salt, was found to competitively inhibit cholate-induced germination by process of having greater affinity for *C. difficile* spores (Sorg and Sonenshein, 2010). In accordance with these findings, small intestinal extracts have been shown to induce the germination of *C. difficile* spores (Giel *et al.*, 2010). Pre-treatment of intestinal extracts with cholesterylamine, a bile salt sequestrant, negated this effect indicating that interaction of spores with bile salts is a pivotal moment in *C. difficile*'s intestinal life residency (Giel *et al.*, 2010). *C. difficile* most likely interacts with bile acid germinants using the germinant receptor, CspC, a

subtilisin-like pseudoprotease that is in control of a unique signalling pathway (discussed in more detail in Section 1.3.3) (Paredes-Sabja *et al.*, 2014).

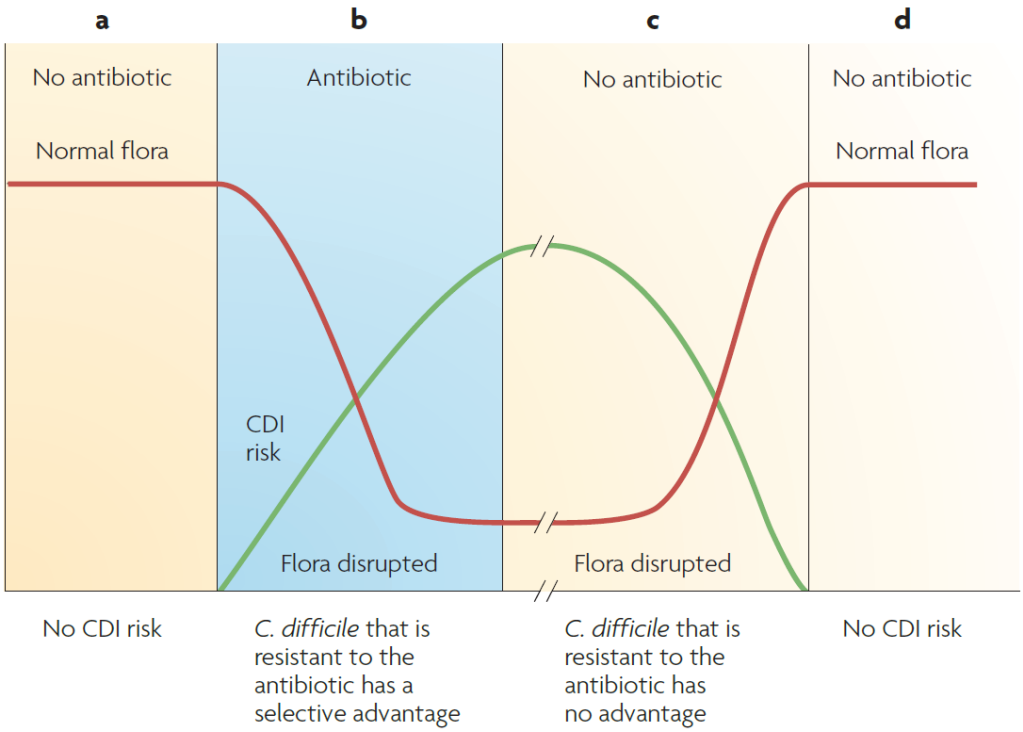


Figure 1.1. The link between antibiotic disruption of the intestinal flora and CDI risk. In the absence of antibiotic therapy**,** the gut microbiota is unperturbed, and the host is resistant to CDI **(a)**. Antibiotic therapy disrupts the intestinal flora and CDI risk increases as resistant *C. difficile* has an opportunity to outgrow **(b)**. The antibiotic level rapidly decreases with cessation of therapy, but the intestinal flora remains perturbed for a variable period of time depending on the antibiotic used **(c)**. During this period susceptibility to resistant or susceptible *C. difficile* is greatest. Once the intestinal flora recovers host colonisation resistance to *C. difficile* is restored **(d)**. (Modified from Rupnik, Wilcox and Gerding, 2009).

Although germination has been demonstrated in both antibiotic and non-antibiotic treated animals, small intestinal extracts from antibiotic treated mice are 3 to 10 fold more effective at germinating *C. difficile* spores (Giel *et al.*, 2010; Koenigsknecht *et al.*, 2015). These findings imply a role for the microbiota, as a protector of gastrointestinal health via regulation of bile salt levels, and therefore providing resistance against CDI. Antibiotic treatment of mice increases the ratio of cholate (germinant) to muricholic acids (germination inhibitor) within the small intestine (Francis, Allen and Sorg, 2013; Koenigsknecht *et al.*, 2015). Antibiotic treatment has also been shown to increase the quantity of taurocholate in the caecum

and decrease the levels of muricholic acids and deoxycholate (Theriot *et al.*, 2014; Buffie *et al.*, 2015; Koenigsknecht *et al.*, 2015). Deoxycholate, a derivative of cholate, like its precursor acts as a germinant for *C. difficile* spores but is toxic to the vegetative form thereby preventing outgrowth (Sorg and Sonenshein, 2008). Muricholic acids have also been shown to inhibit the growth of vegetative *C. difficile* (Francis, Allen and Sorg, 2013). This antibiotic induced change in the caecal bile acid profile results in a 30-fold increase in spore germination when compared to untreated mice (Giel *et al.*, 2010). This is supported by *ex vivo* viability assays, where caecal extracts from untreated mice display decreased *C. difficile* viability while extracts from antibiotic treated animals support growth (Buffie *et al.*, 2015). This ability to support growth is restored in extracts from non-antibiotic treated mice by pre-treating with cholestyramine, signifying that secondary bile acids play an integral role in preventing both germination and outgrowth of *C. difficile*.

The spatio-temporal dynamics of CDI establishment and progression in a mouse model has been eloquently described by Koenigsknecht *et al.* (2015). After the ingestion of *C. difficile* spores by mice, vegetative cells were first detected in the colon after 6 hours signifying that spore germination and outgrowth had occurred within this time. At approximately 15 hours post-ingestion sporulation occurs concomitantly with toxin production. 24 hours post-ingestion of spores the *C. difficile* CFU count had increased by ~5 logs and the highest numbers were found in the large intestine and the caecum. Spores were first detected in the large intestine 24 hours post-ingestion and at 30-36 hours ~20% of viable CFU were in the form of spores. Toxin levels were highest at 24 hours and symptoms of disease presented within 6 hours after detection of toxin. A diagrammatic representation of this cycle is displayed in Figure 1.2.

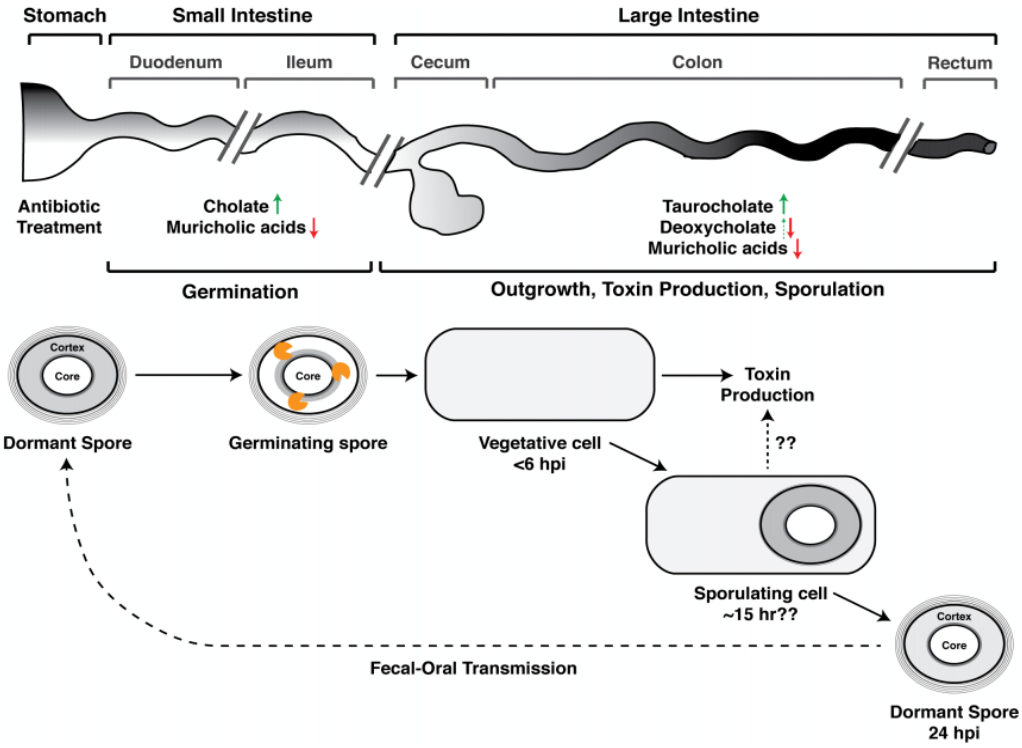


Figure 1.2. The development of CDI. Spore germination occurs within the small intestine in response to cholate derivatives (Wilson *et al.*, 1985; Koenigsknecht *et al.*, 2015)**.** The degradation of the cortex layer in germinating spores is shown followed by rapid outgrowth of vegetative cells at 6-hours post infection. The precise time at which sporulation is induced is unclear, as are the mechanisms behind its co-occurrence with toxin production (Koenigsknecht *et al.*, 2015). The effect of antibiotic treatment on bile acids in specific areas of the gut is shown (Buffie *et al.*, 2015; Koenigsknecht *et al.*, 2015)**.** Note that muricholic acids are murine specific. Green arrows show increase in bile acid germinants while red arrows show decreases in *C. difficile* inhibitory bile acids. (Modified from Shen, 2015).

1.2.4 Virulence factors

1.2.4.1 Toxins

The range in symptoms described in Section 1.2.2 can largely be attributed to two glucosyltransferase toxins: TcdA (308 kDa) and TcdB (269 kDa). *C. difficile* strains also produce a third, binary toxin known as CDT. Toxin A and B are encoded for by genes found on the 19.6 kb chromosomal pathogenicity locus (PaLoc) (Lyerly *et al.*, 1988; Rupnik *et al.*, 2009). The PaLoc contains the toxin genes, *tcdA* and *tcdB*, the putative phage holin *tcdE* and two regulators; *tcdR* which encodes a sigma factor involved in positive transcriptional regulation and *tcdC*, a negative regulator (Figure 1.3). In non-toxigenic *C. difficile* strains, the PaLoc is substituted for a 115 bp non-coding sequence. Both TcdA and TcdB are produced during the late log and

stationary phases of growth (Hundsberger *et al.*, 1997). Toxins A and B are the primary determinants of pathogenicity and virulence in CDI and their actions lead to the symptoms most commonly observed during infection (Thelestam and Chaves-Olarte, 2000; Jank *et al.*, 2007). In a hamster model of CDI, via genetic inactivation of *tcdA* and *tcdB,* it has been demonstrated that the absence of toxins leads to an absence of symptoms (Kuehne *et al.*, 2010).

TcdA and TcdB belong to a family of single-chained proteins named large clostridial toxins (LCTs). LCTs are glucosyltransferases and have between 30 and 90% amino acid sequence similarity, while TcdA and TcdB share 63% homology in their sequences (von Eichel-Streiber *et al.*, 1996; Chaves-Olarte *et al.*, 1997). LCTs contain four functional domains; an amino-terminal catalytic domain, a carboxy-terminal binding domain, a cysteine protease domain and a delivery and receptor-binding domain (von Eichel-Streiber *et al.*, 1996; Rupnik *et al.*, 2009; Chen *et al.*, 2019).

The amino-terminal catalytic domain functions by transferring a glucose from UDP-glucose to the threonine residues on GTPases of the Rho and Ras families in the epithelial cells of the GI tract within the host (Riegler *et al.*, 1995; Thelestam and Chaves-Olarte, 2000). Glycosylation of the Rho and Ras proteins is thought to prevent the exchange of GDP for GTP and/or prevent their interaction with downstream inhibitors and thereby results in their inactivation. These hypotheses have been supported by structural studies demonstrating that the threonine which is to be glucosylated (Thr37) is more accessible in the GDP rather than the GTP state (Ihara *et al.*, 1998). In another study, Rho-GDP was shown to be a preferred target over Rho-GTP (Just *et al.*, 1995). These small GTPases function as messengers between external signals and actin signalling within the eukaryotic cell, either by direct interaction or by means of downstream effectors (Spiering and Hodgson, 2011). The inactivation of these proteins by toxins results in disintegration of the actin cytoskeleton and tight junctions leading to decreased transepithelial resistance and consequently loss of cell shape (Riegler *et al.*, 1995; Thelestam and Chaves-Olarte, 2000). Consequences of this damage to cellular structure is fluid

accumulation, destruction of the intestinal epithelium and the eventual symptoms of CDI. A further consequence of the damage inflicted upon host cells is the release of inflammatory cytokines from epithelial cells, macrophages and mast cells. The disruption of the tight junction permits an influx of neutrophils; this is believed to be directly influenced by TcdA and indirectly by inflammatory cytokines such as IL-8 (Kelly, Pothoulakis and LaMont, 1994; Voth and Ballard, 2005). These factors amplify the inflammatory response observed during CDI.

The receptor binding domains contain multiple "amino acid short repeats" and "amino acid long repeats", collectively named combined repetitive oligopeptides (CROPs). Within this C-terminal repetitive domain, CROPs can consist of 21, 30 or 50 amino acid residues (Sun *et al.*, 2010). Various approaches in CROP sequence analysis has revealed that TcdA has between 30 and 38 contiguous repeats in this region, whereas TcdB appears to contain between 19 and 24 residues (Ho *et al.*, 2005; Greco *et al.*, 2006). The similarity of these sequences with several cell wall binding bacterial proteins has led to speculation that the multiple repeats may increase the affinity and avidity of receptor binding on host epithelial cells (von Eichel-Streiber *et al.*, 1992; Fernández-Tornero *et al.*, 2001).

Due to the homology of the CROP region of the binding domains to the carbohydrate binding region of streptococcal glucosyltransferases, it was hypothesised that toxins may bind to host cell receptors via carbohydrate recognition (von Eichel-Streiber and Sauerborn, 1990). In concordance with this theory, seven potential carbohydrate binding sites have been identified as putative receptors for TcdA (Ho *et al.*, 2005; Greco *et al.*, 2006). In mice, the trisaccharide Gal1(α1–3)Gal(β1–4) GlcNAc comprises part of the TcdA receptor but in humans this receptor is not present (Krivan *et al.*, 1986). In humans, gp96, a glycoprotein found within the heat shock family of proteins, has been shown to act as a receptor for TcdA in human colonocytes (Na *et al.*, 2008). However, whether this acts as the principal TcdA receptor *in vivo* remains to be determined. Until recently the receptor for TcdB remained elusive, however a recent study using CRISPR-Cas9-mediated genome-wide screens identified members of the Wnt receptor frizzled

family (FZDs) as TcdB receptors (Tao *et al.*, 2016). In a mouse model, Tao *et al.* (2016) were able to show that TcdB binds to the Wnt binding site, named the cysteine rich domain, with preferential binding to FZD1, 2 and 7. TcdB blocks Wnt signalling by competing for binding to FZDs, and in FZD7-knockout mice, the colonic epithelium was shown to be less susceptible to TcdB-induced tissue damage *in vivo* (Tao *et al.*, 2016). The size of the role that the CROP region plays in toxin uptake and cytotoxicity has recently been questioned, with the observation that TcdA lacking CROPs is still able to enter the host cell and facilitate cytotoxicity (Olling *et al.*, 2011; Gerhard *et al.*, 2013).

Following binding to the host cell receptor, toxins are internalised after encapsulation by an endosome. The uptake of toxin by host cells seems to be a clathrin-dependent mechanism (Papatheodorou *et al.*, 2010). While the endosome acidifies, the toxin alters in structure thereby exposing the hydrophobic pore domain which subsequently inserts itself within the endosomal membrane leading to the translocation of the protease and glucosyltransferase domains into the cytosol (Reineke *et al.*, 2007). The binding of inositol, a eukaryotic signalling molecule activates the protease domain resulting in proteolytic release of the amino-terminal glucosyltransferase domain into the host cell cytosol, with the rest of the toxin polypeptide remaining attached to the membrane (Reineke *et al.*, 2007; Pruitt and Lacy, 2012). The glucosyltransferase is eventually able, after a cascade of reactions, to inhibit the small GTPases ultimately leading to increased membrane permeability, loss of barrier function and cellular death.

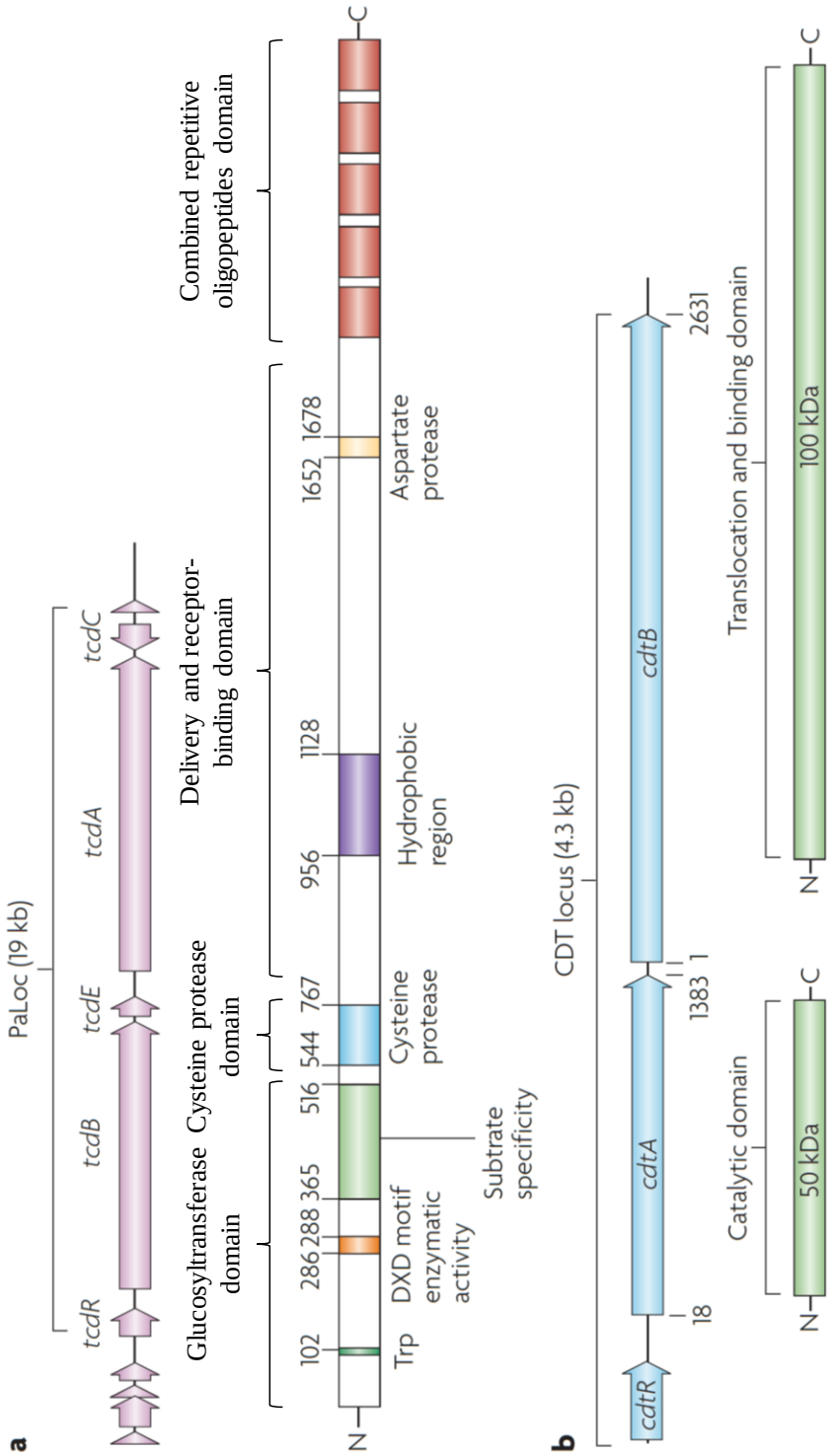


Figure 1.3. *C. difficile* toxins.
a. TcdA (308 kDa) and TcdB (269 kDa) are encoded on the pathogenicity locus**.** In non-toxigenic strains, this region is replaced by a short 115 bp sequence. Both toxins are single chain polypeptides with different functional domains.
b. The binary toxin CDT is encoded on the CdtLoc pathogenicity locus, comprised of the proteins, CdtA and CdtB. CdtA has enzymatic activity while CdtB has a binding function. (Modified from Rupnik, 2009 and Chen *et al.*, 2019).

The binary toxin is the least studied of the three *C. difficile* toxins (Gerding *et al.*, 2014). CDT is frequently observed in strains of *C. difficile* linked to increased severity of CDI. CDT has also been shown to be cytotoxic to Vero cells *in vitro* (Perelle *et al.*, 1997). Nevertheless, studies have demonstrated that *tcda*$^{-}$ *tcdb*$^{-}$ *cdt*$^{+}$ *C. difficile* strains cause no symptoms in hamsters leading many to the conclusion that CDT plays no role in virulence (Perelle *et al.*, 1997; Geric *et al.*, 2006). A more recent finding has cast some doubt on CDT's lack of effect in animals, where 3 of 9 animals infected by an A-B-C+ isogenic 027 strain succumbed to disease. Although the deceased hamsters did not display the typical symptoms of CDI, haemorrhaging and inflammation was observed in the small intestine implicating a role for CDT in *C. difficile* virulence. The binary toxin comprises two separate toxin components; CdtA, an ADP-ribosylating enzyme able to modify actin and CdtB which is able to bind to host cells and translocate CdtA into the cytosol (Gerding *et al.*, 2014). CDT is encoded on the binary toxin locus, CdtLoc, alongside the regulatory *cdtR* gene (Perelle *et al.*, 1997).

1.2.4.2 S-layer proteins

C. difficile vegetative cells are encapsulated by an external proteinaceous layer called the S-layer (Calabi *et al.*, 2001). S-layers form a regularly arranged lattice above the peptidoglycan cell wall of many Gram-positive bacteria. *C. difficile* is unusual in that it expresses two S-layer proteins (SLPs) which vary in size among different strains, and arise due to post-translational cleavage of a single precursor, SlpA (Eidhin *et al.*, 2006). The two SLPs exist as high and low molecular weight species which self-assemble on the cell exterior. Although the exact role of the *C. difficile* S-layer in virulence is yet to be fully understood, several experiments have provided evidence as to how it may affect CDI pathogenicity. SLPs have been shown to play a role in adhesion to enteric cells (Calabi *et al.*, 2001). This observation was complemented by a study demonstrating the capability of isolated SLPs to inhibit binding of *C. difficile* to Caco-2 cells (Merrigan *et al.*, 2013). Purified SLPs from *C. difficile* have been shown to elicit the release of pro-inflammatory and regulatory cytokines from human monocytes, increase T-cell

proliferation and cause the maturation of dendritic cells (Ausiello *et al.,* 2006). In *C. fetus* the S-layer is able to avoid the immune system and phagocytic digestion, providing a possible function for the *C. difficile* S-layer (Sára and Sleytr, 2000). A recent study using a mouse model has demonstrated that SLPs cause maturation of bone marrow derived dendritic cells, which play a key role in cytokine production and helper T-cell induction (Ryan *et al.,* 2011). The study went on to show that SLPs activate innate and adaptive immunity via Toll-like receptor 4 (TLR4) mediation, and in TLR4-defient mice CDI is more severe thereby providing evidence that SLPs play a key role in the immune system's recognition of *C. difficile* (Ryan *et al.,* 2011). It has recently been suggested that S-layers from different ribotypes, in addition to having different sequences also elicit different immune responses (Lynch *et al.,* 2017). S-layers from ribotype 027 and 078 were shown to be able to induce a more potent macrophage and cytokine response when compared to ribotypes 001 and 014 (Lynch *et al.*, 2017). The authors posit that the differing S-layer proteins may play a role in driving the emergence of hypervirulent strains.

1.2.5 Rise of community acquired infections

The Infectious Disease Society of America define CDI as community acquired if it satisfies the following requirements; if symptoms occur in the community or within 48 hours of hospital admission, or after 12 weeks following hospital discharge (McDonald *et al.,* 2007). Incidences of community-acquired *C. difficile* infections (CA-CDI) have almost doubled in the past decade and severity is closely linked to hypervirulent ribotypes 027 and 078, with poor outcomes (Chitnis *et al.,* 2013; Lewis *et al.*, 2016). Data from Europe and North America estimate that CA-CDI comprise 20-27% of diagnosed CDI cases (Centers for Disease Control and Prevention (CDC), 2005; Wilcox *et al.*, 2008). The rise in incidences of CA-CDI is of particular concern to healthcare professionals, especially as it often coincides with increased infection rates among those previously viewed as low risk, such as the young (McDonald *et al.,* 2007). CA-CDI is not associated as strongly with antibiotic treatment as is nosocomial CDI, and as a consequence it appears to present in patients as less severe (Gupta *et al.,* 2014). Although infection is often

less acute, CA-CDI can nevertheless prove fatal (Taori *et al.*, 2014). A 2012 study focussing on community acquired infections found that 40% of patients needed hospitalisation, 20% suffered from severe infection, 20% experienced treatment failure and 28% relapsed (Khanna and Pardi, 2012). Whole genome sequencing performed in Oxfordshire, UK from 2007 until 2011 showed that less than 25% of diagnosed CDI cases had originated from another patient, implicating transmission from a community source (Eyre *et al.*, 2013). So, although CDI is rightfully considered primarily a nosocomial infection, it cannot be considered primarily a nosocomial transmitted infection.

1.3 The spore

1.3.1 Spore structure and function

Bacterial endospores (spores) are dormant, highly resistant, non-reproductive structures produced by bacteria within the Firmicutes phylum (de Hoon *et al.*, 2010). Spore-forming bacteria are able to initiate sporulation in response to adverse environmental conditions, such as nutrient limitation, and are then able to germinate into vegetative cells once conditions become favourable (de Hoon *et al.*, 2010). Sporulation can therefore be considered as a long-term survival mechanism, allowing the bacterium to endure conditions the vegetative cells would otherwise not be able to. When vegetative *C. difficile* cells start replicating in the GI tract, a subset of the population will initiate sporulation. This developmental process generates the metabolically dormant, highly resistant spores that are essential for *C. difficile* to survive excretion from the host. Infected hosts shed large amounts of infectious spores that provide an environmental reservoir for *C. difficile* (Lawley *et al.*, 2009).

The formation of spores by *C. difficile* is therefore integral to the pathogenesis of CDI. With *C. difficile* being an obligate anaerobe, it is the role of the spore to transmit and disseminate CDI between hosts (Jump *et al.*, 2007). *C. difficile* strains which are unable to form spores have been shown to be incapable of persisting within the GI tract and of being horizontally transmitted (Deakin *et al.*, 2012). This

implies that *C. difficile* spores are the sole agent of CDI transmission. Persistence of spores within the GI tract also plays a role in recurrent CDI where a small population of spores that do not germinate during infection are able to survive antibiotic treatment. This residual and persistent population of spores can subsequently germinate and cause disease following the cessation of treatment (McFarland *et al.*, 2002).

Spores are dehydrated and metabolically dormant, and the chromosome is encased in a thick proteinaceous shell (the spore coat). These features make the spore robust, enabling it to be resistant to antibiotics (Baines *et al.*, 2009), disinfectants (Vohra and Poxton, 2011), the host's immune system (Paredes-Sabja and Sarker, 2012b), alcohol (Jabbar *et al.*, 2010), heat and ionizing radiation (Setlow, 2006). Thus, spores are able to survive in the environment for long periods of time prior to infecting a host, germinating into vegetative cells, outgrowing and producing more spores. High numbers of spores are then shed into the environment from the host's faecal matter and the cycle continues.

Transmission electron microscopy (TEM) has revealed that *C. difficile* spores are encapsulated by an exosporium (Panessa-Warren *et al.*, 1997). The exosporium is a loose-fitting structure enveloping the spore and is comprised of various proteins and glycoproteins (Lawley, Croucher, *et al.*, 2009). Of particular interest are the glycoproteins BclA1, BclA2 and BclA3 (Lawley, Croucher, *et al.*, 2009) and the cysteine rich proteins CdeC and CdeM (Calderón-Romero *et al.*, 2018). A *C. difficile* mutant lacking BclA1 has been shown to be less effective at colonising and causing infection within a host implicating this glycoprotein in the initial, pre-spore germination stages of infection (Phetcharaburanin *et al.*, 2014). *bclA1* mutants demonstrate increased germination rates, suggesting that germinants can access the spore more freely due to destabilisation of the exosporium, potentially decreasing spore hydrophobicity and therefore its adhesive properties. CdeC is one of the most abundant proteins found on *C. difficile* spores (Lawley, Croucher, *et al.*, 2009; Díaz-González *et al.*, 2015). CdeC is believed to form a protective layer around the spore, and the absence of the protein leads to increased spore coat permeability,

alongside increased susceptibility to heat, ethanol and phagocytic cells (Barra-Carrasco *et al.*, 2013; Calderón-Romero *et al.*, 2018). CdeM is highly abundant on the spore exosporium and has no known homology to any other protein (Janoir *et al.*, 2013; Díaz-González *et al.*, 2015). The exclusivity of CdeM to *C. difficile*, in addition to its high accessibility on the spore surface makes it a potential candidate as an antibody target for a vaccine, and a mouse infection model using the *C. difficile* UK1 strain has confirmed this hypothesis (Ghose *et al.*, 2016). CdeM is upregulated principally during sporulation and has been shown to be highly expressed throughout infection in experiments using axenic mice (Janoir *et al.*, 2013). A study comparing the fitness of an isogenic *cdeM* mutant to a wild-type strain suggested that CdeM may have a role to play in colonisation and persistence of *C. difficile* spores in a CDI mouse model (Janoir *et al.*, 2013).

The exact function of the exosporium remains unclear however, but it has been suggested that it may play a role in spore persistence and in the adhesion of spores to surfaces (Setlow, 2006). Removal of the exosporium layer by sonication or proteases has been shown to reduce spore hydrophobicity (Escobar-Cortés *et al.*, 2013). Interestingly, a *C. difficile cdeC::erm* mutant, unable to assemble an exosporial layer, demonstrated increased levels of adhesion to epithelial cells, mucin, fibronectin and vitronectin (Mora-Uribe *et al.*, 2016). This translated to an increase in fitness of the *cdeC* mutant compared to wild-type in a mouse infection model (Calderón-Romero *et al.*, 2018). A possible explanation may be gleaned from somewhat contradictory observations; *cdeC* mutants show lower levels of adhesion to a healthy colonic mucosa, yet higher levels to a damaged mucosa. During CDI, toxins and inflammation inflict damage upon the mucosa, including tight junction disruption and epithelial erosion, thereby remodelling it and exposing an increased number of alternative binding sites such as vitronectin and fibronectin. So although CdeC may contribute to higher adhesion to healthy colonic tissue, it seems that in the case of tissue damage mediated by infection an absence of CdeC leads to greater persistence within the host (Calderón-Romero *et al.*, 2018). A possible caveat to studies performed with *cdeC* mutants are the global changes enacted upon the surface of the spore thereby leading to experimentally unforeseen

and uncontrolled for consequences. As an example, in *cdeC* mutant spores increased levels of low molecular weight BclA1, 2 and 3 are detected and as described earlier BclA1 is strongly implicated in the pathogenesis and severity of CDI (Phetcharaburanin *et al.*, 2014).

Beneath the exosporium lies the spore coat. The *C. difficile* spore coat possesses lamaellae similar to those found in spores of *B. subtilis*, but despite this structural resemblance only 25% of the genes encoding spore coat proteins in *B. subtilis* have homologues in *C. difficile* (Henriques and Moran, 2007). Several spore coat proteins have been identified and characterised, including CotA, CotB, CotCB, CotD, CotE, SodA (Permpoonpattana *et al.*, 2011) and most recently CotL (Alves Feliciano *et al.*, 2018). CotA is believed to play a role in spore coat stabilisation and a *cotA* mutant exhibits major structural defects in spore assembly, while displaying increased sensitivity to ethanol and lysozyme (Permpoonpattana *et al.*, 2011). CotB and CotE contain orthologues in other members of the Bacilli and Clostridia (Permpoonpattana *et al.*, 2011). CotCB and CotD are putative manganese catalases and share 70% homology with each other, and with the *B. subtilis* spore coat protein CotJC (Permpoonpattana *et al.*, 2011). While *cotD* mutants show a significantly greater reduction in catalase activity when compared with *cotCB* mutants, activity is not completely abolished suggesting functional redundancy (Permpoonpattana *et al.*, 2013). CotE is a novel, bifunctional protein with peroxiredoxin activity at the amino-terminus, and chitinase activity at the carboxy-terminus (Permpoonpattana *et al.*, 2011). SodA carries superoxide dismutase (SOD) activity and may be involved in polymerising spore coat monomers via oxidative cross-linking, which in turn may provide a role for CotE's peroxiredoxin as a reducer of the SOD by-product, hydrogen peroxide (Permpoonpattana *et al.*, 2013). The chitinase activity carried by CotE has been hypothesised to be involved in macromolecular turnover and the production of nutrients for use by vegetative cells after germination of the spore (Permpoonpattana *et al.*, 2011). CotL is the most recently discovered of the *C. difficile* spore coat proteins. CotL is essential for coat assembly with mutant spores carrying major morphological defects, resulting in the reduction and incorrect localisation of several spore coat proteins (Alves Feliciano *et al.*, 2018). In addition to this, *cotL* mutant spores displayed higher sensitivity to

lysozyme and showed germination impairment, providing further evidence in support of CotL being a *C. difficile* morphogenetic spore coat protein (Alves Feliciano *et al.*, 2018).

An outer membrane exists directly below the spore coat. This membrane forms after spore engulfment by the mother cell during sporulation (as described in Section 1.3.2). Although the outer membrane contains a number of lipids and proteins the precise role of the outer membrane for the dormant spore has not yet been elucidated (Setlow, 2006; Setlow and Johnson, 2013; Loison *et al.*, 2016). Beneath the outer membrane is the cortex, made of peptidoglycan (PG). Precursors made by the mother cell are transported into the intermembrane space and used to synthesise the cortex peptidoglycan (Meador-Parton and Popham, 2000). Interestingly, the spore cortex peptidoglycan is structurally different to that of the vegetative cell, for example, it contains fewer peptide linkages (Popham *et al.*, 1996; Popham, 2002). The cortex plays a role in the heat resistance of spores and it has been proposed to play an active role in the attainment and maintenance of spore dehydration, a crucial attribute which provides spores with their characteristically high resistance to the environment (Gaylarde, 2002; Popham, 2002). The cortex may also play a role in germination and outgrowth (Meador-Parton and Popham, 2000).

Beneath the *C. difficile* spore cortex lies the germ cell wall (GCW). The GCW is comprised of peptidoglycan, but unlike the cortex, this PG is similar to that of the vegetative cell. The GCW is not degraded during germination and serves as the cell wall of the germinating *C. difficile* spore (Atrih *et al.*, 1998). Beneath the GCW lies a second membrane, the spore's inner membrane. The inner membrane contains germination receptors and therefore plays an integral role in spore germination (Cowan *et al.*, 2004; Zheng *et al.*, 2016). The most extensively studied are the highly conserved GerA family of germination receptors, however *C. difficile* has no orthologue and instead responds to germinants via the unique pseudoprotease CspC (Moir, 2006; Francis, Allen, Shrestha, *et al.*, 2013; Setlow, 2014). It has been suggested that interactions between germinants and their receptors alters the permeability of the inner membrane, resulting in an efflux of ions (Setlow, 2014).

The inner membrane also serves as a permeability barrier to damaging chemicals (Setlow and Setlow, 1980; Swerdlow *et al.*, 1981). The inner most layer of the *C. difficile* spore is the core. The core contains all the genetic material, but due to spore dormancy no damage can be repaired, and so protection of DNA is of utmost importance. The most important contributing factors to spore resistance is the low water content, saturation of DNA with small acid soluble spore proteins (SASPs), and the high concentration of dipicolonic acid (DPA). SASPs alter the photochemistry of spore DNA in response to UV, minimising the risks of DNA damage. The high levels of DPA (25% of core dry weight) is chelated with Ca^{2+} and is released upon germination and so is often used as a direct correlate of germination. DPA plays a key role in core dehydration, contributing to the heat and desiccation resistance exhibited by spores (Setlow, 2006).

1.3.2 Sporulation pathway

The process of sporulation, of a vegetative cell entering a metabolically dormant state requires extensive morphological alterations and unsurprisingly is highly regulated, with an estimated 500 genes being involved in the process (Galperin *et al.*, 2012). It remains unclear what signals are required for *C. difficile* vegetative cells to initiate the sporulation process, however in *Bacillus* low nutrient availability and high cell density are essential for initiation of unicellular development (Rao and Higgins, 2016). Recently, RstA was reported as a novel positive regulator involved in the sporulation of *C. difficile* (Edwards *et al.*, 2016). RstA encourages initiation of sporulation through its peptide-interacting domain, while negatively regulating motility and toxin production (Zhu *et al.*, 2018). The authors suggest that RstA may be a global transcriptional regulator, but a detailed pathway has not yet been fully elucidated (Edwards *et al.*, 2016). In some *Bacillus* and *Clostridium* species it has been demonstrated that initiation of sporulation is regulated by several histidine kinases in response to environmental cues resulting in phosphorylation of the master transcriptional regulator Spo0A (Hoch, 1993). Phosphorylated Spo0A acts as both a transcriptional repressor (for other sporulation repressor genes such as *arbB* (Strauch, 1993)) and activator (for other sporulation promoter genes such as *spoIIG* (Satola *et al.*, 1992)). Once phosphorylated Spo0A reaches a threshold

level within the cell, the equilibrium between genes repressed or activated by Spo0A shifts, thereby committing the cell to sporulation (Chung *et al.*, 1994). Spo0A and the histidine kinases are conserved in the *C. difficile* genome, however sporulation regulation is different to that of *B. subtilis* (Underwood *et al.*, 2009). Absence of Spo0A in *C. difficile* mutants prevents the formation of spores, while a mutant devoid of the histidine kinase CD2492 was shown to have a decreased sporulation capacity suggesting that other kinases may be phosphorylating Spo0A. The absence of the *B. subtilis* proteins responsible for the transfer of a phosphoryl group from the histidine kinases to Spo0A (Spo0F and Spo0B) seems to suggest that in *C. difficile* the kinases phosphorylate Spo0A directly, and recent *in vitro* evidence supports this hypothesis (Underwood *et al.*, 2009). Currently, only one *C. difficile* histidine kinase has been shown to autophosphorylate and transfer a phosphoryl group directly to Spo0A; CD1579 (Underwood *et al.*, 2009). It is yet to be determined whether the remaining histidine kinases can directly phosphorylate Spo0A.

Spo0A functions due to its regulation of sporulation-specific RNA polymerase sigma factors, specifically SigE, SigF, SigG and SigK (Fimlaid and Shen, 2015). The sporulation regulatory pathway of *C. difficile* is as follows; (1) Immediately after polar septation, SigF is activated in the forespore when anti-sigma factor SpoIIAB binds to unphosphorylated anti-anti-sigma factor SpoIIAA; (2) SigF activity results in SpoIIR expression, which interacts with the protease SpoIIGA resulting in cleavage of pro-SigE yielding active SigE; (3) Active SigF and SigE, within the forespore and mother cell respectively, cause mother cell engulfment of the forespore; (4) SigE activity results in SpoIIIA-H expression, which via interaction with SigF regulated SpoIIQ forms a channel within the inner and outer forespore membranes. SpoIIIAH and SpoIIQ interact within the intermembrane space after localisation to the asymmetric septum and engulfing membranes; (5) SigE regulated SpoIIID activates SigK in the mother cell (Haraldsen and Sonenshein, 2003; Pereira *et al.*, 2013; Paredes-Sabja *et al.*, 2014; Fimlaid and Shen, 2015; Zhu *et al.*, 2018). Although many factors that control spore formation are shared between *B. subtilis* and *C. difficile*, there are differences. *C. difficile*

produces mature SigK rather than pro-SigK, SigK is responsible for germinant receptor transcription while SigG is in *B. subtilis*, SigE and SigG are dispensable in their activation of SigG and SigK respectively, while SigG can also be activated prior to SigE and SigK (Pereira *et al.*, 2013; Fimlaid and Shen, 2015). These differences indicate that *C. difficile* sigma factor activation is less tightly controlled in comparison to *B. subtilis.*

At the beginning of sporulation, the vegetative cell divides asymmetrically to form a smaller forespore within the larger mother cell separated from each other by a septum. Following this, prespore chromosome segregation takes place ensuring that the prespore carries a full chromosome. Mother cell engulfment swiftly follows, in which the cell wall of the septum between the mother cell and forespore is degraded and the mother cell engulfs the forespore resulting in a prespore within the mother cell cytoplasm encapsulated within two membranes. The spore structures (as described in Section 1.3.1) assemble onto the prespore, followed by the subsequent lysis of the mother cell leading to the release of the mature spore (Errington, 2003). In *B. subtilis*, this entire process takes approximately eight hours (Driks, 2002). In *C. difficile,* sporulation is estimated to take approximately 120 hours to complete (Burns and Minton, 2011).

1.3.3 Germination pathway

Induction of bacterial spore germination is triggered when receptors detect specific germinants and the subsequent outgrowth returns the spore to its vegetative state. In *B. subtilis* the known germinant receptors include GerA, GerB and GerK but these do not appear in the *C. difficile* genome suggesting that a different set of environmental cues are required (Sorg and Sonenshein, 2008). It has been recognised that bile acids are required for *C. difficile* spore germination since the early 1980's (Wilson, 1983). Bile acids are steroid acids synthesised in the liver, stored in the gall bladder and secreted into the small intestine where they facilitate the digestion and absorption of fats. The liver synthesises cholic acid and chenodeoxycholic acid, which can be modified in the GI tract by conjugation to taurine or glycine resulting in a more diverse range of bile salts such as glycocholate

and taurocholate (Sorg and Sonenshein, 2008). Work by Sorg and Sonenshein (2008) has demonstrated that *C. difficile* spores germinate in response to cholate and its derivatives (taurocholate, glycocholate and deoxycholate) with L-glycine acting as a co-germinant. Histidine has also been found to act as a co-germinant in combination with taurocholate and glycine (Wheeldon *et al.*, 2011). It has however been shown that some *C. difficile* strains are able to germinate in rich media without the presence of bile acids, implying that the mechanism of germination receptors is complex (Heeg *et al.*, 2012). Chenodeoxycholate acts as an inhibitor of germination by acting as a competitor of taurocholate and other cholic acid derivatives (Sorg and Sonenshein, 2009).

A recent study by Francis *et al.* (2013) identified CspC as the germinant receptor that recognises bile acids in *C. difficile.* CspC is one of three serine-like proteases packaged into the spore, along with CspA and CspB. Both CspA and CspC encode an incomplete catalytic triad, but despite this loss of activity both are essential for spore germination (Adams *et al.*, 2013). Disruption of CspC using Targetron (Heap *et al.*, 2007) (a system for insertional mutagenesis) prevents spore germination, while a specific SNP mutation (G457R) alters germination specificity allowing germination in the presence of chenodeoxycholate, demonstrating the importance of CspC as a germinant receptor in *C. difficile* spores (Francis, Allen, Shrestha, *et al.*, 2013). CspC is required for Ca-DPA release from spores in response to the presence of glycine and taurocholate, but whether this is due to direct activation of DPA channels or activation of cortex hydrolysis, the mechanism remains unclear (Francis, Allen, Shrestha, *et al.*, 2013). CspA plays a functionally essential role for spore germination, by regulating the levels of CspC in the developing spore (Francis, Allen, Shrestha, *et al.*, 2013; Kevorkian *et al.*, 2016). The only receptor with an intact catalytic triad is CspB, and it has been suggested that it converts pro-SleC into its active, cortex-degrading form (Kevorkian *et al.*, 2016). CspA and CspB are encoded together as a *cspBA* operon in *C. difficile* (Adams *et al.*, 2013).

As is also the case in *C. perfingens,* degradation of the *C. difficile* spore cortex during germination requires the presence of Csps and SleC (Paredes-Sabja *et al.*,

2009b, 2009a; Burns *et al.*, 2010; Adams *et al.*, 2013; Francis, Allen, Shrestha, *et al.*, 2013). However, the signalling pathway resulting in SleC activation differs greatly between these species. In *C. difficile*, during spore formation the CspBA fusion protein undergoes interdomain cleavage resulting in the SleC dependent release and incorporation of CspB into the spore (Adams *et al.*, 2013). CspB then converts pro-SleC into mature SleC. It is hypothesised that CspC activates CspB upon interaction with bile salt germinants. Subsequently, CspB activates SleC by cleaving the N-terminal pro-sequence which then proceeds to hydrolyse the cortex. Cortex degradation leads to the detection of osmotic swelling by the mechanosensing protein SpoVAC within the inner membrane, which next triggers the release of Ca-DPA from the core (Velásquez *et al.*, 2014; Donnelly *et al.*, 2016; Francis and Sorg, 2016). Inactivation of CspC or SleC prevents cortex degradation and Ca-DPA release. The CspC dependent cortex degradation and subsequent Ca-DPA release is the reverse of what happens in *B. subtilis*, where germination occurs from inside-to-out as opposed to outside-to-in as is the case with *C. difficile* (Francis and Sorg, 2016). Once DPA is released from the spore cortex, water replaces it and the hydration of the spore core increases. Following hydrolysis further core hydration results in swelling and germ cell wall expansion. After the water content reaches a threshold level metabolic activity initiates and the spore continues its outgrowth culminating in the vegetative state. The whole process takes approximately 180 min. *in vitro* (Dembek *et al.*, 2013). A depiction of the germination-sporulation cycle is shown in Figure 1.4.

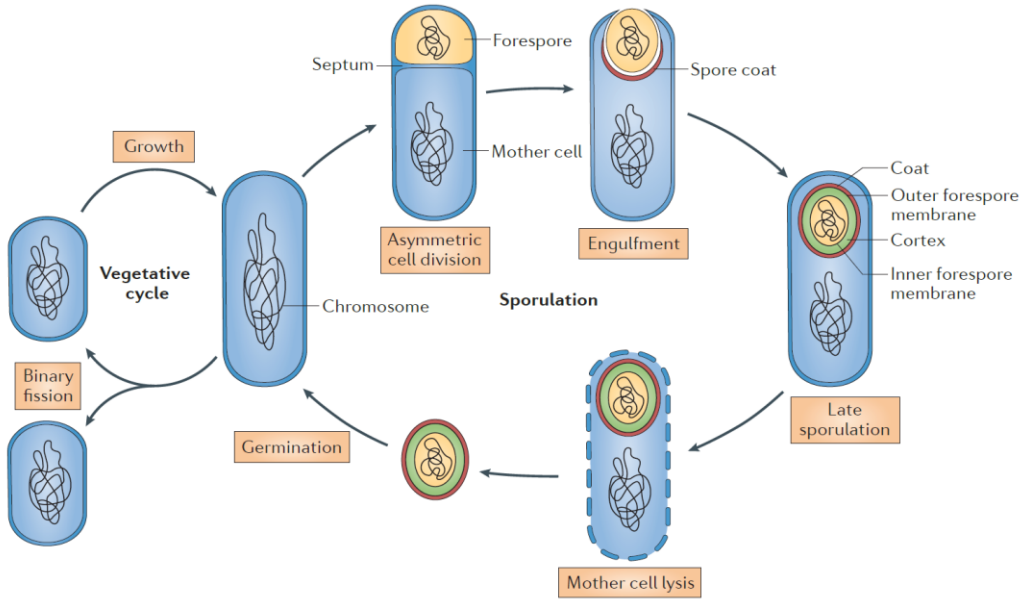


Figure 1.4. The sporulation and germination cycle. Under unfavourable conditions, a vegetative cell may initiate sporulation in order to enter a metabolically dormant, resilient state. Sporulation begins with asymmetric division into a forespore and a mother cell, followed by forespore engulfment by the mother cell resulting in a free prespore within the mother cell cytoplasm. Assemblage of spore structures, and finally mother cell lysis resulting in release of the mature spore completes the process. The spore may then germinate into a vegetative cell and restart the cycle. (Modified from McKenney, Driks and Eichenberger, 2013).

1.4 Control and prevention of CDI

1.4.1 Diagnosis

Due to the methodological issues in diagnosing CDI, the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) recommends a two-step algorithm beginning with either a NAAT or GDH-EIA (Crobach *et al.*, 2016). If the tests come back negative, the patient is deemed CDI negative, but if positive the result is confirmed with a toxin ELISA to yield a positive diagnosis for CDI (Crobach *et al.*, 2016). This two-step algorithm reduces the shortcomings inherent to any individual test.

The primary method used for the diagnosis of CDI is either the isolation of *C. difficile* spores (toxigenic culture-TC) or the detection of toxins from faeces (Kuipers and Surawicz, 2008; Williams and Spencer, 2009). Other methods include glutamate dehydrogenase (GDH) enzyme immunoassays (GDH-EIA) and nucleic acid amplification tests (NAAT). An effective diagnosis combines the presence of

symptoms (e.g. diarrhoea, abdominal pain, fever) with a positive laboratory test (Debast *et al.*, 2014). As the symptoms of CDI also present in many other common illnesses, an accurate case history of the patient is important; symptoms presenting in patients considered high risk such as the young, the elderly and those undergoing antibiotic therapy are considered to more likely be CDI related (Debast *et al.*, 2014; Crobach *et al.*, 2016). Toxins are detected and quantified either using cell cytotoxicity neutralisation assays (CCNA) or with the use of immunodiagnostics, by which ELISA is the primary method (Debast *et al.*, 2014; Peng *et al.*, 2018). Although TC and CCNA are considered to be the gold standard, both methods have limitations. Both methods are time consuming, labour intensive and expensive while TC has low specificity for clinical disease due to its inability to distinguish between toxigenic and non-toxigenic strains of *C. difficile* (Peng *et al.*, 2018). CCNA is analytically sensitive for toxin B, however interpretation of the data is subjective leading to poor reproducibility between laboratories. They are however favourable compared to GDH-EIA, which are not specific due to the commonality of GDH in non-toxigenic Clostridial species (Surawicz *et al.*, 2013). NAATs have excellent specificity and sensitivity, simplified data reporting, and are rapid. NAATs are therefore considered the most cost-effective method of diagnosing CDI (Schroeder *et al.*, 2014). NAATs target the encoding genes of TcdA and TcdB, resulting in their main disadvantage; no differentiation between active toxin production and *C. difficile* colonisation without the production of toxin (Dunwoody *et al.*, 2018).

1.4.2 Prevention

1.4.2.1 Hospital management and hygiene

Since the turn of the century and the spate of CDI outbreaks, considerable effort has been made to improve the conditions of hospital environments as a means of controlling CDI. Although these simple preventative measures draw upon the 16th Century germ theory of disease, a theory brought into practice by Joseph Lister in the 1870s through the use of aseptic surgical techniques, it would not be until 2004

that the UK government started the "Cleanyourhands" campaign leading to a decrease in the CDI rate (Stone *et al.*, 2012).

Studies in mice have demonstrated the ease and rapidity of CDI transmission and so the implementation of standardised regimens of glove use, disinfection, hand washing and education within hospitals was paramount (Department of Health and the Health Protection Agency, 2008; Lawley *et al.*, 2009). Due to the high number of spores excreted in faeces by patients during the 'supershedding' state of CDI the environment can become inundated with highly resistant spores, significantly increasing the probability of nearby susceptible patients developing the disease. Alcohol gel hand rub has been shown to be ineffective at removing *C. difficile* spores, while washing hands with soap and water has been shown to correlate with a decrease in CDI cases (Stone *et al.*, 2012). In mice, disease persistence only requires 7 spores/cm^2 and spores are able to persist on surfaces for at least five months, highlighting the importance of keeping equipment and surfaces clean (Gerding *et al.*, 2008; Stabler *et al.*, 2009). Due to the chemically resistant properties of *C. difficile* spores, only hydrogen peroxide and chlorine disinfectants have been demonstrated to destroy spores (Gerding *et al.*, 2008). Interestingly, recent work provides evidence for the use of alcohol-based sanitisers containing taurocholic acid to induce germination thereby rendering spores susceptible to disinfection (Nerandzic and Donskey, 2017).

1.4.2.2 Probiotics

CDI is the result of a perturbed microbiota, so the ingestion of live bacteria (probiotics) as both a prophylaxis and a treatment holds promise. The number of bacteria inhabiting the GI tract is estimated to equal the number of human cells in an entire human body (~$1x10^{13}$) (Sender *et al.*, 2016). The healthy adult microbiota is diverse, containing as many as 1000 different species all interacting with each other and their host to reach an equilibrium. Antibiotic treatment decimates this gut population, significantly reducing diversity and total numbers potentially causing symptoms such as diarrhoea, nausea and stomach cramps (Phillips, 2009). There therefore appears to be a niche for the ingestion of probiotics to replenish lost

numbers and prevent opportunistic pathogens from causing illness during this period of a weakened microbiome. The lack of current widespread clinical use may be attributed to an absence of standardisation across trials and a superficial understanding of what makes a good probiotic (Hell *et al.*, 2013). Additionally, it remains unclear as to the exact mechanism by which commensal bacteria prevent colonisation by *C. difficile.* Hypotheses include competition for binding sites, direct inhibition by antimicrobials/bacteriocins, bile acid modification and competition for food (Borriello, 1990; Dubois *et al.*, 2019; Buffie *et al.*, 2015; Collins *et al.*, 2018; Hryckowian *et al.*, 2018) .

There have been numerous studies in mice and hamsters showing the efficacy of probiotics in the reduction of incidences and severity of CDI. *Lactobacillus salivarius, Lactobacillus reuteri, Lactococcus lactis* and *Bacillus clausii* secrete compounds which directly inhibit *C. difficile* and have been shown to partially protect against CDI (Corr *et al.*, 2007; Rea *et al.*, 2007; Mills *et al.*, 2018). *L. reuteri* produces the *C. difficile* toxic antimicrobial reuterin via glycerol fermentation and the bacterium has been shown to have a high level of resistance against vancomycin, metronidazole and fidaxomicin making it an attractive treatment option for patients receiving antibiotic therapy (Spinler *et al.*, 2017). *B. amyloliquefaciens* has also been shown to have direct inhibitory action against *C. difficile,* and dosing vegetative cells to mice conferred protection against CDI (Geeraerts *et al.*, 2015). Mice dosed with *B. amyloliquefaciens* lost less weight, had significantly lower toxin A and B, and displayed less colon damage when compared to naïve and *S. boulardii* treated mice. Human trials have been conducted with the yeast *S. boulardii* (McFarland *et al.*, 1994; Surawicz *et al.*, 2000), *Lactobacillus rhamnosus* GG (Pochapin, 2000), *Lactobacillus acidophilus* and *Bifidobacterium bifidum* (Plummer *et al.*, 2004) and *Lactobacillus plantarum* 299v (Wullt *et al.*, 2003). These studies all demonstrate a significant effect on the incidence and relapse of CDAD when used in conjunction with antibiotics (Gougoulias *et al.*, 2007). Mice pre-fed with *S. boulardii* show enhanced intestinal IgA responses to orally dosed Toxoid A, providing a possible mechanism of protection (Qamar *et al.*, 2001). A recent double blind, randomised controlled trial study assessed the

effectiveness of *S. boulardii* at preventing primary AAD in hospitalised patients receiving antibiotic therapy (Ehrhardt *et al.*, 2016). The study included 477 participants but failed to find an effect, although this could be due to the study being underpowered because of fewer than anticipated patients. A phase 2, probiotic adjunct trial found that an oral probiotic capsule containing a cocktail of *Lactobacillus acidophilus* NCFM, *Lactobacillus paracasei* Lpc-37, *Bifidobacterium lactis* Bi-07 and *B. lactis* Bl-04 significantly improved diarrhoea outcomes (Barker *et al.*, 2017). There was however no reduction in CDI recurrence or functional improvement over time and the authors suggest that a larger scale randomised controlled study is necessary.

Another interesting approach was recently shown by Buffie *et al.* (2015), who used 16S rRNA sequencing in mice treated with various antibiotics to identify specific taxa correlated with resistance to CDI. The organism providing the greatest protection- *C. scindens*, conferred resistance by synthesising secondary bile acids through the action of its 7α-dehydroxylase enzyme (for more details see Section 1.5.3). The study showed that upon administration to mice, *C. scindens* increases resistance to CDI in a secondary bile salt dependent manner. This suggests that the synthesis of secondary bile acids may play an integral role in microbiota-conferred CDI resistance. This modulation of secondary bile acid levels could be accomplished by oral administration of bacteria with 7α-dehydroxylase activity or by direct administration of secondary bile acids, with the latter having been shown to be successful in a single case of ursodeoxycholate treatment of CDI linked recurrent ileal pouchitis (Weingarden *et al.*, 2016).

1.4.2.3 Developing a vaccine

Vaccination holds great promise at severely diminishing the risk of CDI within high risk populations. However, as of yet a vaccine does not exist and most research effort have targeted toxins A and B and aim to elicit a long term, adaptive immune response (de Bruyn *et al.*, 2016; Chai and Lee, 2018). Pfizer is currently in phase 3 trials of their vaccines, but their data has not been published (NIH, 2017). Sanofi Pasteur developed a vaccine, “Cdiffense”, a formalin-inactivated TcdA and TcdB

vaccine shown to elicit a dose dependent IgG response (Anosova *et al.*, 2013). A recent Phase 3 clinical trial of Cdiffense was prematurely terminated due to a low probability of attaining its primary outcome (McKee, 2017). A Phase 2 trial by Valneva, termed VLA84, showed promising results, but they are yet to continue with their study (Valneva, 2015). A potential problem inherent to toxoid vaccines is their inability to prevent colonisation (Siddiqui *et al.*, 2012).

To prevent colonisation, it is considered essential to target the surface proteins of either the vegetative cell or the spore. A study by Pizarro-Guajardo *et al.* (2018) analysed the spore proteome of *C. difficile* strain R20291 and provided a list of five immunoreactive proteins; CotE, CotA, CotCB, CdeC and a cytosolic methyltransferase. There are yet to be any studies determining the efficacy of these spore coat protein targets *in vivo*. Antibodies to the flagellar proteins FliC and FliD, cell wall adhesin Cwp66, cell wall cysteine protease Cwp84 have all been found in patient sera indicating their potential as vaccine candidates (Pechine *et al.*, 2005). Cwp84 antigen showed promise, with vaccinated hamsters showing a 33% increase in survival (Péchiné *et al.*, 2011). Immunisation with the surface layer protein SlpA led to only a low antibody response in hamsters (Ní Eidhin *et al.*, 2008). The surface polysaccharides of *C. difficile* are also being considered as a potential vaccine candidate. PSII and LTA are found on the surface of all *C. difficile* strains as of now, and antibodies against both have been found in patient sera (Ganeshapillai *et al.*, 2008; Oberli *et al.*, 2011; Reid *et al.*, 2012; Martin *et al.*, 2013). Although polysaccharides are not able to elicit a T-cell response on their own, they can be conjugated to a carrier protein in order to do so. Both LTA and PSII Have been successfully conjugated to carrier proteins (Oberli *et al.*, 2011; Cox *et al.*, 2013). Work is underway to determine if these vaccine candidates can prevent colonisation or CDI. It is likely that an ideal vaccine would target both the surface antigens of *C. difficile* and the toxins, for maximal benefit.

1.4.3 Treatment

1.4.3.1 Antibiotics

The first line of treatment for CDI is antibiotic therapy. For primary and recurrent CDI, the Infectious diseases Society of America (IDSA) now recommends vancomycin or fidaxomicin over metronidazole (McDonald *et al.*, 2018). Vancomycin is a glycopeptide antibiotic that exerts its bacteriostatic effects against *C. difficile* by inhibiting cell wall synthesis (Reynolds, 1989). Two large, multicentre, randomised controlled trials both found vancomycin to be superior in treating any severity of CDI compared to metronidazole, with even more prominent differences in those suffering from severe disease (Johnson *et al.*, 2014). Fidaxomicin has been approved for treatment in several countries in the past few years. Fidaxomicin is a macrocyclic lactone antibiotic which inhibits the σ^{70} factor thereby inhibiting transcription of any gene dependent on this factor and exerting a bactericidal effect upon *C. difficile* (Tupin *et al.*, 2010). A metanalysis comparing fidaxomicin to vancomycin showed both to have equal clinical cure rates, although fidaxomicin may possibly be more effective at reducing persistent diarrhoea and death (Crook *et al.*, 2012). The relapse rates for fidaxomicin are also lower. This could possibly be due to its narrower spectrum of activity having less of an effect on the gut microbiota and/or its ability to prevent *C. difficile* sporulation (Babakhani *et al.*, 2012; Crawford *et al.*, 2012). A possible barrier to widespread fidaxomicin use is the high price; treatment with fidaxomicin costs twice as much as treatment with vancomycin (Simon, 2014).

1.4.3.2 Faecal microbiota transplantation (FMT)

Faecal microbiota transplantation (FMT) is a type of bacteriotherapy that focuses on replenishment of healthy gut microbiota from screened donors to recipients with a dysregulated microflora to re-establish a protective gut microbiome (Shahinas *et al.*, 2012). FMT has proved to be a very efficacious treatment for recurrent CDI, with success rates of ~90% (Bakken *et al.*, 2011). FMT involves the transplant of screened, processed faecal matter from a healthy donor to a diseased patient. A suggested mechanism for the beneficial effects of FMT in CDI patients rests on the

theory of bacterial interference; the use of probiotic bacteria to compete for the niche required by pathogenic bacteria to outgrow and cause disease (Khoruts and Sadowsky, 2016). Supporting evidence for this theory was presented in a publication by Hryckowian *et al.* (2018), where it was found that a diet high in microbiota-accessible carbohydrates (MACs) led to outgrowth of MAC-utilising taxa and an associated decrease in *C. difficile* fitness. Interestingly, the sterile faecal filtrate from a healthy donor was shown to have a similarly high efficacy compared to conventional FMT for CDI treatment suggesting that soluble components within the filtrate (such as antimicrobials, enzymes, bacteriophages) may be an integral part of FMT's efficacy, rather than the presence of the vegetative cells themselves (Ott *et al.*, 2017). This could provide a valuable alternative for immunocompromised patients, where the transfer of live bacteria may pose a risk. Valerate, a short-chain fatty acid with inhibitory effects on *C. difficile* is depleted during antibiotic therapy and while antibiotic cessation does not increase its level, FMT does elevate levels, implicating this metabolite in FMT efficacy (McDonald *et al.*, 2018). Recent studies have also demonstrated rapid, but sustained changes in the gut bacteriophage profiles following FMT; providing another possible mechanism, but more work in this area is needed (Draper *et al.*, 2018; Zuo *et al.*, 2018).

The possibility of identifying the microorganisms within FMT which are responsible for the efficacy, culturing them and delivering them to patients directly without the need for stool donors is an enticing prospect. A study by Lawley *et al.*, (2012) attempted this and identified a culturable cocktail of phylogenetically diverse bacteria from healthy mouse faecal samples that were capable of re-establishing a healthy gut microbiota and clearing CDI in mice. There have been several clinical studies investigating the efficacy of encapsulated FMT, and these studies are summarised in a recent meta-analysis (Iqbal *et al.*, 2018). The authors summarise that the efficacy of freeze-dried encapsulated FMT is comparable to that of conventional FMT in the treatment of recurrent CDI and is safe and cost effective. However, randomised-controlled trials are required to investigate possible adverse effects, and compare efficacy to current oral antimicrobial

treatments (Iqbal *et al.*, 2018). Further studies to ascertain the best route for administration are needed to allow for commercialisation and wider encapsulated FMT use.

The Infectious Disease Society of America (IDSA) now recommends treating with FMT after antibiotic failure for at least two recurrences of CDI (McDonald *et al.*, 2018). The only current barriers to the widespread and universal use of FMT include the need for trained personnel to administer treatment, the invasiveness of delivery and aesthetics. The incipient clinical introduction of encapsulated FMT would remove these barriers.

1.5 The gut microbiome

1.5.1 Role of the microbiota

The human gastrointestinal tract is a multifaceted system starting from the oral cavity, and extending through the stomach and the intestines, and terminating at the anus. The GI tract and the organisms which inhabit it perform a wide array of functions, as is shown in Figure 1.5. The GI tract lumen surface is covered by a thick mucus layer that acts as a protective barrier against the harsh internal environment. Due to the abundance of mucus, and its monomer mucin within the GI tract, the microbiota needs to interact with this mucosal barrier in a variety of ways so that its normal processes can be fulfilled (Turroni *et al.*, 2012). The composition, diversity and density of the microbiota varies along the GI tract, with major populations predominating in areas based on their performed functions and by their suitability for the local environment. Shortly after birth, bacteria from the mother and the nearby environment rapidly colonise the gut of the infant (Sommer and Bäckhed, 2013). The colonisation and establishment of the gut microbiota within the infant is vital for the health of the adult, and the normal function of the gastrointestinal tract (Turroni *et al.*, 2012). It takes approximately one to two years from birth for the gut microbiota to establish itself to resemble that of an adult (Sommer and Bäckhed, 2013). Contrary to previous thought, the gut flora does not merely maintain a commensal relationship with the host, but a mutualistic,

symbiotic one (Sears, 2005). Although humans (Steinhoff, 2005) and mice are capable of surviving without a microbiota, so named 'gnotobiotic mice' demonstrate an underdeveloped immune system, thinner intestinal walls, smaller hearts, liver and lungs resulting in diminished cardiac output and blood flow to organs and negative impacts on reproductive performance (Nicklas *et al.*, 2015).

Fungi, archaea, viruses and bacteria can all be found within the GI tract. The roles of viruses and fungi are yet to be fully elucidated, however they are implicated in host physiology and immunity in addition to seemingly playing a role in microbiota dynamics (Hillman *et al.*, 2017). The function of bacteria within the GI tract is better understood; with links to vitamin production, immune system enhancement and pathogen defence, histological development, ion absorption, the production of short chain fatty acids (SCFA) and metabolites via the fermentation of non-digestible foods (Guarner and Malagelada, 2003; Flint *et al.*, 2007; Hillman *et al.*, 2017; Corfield, 2018). Several members of the gut flora can produce SCFAs through fermentation; SCFAs stimulate the production of innate immune cells thereby increasing the resistance of the host to pathogens (Levy *et al.*, 2016). Research has discovered that imbalances of the gut microbiome are strongly associated with obesity (Ley, 2010).

Initial work on the microbiota hypothesised that its key function was metabolism of indigestible ingested compounds, development and maintenance of the intestinal epithelium and directly defending against pathogens. Contemporary study, has however focussed more on microbiota-mediated development of the immune system and the gut-brain axis (Wang and Kasper, 2014). Pathogens are directly inhibited by the microbiota through various mechanisms including competition for space/nutrients and the secretion of antimicrobials. When the microbiota is disrupted, opportunistic enteropathogens such as *C. difficile*, *Shigella*, *Staphylococcus aureus* and *Escherichia* can outgrow and cause disease (Yoon *et al.*, 2014). As the gut flora establishes itself, the intestinal epithelium and the secreted mucosal barrier develop which supports the microbiota by acting as an anchoring point while providing a barrier against pathogenic microorganisms

(Sommer and Bäckhed, 2013). The establishment of the gut microbiota also coincides with the development of gut-associated lymphoid tissue (GALT), which can detect and subsequently attack pathogens (Faderl *et al.*, 2015). Due to this concomitant establishment, GALT does not consider the gut flora, or their metabolites as threats (Sommer and Bäckhed, 2013). Specific bacterial species have been shown to stimulate the immune system and elicit the production of inflammatory and anti-inflammatory cytokines; *Bacteroides fragilis* and some *Clostridia* species elicit an anti-inflammatory response while segmented filamentous bacteria induce Th17 cell differentiation in the small intestine (Reinoso Webb *et al.*, 2016). *Bacteroidetes* and some *Clostridia* can induce regulatory T cells (Tregs) (Reinoso Webb *et al.*, 2016). Tregs can suppress the immune response, promote repair of the epithelium and enhance tolerance to microorganisms (Atarashi *et al.*, 2011; Maloy and Powrie, 2011). The gut microbiota is also able to regulate antibody production by promoting B cells to class switch to IgA via NF-kB signalling (Mantis *et al.*, 2011; Sommer and Bäckhed, 2013). IgA plays an important maintenance and regulating role in the gut and can diversify the gut flora and help in the removal of unwanted pathogenic bacteria via immune exclusion (Mantis *et al.*, 2011).

The gut microbiota also plays an important role in the metabolism of the host. In humans, the gut flora is responsible for the breakdown of indigestible polysaccharides, such as plant cellulose, sugars, certain fibres and starches (Clarke *et al.*, 2014). It has been demonstrated that gnotobiotic mice require a caloric intake 30% higher than conventional mice to maintain the same weight, highlighting the important of the gut microbiota in metabolism and digestion (Bäckhed *et al.*, 2004; Clarke *et al.*, 2014). The most common SCFAs produced by bacteria are butyric acid, lactic acid, acetic acid and propionic acid; all of which are a source of energy and nutrients for host cells (Beaugerie and Petit, 2004; Gibson, 2004). The gut microbiota is also able to synthesise vitamins, such as folate and biotin and aides in the absorption of minerals such as calcium and magnesium (O'Hara and Shanahan, 2006). The gut flora has also been shown to be able to metabolise and remove

xenobiotics, including carcinogens, drugs, toxins and phytochemicals (Sousa *et al.*, 2008).

The gut-brain axis describes the biochemical interactions between the central and enteric nervous systems. Recent work has begun to describe the influence the gut microbiota has on these interactions (Carabotti *et al.*, 2015). This interaction is bidirectional through means of endocrine, neural, humoral and immune links. The gut microbiota is implicated in CNS interactions resulting in modulation of neuro-endocrine systems associated with anxiety, memory and stress responses (Carabotti *et al.*, 2015). These effects are often strain specific, thereby providing an avenue for potential therapeutic use of probiotics in treating certain neurological conditions (Wang *et al.*, 2016). Probiotics may also be important in resolving the detrimental effect the CNS can have on the microbiota (Wang *et al.*, 2016). For example, it has been shown that norepinephrine release during surgery promotes the growth of *Pseudomonas aeruginosa* which can lead to sepsis (Alverdy *et al.*, 2000). A 2016 study systematically reviewed 38 studies to examine the effects of probiotics on CNS function in human and animals (Wang *et al.*, 2016). Various species within the genera *Bifidobacterium* and *Lactobacillus* showed potential in the treatment of various psychiatric disorders, including OCD, autism spectrum disorder, depression and anxiety.

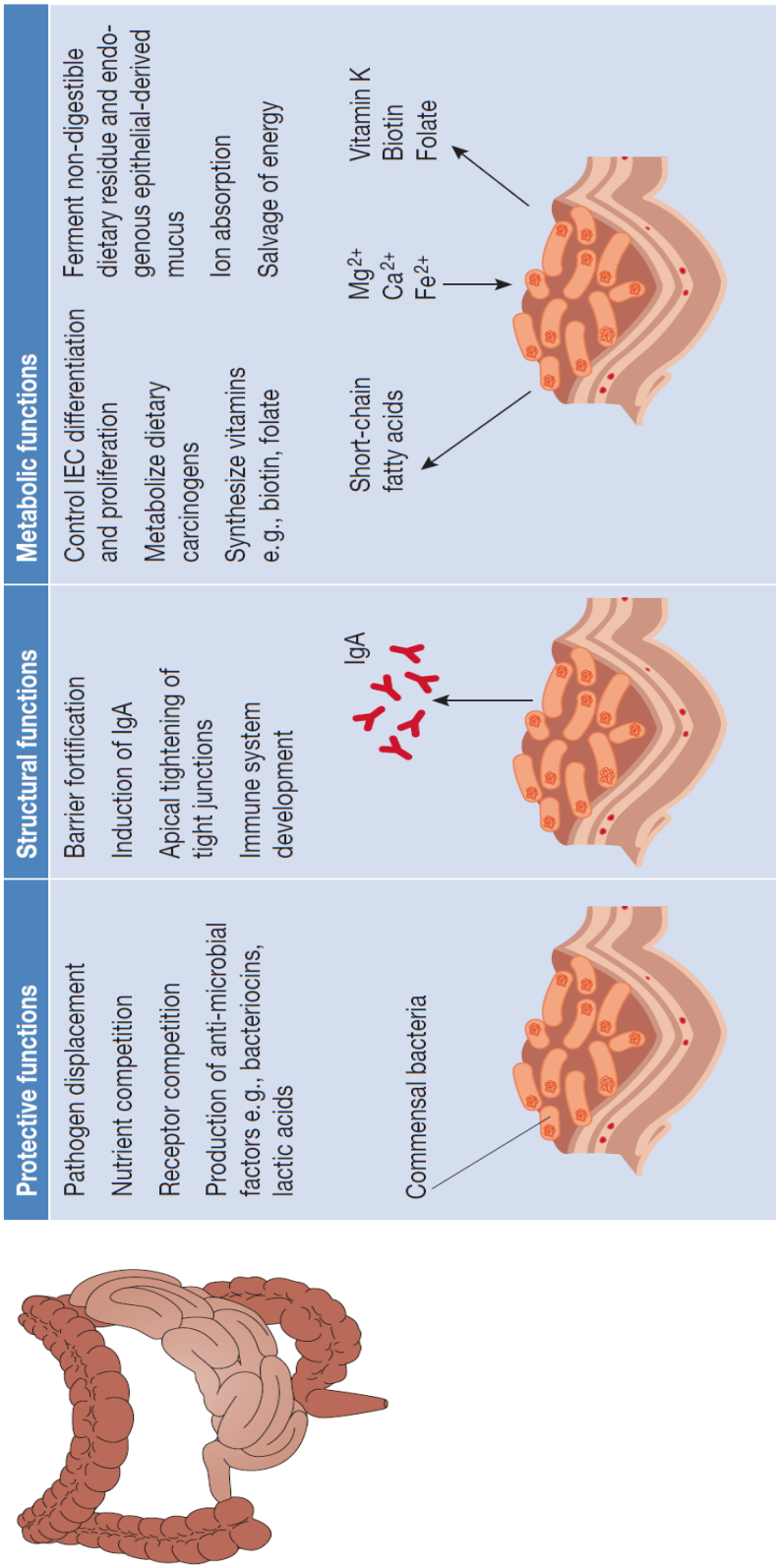


Figure 1.5. Functions of the intestinal microbiota. The gut flora exerts a multitude of protective, structural and metabolic functions upon the mucosal intestinal epithelium. **(**Modified from O'Hara and Shanahan, 2006).

The largest proportion of gut microbes are located in the large intestine. The small intestine only contains a very small number of microorganisms, predominantly Gram-positive cocci and rod-shaped bacteria (Nelson and Mata, 1970). As one moves down the small intestine into the distal small intestine, the more favourable alkaline conditions allow for support of more Gram-positive bacteria, including *Enterobacteriaceae* (Donaldson *et al.*, 2016). Microbes present in the small intestine hold a wide variety of functions, such as the expression of regulatory signals promoting development of the gut and vitamin metabolism (Sommer and Bäckhed, 2013). The large intestine on the other hand, is considered to be the largest reservoir for bacteria in humans (Mcbain and Macfarlane, 1998). Obligate anaerobes such as *Bifidobacterium* and *Bacteroides* comprise 99% of the flora within the large intestine, and 60% of faecal dry mass (Guarner and Malagelada, 2003). This makes faecal matter ideal for identifying the gut flora through nucleic acid extraction and 16S rRNA gene sequencing. 16S rRNA sequencing has revealed there to be up to 1000 different bacterial species living within the gut, with approximately 30 to 40 species comprising 99% of the population (Beaugerie and Petit, 2004; Sears, 2005).

1.5.2 Effect of antibiotics

The administration of broad-spectrum antibiotics can have dramatic effects upon the host, by way of gut microbiota alteration, both in quantity and diversity (Carman *et al.*, 2004). As described in detail in Section 1.2.3, antibiotic therapy perturbation of the gut flora can lead to the outgrowth of antibiotic-resistant bacteria within the gut potentially resulting in CDI. AAD is a more common occurrence than CDI; the incidence rate of AAD is between 5% and 25% (Beaugerie and Petit, 2004). AAD can take place when the diversity and number of gut flora species is reduced, diminishing the host's ability to metabolise bile acids and ferment carbohydrates (Theriot and Young, 2013). The increased amount of carbohydrates can absorb water resulting in runny stool. Other factors contributing to AAD development include the toxic effects of antibiotics and the overgrowth of pathogenic organisms, such as *C. difficile*.

An antibiotic initiated reduction in native bacterial species can lead to the emergence and outgrowth of pathogens such as *C. difficile* and *Salmonella kedougou*, which can result in AAD (Guarner and Malagelada, 2003; Beaugerie and Petit, 2004). The successful treatment of these infections with FMT indicate the maleficent effects antibiotics can have upon the microbiota, and the importance of restoring these bacterial species to the gut, principally *Bacteroides* and *Firmicutes* (Brandt *et al.*, 2011). In addition to altering the microbiota population of the GI tract, antibiotics can also affect caloric intake via carbohydrates, intra-community metabolic interactions and homeostasis for immune, hormonal and metabolic systems within the host (Carman *et al.*, 2004). Studies have demonstrated that combining probiotics (including *Lactobacillus* and *Saccharomyces* species) with antibiotic therapy may help to prevent the onset of AAD and CDI (Brandt *et al.*, 2011).

1.5.3 Control of CDI

The principle mechanism by which the microbiota provide host resistance to CDI is through bile acid modulation. Studies have shown that bile acids not only play a direct role in CDI, but perhaps also an indirect one by preventing the re-establishment of the normal microflora and subsequent CDI colonisation resistance (Kurdi *et al.*, 2006). Both cholate and taurocholate have been implicated as growth inhibitors of several groups of bacteria and increased levels during antibiotic treatment provides a competitive advantage to *C. difficile* (Tannock *et al.*, 1997; Kurdi *et al.*, 2006). The link between microbiota health and bile acid composition is most acutely observed when analysis is performed on recipients of faecal microbiota transplantation (FMT). Prior to FMT, primary bile acid levels are elevated, and secondary bile acids are almost undetectable in faecal samples (Weingarden *et al.*, 2014). In, contrast post-FMT samples contained mainly secondary bile acids, as is the case with samples from non-CDI donors (Weingarden *et al.*, 2014).

Secondary bile acids are formed by two bacterial enzymatic reactions; deconjugation within the small intestine and dihydroxylation/epimerisation within

the large intestine (Winston and Theriot, 2016). Conjugated bile acids are deconjugated by extracellular bile salt hydrolases (BSH), which are produced by a large variety of bacteria (Ridlon *et al.*, 2006; Jones *et al.*, 2008; Winston and Theriot, 2016). There have been 3 major phyla implicated in BSH production according to metagenomic screening: Firmicutes (30%), Bacteroidetes (14.4%) and Actinobacteria (8.9%) (Jones *et al.*, 2008). BSHs have been shown to protect against colonisation by *C. difficile* by increasing competition within the lower GI tract through detoxifying bile acids deleterious to microflora (Ridlon *et al.*, 2006). The second enzymatic reaction results in oxidisation and epimerisation of specific hydroxyl groups by three bacterial hydroxysteroid dehydrogenases (HSHD): 3-α, 7-α, and 12-α (Ridlon *et al.*, 2006; Winston and Theriot, 2016).

HSHDs are able to create 27 unique metabolites from the dehydrogenation of cholate alone (Ridlon *et al.*, 2006). 7α-dehydroxylation is performed by only a small proportion of the microbiota. All bacteria with 7α-dehydroxylation activity are anaerobic and the majority are *Clostridium* species within the Firmicutes phylum. The removal of the 7α-dihydroxyl group of primary bile acids is complex and requires several intracellular enzymatic steps but ultimately result in the formation of secondary bile acids; lithocholate from chenodeoxycholate and deoxycholate from cholate (Winston and Theriot, 2016). From here the microbiota can further modify deoxycholate and lithocholate into products such as taurodeoxycholate and ursodeoxycholate. The various bile salts, their location in the gastrointestinal tract and their effect on *C. difficile* is shown in Figure 1.6.

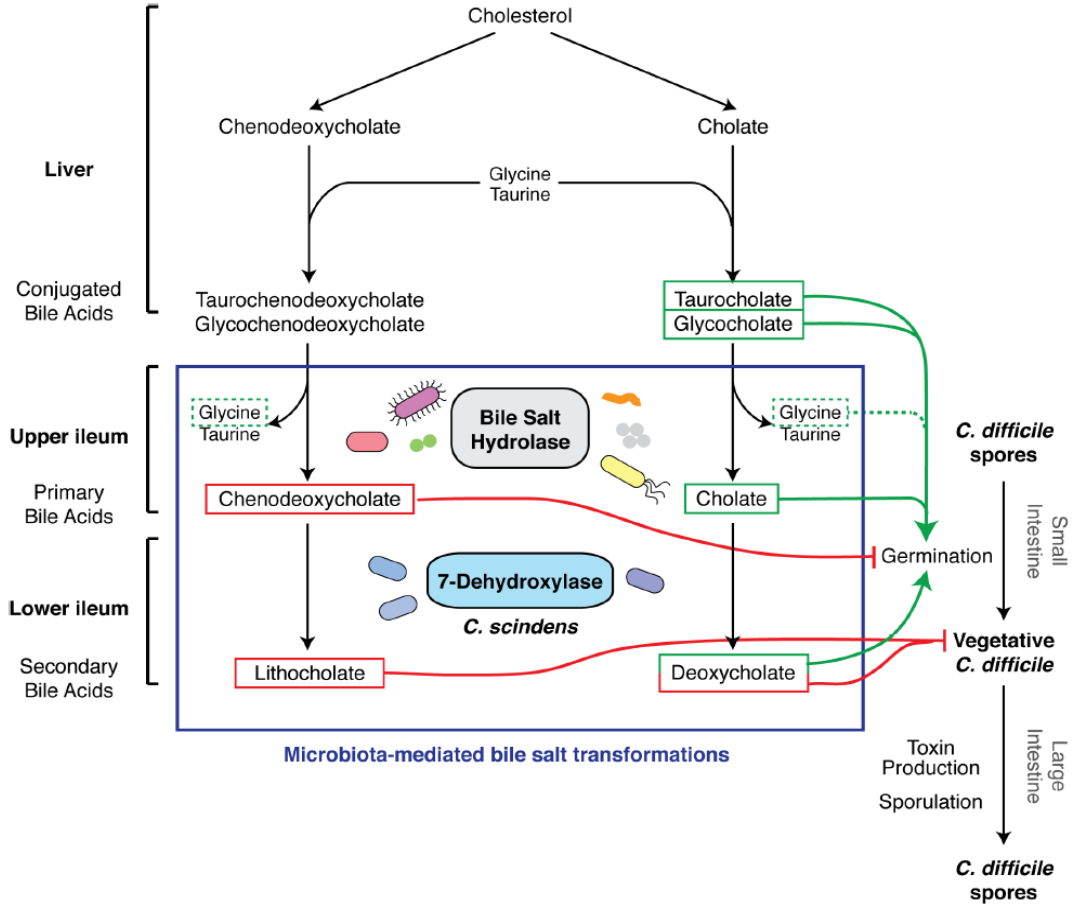


Figure 1.6. Bile acid metabolism in relation to *C. difficile* life cycle. Bile acids are synthesised from cholesterol in the liver and are released as conjugated bile acids. The gut flora in the ileum deconjugate bile acids to primary bile acids-cholate and chenodeoxycholate using bile salt hydrolases. Subsets of bacteria secrete the enzyme 7α-dehydroxylase which digests primary bile acids resulting in secondary bile acids such as deoxycholate and lithocholate. Secondary bile acids are toxic to *C. difficile* vegetative cells, while cholate derivatives induce germination in *C. difficile* spores. Glycine acts as a cogerminant. (Modified from Shen, 2015).

A prediction made by Giel *et al.* (2010) based on deoxycholate inhibition of *C. difficile* growth and the observed decrease in 7α-dehydroxylation activity following antibiotic treatment was recently supported in a pioneering study by Buffie *et al.* (2015). Buffie *et al.* (2015) was able to correlate resistance to CDI with the presence of *C. scindens* and increased levels of secondary bile acids (principally deoxycholate) within allogeneic stem cell transplant patients infected/not infected by CDI using microbiome and metabolome systems analysis. Partial protection against CDI in mice was demonstrated by the adoptive transfer of *C. scindens.* This protection correlated with deoxycholate levels within the mice.

CHAPTER 2

Materials and Methods

2.1 General methods

General methods for working with *B. subtilis* are described elsewhere (Smith, 1991). The same methods were used for working with *B. licheniformis* and *B. amyloliquefaciens.* Work with *E. coli* including cloning are as described by Sambrook and Russell (Wood, 1983).

2.2 Bacterial strains

630 is a toxigenic (*tcdA*$^+$ *tcdB*$^+$) strain of *C. difficile* isolated from a patient with pseudomembranous colitis during an outbreak of CDI (Wüst *et al.*, 1982) and 630Δ*erm* is an erythromycin sensitive derivative of 630 (Hussain *et al.*, 2005) used for the creation of ClosTron mutants. Both strains were obtained from Neil Fairweather (Imperial College, UK). Two previously described *cotE* ClosTron mutants, isogenic to 630Δ*erm*, were used: *cotE*$^-$ (*cotE::CT220s*) and *cotEC*$^-$ (*cotE::CT1203s*) (Permpoonpattana *et al.*, 2013). A *sigK* ClosTron mutant JP051 (*sigK::CT266s*) carrying an insertion after codon 87 of the coding open reading frame (ORF) was previously constructed in this laboratory. The *cotE* mutants were complemented with wild type copies of the respective genes using pRPF185 as described previously (Permpoonpattana *et al.*, 2013). Other strains of *C. difficile* including the hypervirulent strain R20291 and the high toxin producing strain VPI 10463 were laboratory stocks. PY79 is a prototrophic (Spo$^+$) laboratory strain and a lab stock, while other *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis* strains were isolated from soil or from humans. For strains and plasmids used in this study please refer to Table 2.1.

Unless stated otherwise, *C. difficile* was routinely cultured in BHIS broth (for 1 litre: 37 g BHI (Oxoid), 5 g Yeast Extract (BD Bacto), 1 g L-Cysteine (Sigma)) or on BHISS agar (as before with 1 g sodium taurocholate (Sigma) and 15 g agar

(Sigma)). TY broth comprised 30 g tryptose (BD Bacto), 20 g yeast (BD Bacto) and 1 g sodium thioglycolate (Sigma) per litre. *C. difficile* strains were grown at 37°C in an anaerobic cabinet (Don Whitley), maintained with an anoxic atmosphere (10% CO_2, 10% H_2, 80% N_2). Unless stated otherwise, *Bacillus* was routinely cultured in BHIB broth (for 1 litre: 37 g BHI (Oxoid), 5 g Yeast Extract (BD Bacto)) or on DSM agar (for 1 litre: nutrient broth 8 g (Bacto), KCl 1 g (Sigma), 0.25 g $MgSO_4.7H_2O$ (Sigma), 15 g agar (Sigma) and following autoclaving 1 ml of each of the following filter sterilised solutions were added: 1 M $Ca(NO_3)_2$, 10 mM $MnCl_2$ and 1 mM $FeSO_4$.

To store a strain (*C. difficile* or *Bacillus*), a single colony was used to inoculate 10 ml of the appropriate media (that favoured sporulation) and incubated overnight at 37ºC. 1 ml of the culture was added to 1 ml of autoclaved 50% glycerol (left in the anaerobic cabinet overnight prior to use for *C. difficile* strains). The sample was vortexed before storage at -80°C.

2.3 Sporulation of *C. difficile*

Spores of *C. difficile* were prepared by growth on SMC agar plates using an anaerobic incubator as described previously (Permpoonpattana *et al.*, 2011). A single colony from a BHISS streaked plate was used to inoculate 10 ml of TGY medium (for 1 litre: 30 g tryptic soy broth (Sigma), 20 g glucose (Sigma), 10 g yeast extract, 1 g L-cysteine) and incubated overnight at 37ºC. 1 ml of TGY culture was then sub-cultured into SMC broth (for 1 litre: 90 g peptone (Sigma), 5 g proteose peptone (Sigma), 1 g $(NH_4)_2SO_4$ and 1.5 g Trizma base (Sigma)) containing 0.1% L-cysteine (modified from Wilson, Kennedy and Fekety, (1982)) incubated overnight and then plated onto SMC agar. Plates were incubated anaerobically for 7 days at 37ºC, sporulation efficiency was confirmed using phase-contrast microscopy and spore crops harvested and maintained at 4ºC until purification. Spores of the complemented strains were prepared in the same way, but all media, including agar plates was supplemented with 15 µg/ml thiamphenicol and 500 ng/ml anhydrous tetracycline (ATC).

Table 2.1. Strains used in this study

Strain	Identity	Plasmid	Source
E. coli BL21 (DE3)	*E. coli*	pET28b- CotEN (Met1-K280)	Permpoonpattana *et al.*, 2013
E. coli BL21 (DE3)	*E. coli*	pET28b- CotEC (Pro361-Phe712)	Permpoonpattana *et al.*, 2013
630	*C. difficile*		Neil Fairweather
630Δ*erm*	*C. difficile*		Neil Fairweather
630Δ*erm cotE::CT220s* (*cotE*-)	*C. difficile*	pMTL007C-E2	Permpoonpattana *et al.*, 2013
630Δ*erm cotE::CT1203s* (*cotEC*-)	*C. difficile*	pMTL007C-E2	Permpoonpattana *et al.*, 2013
630Δ*erm sigK::CT266s*	*C. difficile*	pRPF185	Neil Fairweather
R20291	*C. difficile*		Neil Fairweather
VPI 10463	*C. difficile*		Neil Fairweather
SH1	*C. difficile*		Neil Fairweather
SH101	*C. difficile*		Neil Fairweather
SH102	*C. difficile*		Neil Fairweather
R20291	*C. difficile*		Neil Fairweather
CD196	*C. difficile*		Neil Fairweather
SH104	*C. difficile*		Neil Fairweather
CD10	*C. difficile*		Neil Fairweather
SH242	*C. difficile*		Neil Fairweather
SH200	*C. difficile*		Neil Fairweather
SH203	*C. difficile*		Neil Fairweather
SH218	*C. difficile*		Neil Fairweather
SH210	*C. difficile*		Neil Fairweather
SH213	*C. difficile*		Neil Fairweather
SH215	*C. difficile*		Neil Fairweather
SH220	*C. difficile*		Neil Fairweather
SH222	*C. difficile*		Neil Fairweather
SH231	*C. difficile*		Neil Fairweather
SH236	*C. difficile*		Neil Fairweather
SH239	*C. difficile*		Neil Fairweather
SH103	*C. difficile*		Neil Fairweather
SH3	*C. difficile*		Neil Fairweather
PY79	*B. subtilis*		*Bacillus* Genetic Stock Centre
10A1	*B. amyloliquefaciens*		*Bacillus* Genetic Stock Centre
SG57	*B. amyloliquefaciens*		Hong *et al.*, 2009
SG83	*B. subtilis*		Hong *et al.*, 2009
SG137	*B. amyloliquefaciens*		Hong *et al.*, 2009
SG140	*B. subtilis*		Hong *et al.*, 2009
SG185	*B. amyloliquefaciens*		Hong *et al.*, 2009
SG277	*B. amyloliquefaciens*		Hong *et al.*, 2009
SG297	*B. amyloliquefaciens*		Hong *et al.*, 2009
SG378	*B. amyloliquefaciens*		Hong *et al.*, 2009

2.4 Spore purification

The harvested spore pellet was further purified using centrifugation through a 20% (v/v) to 50% (v/v) Histodenz gradient (Sigma) as described elsewhere (Phetcharaburanin *et al.*, 2014). Following centrifugation through the Histodenz gradient, spores were washed five-times in ice cold dH_2O. Spore CFU was determined by heat treatment (60°C, 20 min.) and plating on BHISS agar plates.

2.5 Spore coat extractions (Chapter 3)

Spores (1×10^8) and vegetative cells were heated at 80°C for 20 min. and then pelleted by centrifugation and suspended in 40 µl of Bolt extraction buffer (Thermo), 10 µl LDS Sample Buffer, 4 µl Bolt Reducing Agent in 26 µl of dH_2O containing 0.5 mM PMSF and 0.052 mg of benzamide and incubated for 10 min. at 95°C. The suspensions were then centrifuged (10,000 g, 4°C, 5 min.) and supernatants kept on ice until SDS-PAGE analysis.

2.6 Antibody production (Chapter 3)

Plasmids (pET28b-derived) containing segments of CotE (tagged with poly-His at the C-termini) were used to express N (Met1-K280) and C (Pro361-Phe712) terminal segments of CotE in *E. coli* (Permpoonpattana *et al.*, 2013). High levels of expression were obtained upon IPTG induction (300 µm) and purification of proteins was accomplished by passage of the cell lysate through a HiTrap chelating HP column (GE Healthcare) on a Pharmacia AKTA liquid chromatography system. Polyclonal antibodies were raised in mice immunised via the intra-peritoneal route with 2 µg of purified recombinant proteins on days 1, 14 and 28.

2.7 Western blotting of spore coat proteins (Chapter 3)

Western blotting was carried out as described previously (Permpoonpattana *et al.*, 2013). Spore coat proteins were fractionated by size on a 12% SDS-PAGE gels using approximately 10 mg of protein per lane. The gels were transferred onto an

Immobilon-P PVDF Membrane (Merck) using a Trans-Blot® Turbo™ Transfer System (Bio-Rad). Membranes were dehydrated in 100% MeOH and allowed to dry for 1 hour at RT. The blot was probed with polyclonal antibodies (anti-CotEC or anti-CotEN at a 1/4000 dilution). Membrane were washed 3x with PBS + Tween-20 (0.05%) (v/v) (PBST) and detected with a goat anti-mouse HRP secondary antibody (1/2000 dilution) and developed using Amersham ECL Western blotting substrate (GE healthcare) on Amersham hyper-film ECL (GE healthcare).

2.8 Southern blotting (Chapter 3)

Genomic DNA was isolated from cultures of 630Δ*erm*, CotE$^-$ and CotEC$^-$ vegetative cells grown in BHIS using a phenol-chloroform extraction method, as described previously (Wren and Tabaqchali, 1987). The probe was prepared by amplifying a 500 bp sequence found within the *C. difficile erm*^{R} gene of the ClosTron plasmid, pMTL007C, used for gene constructions. Primers used were ERMF and ERMR (Table 2.1). The amplification product was purified using the QIAquick PCR purification kit (Qiagen) and labelled with Dig-High Prime following the manufacturer's instructions. Genomic DNA was digested with HindIII and run on an agarose gel (0.8% w/v). DNA was transferred onto a Hybond-N+ membrane (Amersham) using 0.4 M NaOH as transfer buffer. The probe was denatured (100^{o}C for 10 min.) and added to Dig High prime hybridisation buffer and the membrane was hybridised overnight at 42^{o}C. The membrane was washed, blocked, detected and developed following the manufacturer's instructions; DIG High Prime DNA labeling and Detection Kit I (Roche).

2.9 Germination assays (Chapter 3)

Spore germination rates were determined using preparations of Histodenz-purified spores of wild type and CotE mutants. Spores (10^6) were resuspended in BHIS medium containing 0.1% (w/v) sodium taurocholate, incubated at 37^{o}C anaerobically and at appropriate time points (0-60 min.) sampled for the number of heat-resistant (60^{o}C, 20 min.) or ethanol-resistant (EtOH) (50% v/v for 30 min.)

CFU. The percentage of initial counts at each time point were calculated and plotted.

Table 2.2. Primers used in this study

Primer	Direction	Sequence (5' to 3')
ERMF	Forward	CGAAATTGGAACAGGTAAAGG
ERMR	Reverse	GCTTCCAATATTTATCTGGAACATC
HPRTF	Forward	GTAATGATCAGTCAACGGGGGAC
HPRTR	Reverse	CCAGCAAGCTTGCAACCTTAACCA
IL-6F	Forward	ACCGCTATGAAGTTCCTCTCTG
IL-6R	Reverse	AATTAAGCCTCCGACTTGTGAA
MIP-2αF	Forward	GCTGTCAATGCCTGAAGACC
MIP-2αR	Reverse	CCTTGAGAGTGGCTATGACTTC
TNF-αF	Forward	CACCACGCTCTTCTGTCTACTG
TNF-αR	Reverse	TGAGAAGATGATCTGAGTGTGA

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

The surface hydrophobicity of spores was determined using the spore adhesion to hydrocarbon (SATH) assay using *n*-hexadecane as the hydrocarbon (Seale *et al.*, 2008). Histodenz-purified spores were washed in 1 M NaCl in dH2O by centrifugation at 16,000 g for 10 min. and resuspended in 0.1 M NaCl at a density of 1 x 10^8 spores/ml. Spore suspensions (2 ml) were added to 1 ml *n*-hexadecane (Sigma) and vortexed for 1 min., followed by 10 min. incubation at 37°C, and vortexed again for 30 seconds. The absorbance of the aqueous phase was measured at OD_{600}. The mean of two measurements was determined. The percent hydrophobicity (*%H*) was determined from the absorbance of the original spore suspension (*Ai*) and the absorbance of the aqueous phase after incubation with hexadecane (*Af*) according to the following equation: $\%H = [(Ai - Af) / Ai] \times 100$.

2.11 Cell culture (Chapter 3)

HT29 cells (ATCC, Manassas, USA), a colonic epithelial cell line was seeded at 2 x 10^6 cells in a 75 cm^2 flask and grown in McCoy's 5A medium (Sigma) supplemented with 1% L-glutamine (v/v) (Sigma), 1% (v/v) penicillin and streptomycin (Sigma), and 10% foetal bovine serum (FBS) (v/v) (Sigma). HT29-MTX cells (PHE, UK), a mucus secreting colonic epithelial cell line, was seeded at 2 x 10^6 cells in a 75 cm^2 flask and grown in Dulbecco's modified Eagle medium (DMEM) (Life technologies, UK) supplemented with 1% (v/v) L-glutamine, 1% (v/v) penicillin and streptomycin, 1% (v/v) non-essential amino acids (all Sigma) and 10% (v/v) FBS. In both cases 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.

To confirm the presence of mucus on HT29-MTX cells monolayers were washed with 3% acetic acid for 3 min. 1% (w/v) Alcian blue solution (Sigma) pH 2.5 was added to monolayers for 30 min. at RT. Monolayers were briefly rinsed with 3% acetic acid and then dH_2O to remove excess Alcian blue solution. Nuclear fast red solution (Sigma) was added to monolayers for 5 min. and the excess removed by washing with distilled dH_2O. Coverslips were mounted and sealed using clear nail varnish. Slides were visualised using an EVOS FL digital microscope (Life technologies, UK) with mucus visualised by blue staining of polysaccharide residues. Experiment performed in collaboration with fellow student, Saba Anwar.

2.12 Cytotoxicity assays (Chapter 3)

Assays were based on those that have been described previously (Kuehne *et al.*, 2010). Overnight cultures of *C. difficile* in TY broth were used to inoculate a starting culture in TY medium at a starting optical density at 600 nm of 0.05. Samples were taken at 6, 12, 24 and 48 hours, centrifuged (10,000 g, 10 min.) and supernatants filter-sterilised through 0.2 μm filters. The samples were then normalised proportionally according to the OD taken at collection using PBS. Samples were serially diluted in PBS and 10 μl was added to 90 μl of DMEM (2%

v/v FCS) media above confluent HT29 (for TcdA) or VERO (for TcdB) cells giving a 1:10 dilution. Cells were incubated for 24 hours at 37ºC, 5% CO_2 before being scored for toxin endpoints using an Olympus CKX41 light microscope. End point titers were taken at the point where cell rounding was less than 50%.

2.13 Induction of cytokines (Chapter 3)

J774A.1 macrophages (1 x 10^6) were seeded into a 24-well plate in DMEM complete medium and incubated overnight at 37ºC (at 5% CO_2). To investigate the capability of the recombinant proteins to elicit an inflammatory response, the seeded cells were washed twice with DMEM and then rCotEN, rCotEC or LPS were diluted in DMEM to final concentrations ranging from 1-10,000 ng/ml and added to wells. The plate was incubated for 5 hours prior to harvesting cells. For analysis using spores, seeded cells were washed with DMEM twice and replaced with spores (630Δ*erm*, CotE$^-$ or CotEC$^-$) re-suspended in DMEM (MOI of 10:1) with one well retained as a 'media only' control. The plate was spun at 1,500 g for 5 min. to sediment material and was then incubated at 37ºC at 5% CO_2 for 3 hours. All media was removed after incubation and 500 µl of TRI reagent (Sigma) was added. Total RNA was isolated according to the manufacturer's protocol for TRI reagent. RNA was then frozen at -80ºC prior to use. Possible DNA contamination was removed with the use of DNase 1 (Sigma) and RNA quantified (NanoDrop 2000, Thermofisher) prior to cDNA synthesis. The 'High Capacity' cDNA reverse transcription kit (Applied Biosystems) was used to prepare cDNA. PCR amplification of specific genes was performed using a Rotor Gene 6000 and SYBR Green JumpStart Taq ReadyMix (Sigma). Primers for IL-6, MIP-2α and TNF-α were analysed with the housekeeping gene HPRT (hypoxanthine guanine phosphoribosyltransferase) being used as an endogenous control. Amplification of these genes was quantified using the plasmid pCCy17. pCCy17 is a mouse multi-gene quantification plasmid containing two housekeeping genes (β-actin & HPRT) and provided by Dr Ian Teo, Imperial College, London. Gene expression was analysed by comparing the mRNA copy numbers of a specific cytokine gene versus that of HPRT. Primer sets used are described in Table 2.1.

2.14 Spore adhesion assays (Chapter 3)

The method was performed as described previously (Hong, Hitri, *et al.*, 2017). HT29-MTX and HT29 cells were seeded at 4 x 10^4 cells/well in 24-well plates for 14 days at 37°C with 7.5% carbon dioxide with regular changes of culture media (DMEM for HT29-MTX cells or McCoy's 5A media for HT29 cells) at 37°C in an atmosphere of 5% CO_2. Mucus production was checked using Alcian blue staining. On the day of experimentation, cell monolayers were washed once with Dulbecco's Phosphate-Buffered Saline (DPBS) (Life technologies, UK) and monolayers were incubated with 500 µl/well of culture medium (without supplements) (DMEM for HT29-MTX cells or McCoy's 5A media for HT29 cells) containing *C. difficile* spores at a multiplicity of infection (MOI) of 100 for 2 hours at 37ºC, 5% CO_2. Non-adherent spores were removed by washing with PBS 5-times and the cells were lysed with Triton X-100 (0.1% w/v) in PBS for 10 min. For assessing the total count of adherent *C. difficile*, samples were serially diluted and plated on BHISS plates. For measurement of adherent *C. difficile* spores, samples were heat treated at 60ºC for 20 min. Plates were incubated anaerobically for 48 hours and % of adhesion calculated using the following formula: % of adhesion = (CFU count / initial number of spores added) x 100. For antibody-blocking adhesion experiments; the same procedure as described above was used, but spores were pre-incubated with anti-CotEC antibody (1/10) for 30 min. at 37°C before being added to the wells. Data were presented as the percentage proportion of binding demonstrated by that of spores of strain 630. Experiments were repeated three-times in triplicate. Error bars represent standard deviation. Statistical significance between groups was calculated using an unpaired *t*-test.

2.15 Spore binding to pig mucus explants (Chapter 3)

Intestinal tissue was removed from the terminal ileum and caecum of a four-week-old piglet. Tissue was pre-incubated at 40ºC and then segments (2 cm^2) were placed in 6-well plates. For each sample well 1 ml of a suspension of spores (2 x 10^7) in PBS was added, while controls received PBS only. Plates were centrifuged (1,300 g, 2 min.) and 1 ml of PBS was added to the samples (ileum, caecum). Plates were

incubated anaerobically for 30 min., 1 hour or 3 hours at 37^{o}C. Plates were then washed twice with 1 ml PBS after which the mucus layer was removed and stored at -20^{o}C till use. Adherent viable counts were determined by serial dilution and plating on BHISS plates.

2.16 Spore binding to ligands (Chapter 3)

Ligands, conjugated to human serum albumin (HSA), were synthesised by Dextra (Reading, UK) and were dissolved in PBS (pH 7.4) and used to coat 96-well microplates at a concentration of 10 μg/ml (overnight at 4^{o}C). For mucin, porcine stomach type III (Sigma), a solution was made fresh in PBS (pH 7.4) and used to coat plates at 1 μg/ml (overnight at 4^{o}C). Plates were washed three-times with PBST + Tween-20 (0.05%) (v/v) (PBST) at RT and then blocked using 2% (w/v) BSA in PBS (1 hour, 30^{o}C) followed by three further washes with PBST. Recombinant proteins (rCotEN or rCotEC) were diluted in 1% (w/v) BSA to a concentration of 500 nM and then diluted 2-fold on the coated plates. Plates were incubated for 2.5 hours at 30^{o}C and then washed 3-times with PBST. For detection, primary mouse antibodies to rCotEN and rCotEC were used at a 1/1000 dilution with a 1-hour incubation at 30^{o}C. Plates were then washed three-times and a secondary goat, anti-mouse IgG (DAKO) was used for detection of bound primary antibody (1 hour at RT). Plates were developed with tetramethyl benzidine (TMB) (Sigma) to develop colour and the reaction was stopped with 2 M sulphuric acid and absorbance read at OD_{450}.

2.17 Mucin degradation using amido black staining (Chapter 3)

Three millilitres of sterile molten 0.5% (w/v) agarose containing 0.5% (w/v) of mucin (porcine type III, Sigma M1778) was overlaid onto sterile microscope slides (76 × 26 mm) rapidly. The sterile slide was placed into a petri dish and the agarose left to solidify, after which 3-mm-diameter holes were punched out of the medium. Ten microliters of spores (1×10^{10}/mL) was added to the wells. The petri dish was closed and sealed with parafilm and incubated for 48 hours at 37°C. Before staining with amido black solution for 30 min. (Sigma), the agarose was compressed to a

thin layer using a 500 g weight (30 min. with Whatman No. 3 between agarose and weight). The slide was then de-stained several times with 7% (v/v) acetic acid. Degradation exhibited as zones of clear agarose on a black agarose (no-degradation) background.

2.18 Mucin degradation using amido black staining using RP-HPLC (Chapter 3)

A freshly made solution of mucin was labelled fluorescently with Rhodamine B isothiocyanate (RBITC, mixture of isomers, Sigma-Aldrich) and purified by gravity-flow chromatography using 10DG Desalting Columns filled with Bio-Gel® P-6DG Gel (Bio-Rad) and equilibrated in PBS pH 6.4 buffer prior to the experiment. Equal aliquots of mucin spiked with the fluorescently labelled RBITC-mucin in PBS (pH 6.4) were added to purified wild type and mutant *cotEN*$^{-}$ spores of *C. difficile*. Suspensions were incubated for 36 hours at 37^{o}C under anaerobic conditions with continuous gentle agitation. Mixtures were pelleted (10,000 g, 10 min.). SDS was then added to all samples (final concentration 0.2% w/v) to stop any further interactions and to ensure stability and solubility. Before HPLC analysis samples were again pelleted. A TSKgel® Size Exclusion HPLC Column (PW_{XL}-Type, L. x I.D. 300 mm x 7.8 mm) was used to simultaneously exchange buffer in mucin samples and then to conduct reverse phase separation of the absorbed and washed mucins by gradient elution using mobile phases with decreasing salt concentration and increased concentration of organic modifier. A FLEXAR programmable HPLC setup included a quaternary solvent delivery system, autosampler and a column oven (30^{o}C). The eluted mucins were detected using an Altus A-10 FL fluorescence detector (excitation was at 550 nm, emission was at 575 nm). All samples were injected in 50mM PBS pH 6.4 (0.4 ml/min.) and the isocratic flow was maintained for up to 30 min. to allow elution of hydrophilic sample constituents. Under these conditions mucins remain associated with weakly hydrophobic hydroxylated polymethacrylate PW_{XL} polymer stationary phase, thus allowing an extended washing and equilibration cycle (column eluates were monitored by measuring fluorescence excitation 550 nm, emission at 575 nm). For

gradient elution the mobile phase was changed from 0/0/100 to 100/0/0 A/B/C in 25 min. (eluent A: 100% Methanol (MeOH); eluent B: dH_2O; eluent C: 50mM PBS, pH 6.4). The column was regenerated and re-equilibrated after each separation by washing with individual eluents A, B and C (15 min. each wash). This experiment was performed under the guidance and expertise of Dr. Mikhail Soloviev.

2.19 Animal experiments using wild type and CotE mutants (Chapter 3)

All animal procedures were performed under the Home Office project license PPL 70/8276. For Golden Syrian hamster experiments females, aged 16-18 weeks were used (Harlan UK Ltd.). For challenge, animals were transferred to individually ventilated cages (IVCs) and dosed by intra-gastric gavage (i.g.) with clindamycin (clindamycin-2-phosphate, Sigma; 30 mg/kg body weight). After 16 hours they were dosed i.g. with spores or vegetative cells (10^6) of wild type or mutants. Animals were monitored for symptoms of disease progression and culled upon reaching the clinical endpoint. The symptoms of CDI were scored as severe/clinical endpoint (wet tail >2 cm, high lethargy), mild (wet tail < 2 cm), or healthy. Faeces was sampled periodically, and caeca taken from culled animals. For mice a non-fatal colonisation model was used referred to as the 'Holloway model' (Colenutt and Cutting, 2014). C57 BL/6 mice (6-8 weeks old, female) animals were dosed i.g. with clindamycin (30 mg/kg) and transferred to IVCs. One day later animals were given a single dose (i.g.) of spores or vegetative cells (10^6) of wild type or mutants and faecal samples were sampled periodically afterwards. ID_{50}s were determined using the non-fatal murine model as described previously (Phetcharaburanin *et al.*, 2014).

2.20 *In vivo* toxin production (Chapter 3)

Clindamycin-treated mice (n= 6/gp) were dosed (i.g.) with 10^3 spores of wild type *C. difficile* 630 or one of the two *cotE* mutants 24 hours post clindamycin treatment (30mg/kg). Faeces was collected at 24, 48- and 72-hours post-infection. The extraction of toxins A and B from faecal samples is described below in Section

2.22. Levels of toxin A and toxin B were determined by ELISA in faeces sampled at 24, 48- and 72-hours post-infection. The presence of functional toxins A and toxin B was also verified using cytotoxicity assays using HT29 (toxin A) and VERO (toxin B) cells.

2.21 Evaluation of GlcNAc and glucosamine in a CDI murine model (Chapter 3)

The non-fatal colonisation model, the 'Holloway model' model was used (Figure 3.23) with a challenge dose of 10^2 spores of CD630 per mouse. From day -2 until the end of the experiment, mice received sterile drinking water with solublised N-Acetyl-D-glucosamine (Sigma) or glucosamine hydrochloride (Sigma) at various concentrations (20, 50 or 100 mg/ml) ad libitum, while naïve mice received sterile drinking water only. Average water consumption was measured for each group and quantity of GlcNAc/glucosamine ingested calculated. At 24 hours post challenge mice were culled and caecum samples were tested for toxins using ELISA and EtOH resistant CFU counts.

2.22 Analysis of correlates of infection (Chapter 3 and 4)

The infectivity of spores (expressed as the 50% infectious dose, ID_{50}) was determined in mice using Histodenz-purified spores (doses of 10^1, 10^2 and 10^3 CFU) of 630 or *cotE* mutants as described elsewhere (Phetcharaburanin *et al.*, 2014). Caeca were removed 24 hours post-infection and colonisation was defined by the presence of toxins and spore CFU counts 2-logs higher than the initial dose. Colonisation in mice and hamsters was assessed from both counts of *C. difficile* spores and the presence of toxins in faecal and/or caecum samples. Caecum samples were homogenised in PBS containing a protease inhibitor (Pierce) and incubated for 30 min. at 4^{o}C. Homogenates were centrifuged (10,000 g) and the supernatant collected for toxin analysis by ELISA as described elsewhere (Phetcharaburanin *et al.*, 2014). The pellet was resuspended in 70% v/v ethanol and serially diluted and plated. Spore counts were determined by 30 min. treatment of homogenised faeces in ethanol (70% v/v) followed by serial dilution and plating on BHISS agar. The

presence of TcdA and TcdB was determined by sandwich ELISA as described elsewhere (Phetcharaburanin *et al.*, 2014) and each sample was validated for functional toxin using toxin cytotoxicity assays using HT29 (TcdA) and VERO (TcdB) cells as described previously (Permpoonpattana, Hong, *et al.*, 2011).

2.23 Characterisation of intestinal spore formers in mice and hamsters (Chapter 4)

C57BL/6 mice (6 weeks, female) housed in groups of 4/cage were kept in IVCs for 10 days prior to being dosed with clindamycin (30 mg/kg). Freshly voided faeces was collected 24 hours before and after clindamycin treatment and then homogenised in PBS, heat-treated (68^{o}C, 1 hour) serially diluted and plated on LB for aerobes or BHISS agar for anaerobic bacteria, a medium used for culture of the human gut microbiota (Lau *et al.*, 2016). Plates were incubated aerobically or anaerobically at 37^{o}C for 2 days. 500 colonies were randomly picked and restreaked. The presence of spores in colonies was checked microscopically and each colony was grown for 16 hours at 37^{o}C in liquid culture (2 ml) using aerobic or anaerobic conditions as required before sub-culturing (1/100) overnight in the same conditions. The cell free supernatant was then obtained using centrifugation and filtering through a 0.45 μm syringe filter. Activity against CD630 was determined using a microdilution assay (see below). Biosurfactant activity was determined using an oil displacement assay (Youssef *et al.*, 2004). *gyrA* sequencing used protocols and primers previously described for *Bacillus* (Chun and Bae, 2000). Antibiotic minimal inhibitory concentrations were made using a microdilution method as stipulated by the Clinical and Laboratory Standards Institute (CLSI, 2019).

2.24 *In vitro* analysis of anti-*C. difficile* activity (Chapter 4 and 5)

2.24.1 Agar diffusion assay

Aerobic *Bacillus* strains were grown in LB medium at 37^{o}C for 16-18 hours while anaerobic spore formers were grown in BHISS broth overnight in an anaerobic

chamber. Samples were centrifuged (microfuge, 10,000 g, 10 min.) and supernatants filter-sterilised (0.45 μm syringe filter) and stored on ice till use. TGY agar plates (for 1 litre: 30 g tryptic soy broth, 20 g glucose, 10 g yeast extract, 1 g L-cysteine and 15 g agar) were pre-reduced and after spreading with an overnight *C. difficile* culture (~100 μl) allowed to dry for 30 min. after which 4-6 wells were cut per plate. Plates were reduced for 4 hours in an anaerobic chamber before use. 5 mm diameter wells were cut in the TGY agar and 50 μl of *Bacillus* supernatants applied to labelled wells and the plates incubated at 37^{o}C for 48 hours in an anaerobic chamber and the diameter of the zones of inhibition measured.

2.24.2 Microdilution assay

Indicator culture:

A single colony of the relevant *C. difficile* strain was inoculated into 10 ml of BHIS and incubated overnight at 37^{o}C in an anaerobic chamber. The overnight culture was then sub-cultured 1:100 into BHIS (typically 0.1 ml into 10 ml BHIS) and incubated at 37^{o}C for 6 hours after which the culture was ready for use.

Plate set up:

180 μl of sterile BHIS was pipetted into the first row of a 96-well U-bottom microplate (Sigma) and 100μl into each subsequent row. 20 μl of the sample to be tested was pipetted into the first row (1:10 dilution factor) and serially diluted in a 2-fold dilution series until the last row (1:1280 dilution factor) on the microplate. For one serial dilution a 'media only' control was also pipetted into a single well on the first column. 10 μl of the 6 hours *C. difficile* 'indicator culture' was pipetted into each well and the plate was incubated overnight at 37^{o}C in an anaerobic chamber. After overnight growth the microplate contents were agitated on a rotary plate shaker at 200 rpm for 2 min. after which the OD_{600} was measured using a microplate plate reader. Positive inhibitory activity was defined as an OD_{600} < 50% of the CD630 media only control.

2.24.3 Co-culture assays

C. difficile strains were grown in BHIS medium overnight at 37ºC under anaerobic conditions. The following day 9 ml BHIS was inoculated with 90 µl of overnight culture and incubated at 37ºC until the optical density reached ~ 0.2-0.3 OD_{600}. At this point 1 ml of a freshly prepared supernatant was aseptically added to the growing *C. difficile* cultures and growth resumed, and the OD_{600} recorded hourly. For cell viability counts, aliquots were taken alongside OD readings, serially diluted and plated on BHISS agar plates. For experiments involving HPLC separated fractions, vacuum evaporated (Genevac EZ-2) fractions were resuspended in 1 ml of PBS, and the quantity resuspended normalised to the original filtrate, using activity previously determined by microdilution assay. In the case of the SG277 SEC, surfactin and $AmSO_4$ fractions, samples were normalised by dilution with PBS so that activity (in microdilution assay) was equivalent to that of the supernatant (Figure 5.14). The same volume for each sample as was used for the supernatant (1 ml) was then used in the co-culture assay. For experiments involving a variety of *C. difficile* strains (Table 5.4), *C. difficile* cultures were grown for 10 hours at 37ºC in BHIS and 18 ml was moved to a falcon tube followed by an addition of 2 ml of SG277 filtrate. The cultures were incubated for 18 hours at 37ºC after which the OD_{600} was read to assess the inhibitory activity against the strains.

2.25 *In vivo* analysis of allochthonous intestinal aerobic spore formers (Chapter 4)

C57BL/6 mice (6 weeks, female) (n = 6) were housed in either conventional cages or IVCs with three animals per cage. Faeces was sampled every 10 days, homogenised in PBS, and the total counts or heat-resistant (68ºC, 1 hour) spore counts were determined following serial diluting and plating on LB agar (37ºC, O/N). Colonies were quantified and calculated to give values per gram of faecal matter.

2.26 *Ex vivo* germination of *C. difficile* spores in intestinal contents (Chapter 4)

C57BL/6 mice (6 weeks, female) housed in groups of four/cage were dosed with clindamycin (30 mg/kg) or left untreated. 24 hours post dosing with clindamycin, mice from the treated and non-treated cages were culled and the contents removed from the small intestine and weighed. Intestinal contents were diluted 1:2 (w/v) in saline (with *Clostridium difficile* supplement (CD supplement); cefoxitin and D-cycloserine, Sigma), homogenised, centrifuged (21,000 g, 15 min.) and the supernatant was removed. Centrifugation was repeated twice more. CD630 spores were added to a final concentration of 10^6 CFU/ml to the samples and they were incubated at 37^{o}C in anaerobic conditions. Total counts and heat resistant counts (60^{o}C, 20 min) at different time points were plated on BHISS supplemented with CD supplement.

2.27 *In vivo* germination of *C. difficile* spores (Chapter 4)

C57BL/6 mice (6 weeks, female) housed in groups of three/cage were dosed with clindamycin (30 mg/kg) or left untreated. Three untreated and three treated mice were dosed i.g. with 10^7 CD630 spores and a single mouse culled at each time point (1, 2 and 3 hours). The stomach, small intestine and large intestine contents were removed, added to pre-reduced PBS (1 ml), homogenised, serially diluted and plated on BHISS (CD supplement) for total counts. Homogenised sample was then heat treated (60^{o}C, 20 min.) and plated on BHISS (CD supplement). The germination rate was calculated as follows: 100 – (Heat resistant count/Total count x 100).

2.28 *Ex vivo* analysis of anti-*C. difficile* activity of intestinal contents (Chapter 4)

2.28.1 Intestinal contents extraction

C57BL/6 mice (6 weeks, female) housed in groups of four/cage were dosed with clindamycin (30 mg/kg) or left untreated. 24 hours post dosing with clindamycin, mice from the treated and non-treated cages were culled and the contents were removed from the small intestine and weighed. Intestinal contents were homogenised in 1 ml dH_2O and shaken vigorously for 1 hour at RT. After incubation, 9 ml of 100% MeOH was added to the sample and incubated for 3 hours at RT before being centrifuged (10,000 g, 15 min.). The supernatant was removed and evaporated in a vacuum evaporator for 24 to 36 hours until only a dry pellet remained. The pellet was resuspended in saline at a ratio of 1:3 (w/v) according to the original weight. The samples were incubated for 3 hours at RT prior to being centrifuged at 21,000 g for 10 min. and the supernatant removed. Centrifugation was repeated twice more under the same conditions and the supernatant was removed and stored at -20^{o}C.

2.28.2 Spore recovery assay

The assay was performed at 37^{o}C in anaerobic conditions. Intestinal contents samples were diluted 1:1 in BHIS (+ 2% sodium taurocholate and CD supplement) and spores were added to a final concentration of 10^6 CFU/ml. Samples were taken at 0, 1, 2 and 3 hours and total CFU plated on BHISS agar supplemented with CD supplement. Heat resistant counts (60^{o}C, 20 min.) were also enumerated to confirm that germination was occurring. Saline and supernatant from SG277 O/N BHIB culture (0.45 µm filter) were utilised as negative and positive controls. SG277 filtrate was diluted 1:5 in saline which was then diluted 1:1 in BHIS + 2% (w/v) sodium taurocholate before addition of spores.

2.29 RP-HPLC analysis of small intestinal contents (Chapter 4)

Following extraction of intestinal contents (2.28.1), samples (1.5 ml) were diluted with 100% MeOH to a final concentration of 20%. All the following buffers used with Sep-Pak C18 cartridges (Waters) were acidified with acetic acid to a final concentration of 0.1%. Sep-Pak C18 cartridges were activated with 10 ml of 100% MeOH before being equilibrated with 10 ml of 20% (v/v) MeOH. The sample was then pushed through the column gently and collected. This was repeated twice more. The column was washed with 10 ml of 20% (v/v) MeOH and elution was performed with 2 ml of 100% MeOH. The eluted fractions were evaporated for 3 hours using a vacuum evaporator. Dried samples were resuspended at a 10:1 (w/v) ratio in 60% (v/v) MeOH according to the original weight of the intestinal contents. Samples were then either tested for activity or 50 µl (representing 0.5 g intestinal contents) was analysed using RP-HPLC (2.31). All fractions were identified using MALDI-TOF analysis (2.31) and peaks within regions corresponding to different lipopeptides were combined and tested for activity. Activity was measured using the microdilution assay, with dilution values representing the final dilution of the newly resuspended sample at which activity is still present.

2.30 Prophylactic treatments in animals (Chapter 4)

For animal experiments, methods were followed from that described previously (the Holloway model) in Section 2.19 and 2.22 (Permpoonpattana *et al.*, 2011; Hong, Ferreira, *et al.*, 2017).

2.30.1 Preparation of prophylactic treatments

SG277 and SG378 were grown overnight (18 hours, 37^{o}C) in 100 ml BHIB and after centrifugation the pellet suspended in 2 ml of PBS or supernatant to give an approximate 200 µl dose of 5 x 10^{9} CFU. For strains comprising the cocktail of bacteria isolated from mouse faeces with *in vitro* activity against *C. difficile* (*B. amyloliquefaciens, B. licheniformis, B. subtilis*), a single colony of each was resuspended in individual flasks containing 25 ml BHIB and grown overnight (18

hours, 37ºC), and after centrifugation the resultant pellets were mixed and resuspended in 2 ml of PBS to give an approximate 200 µl dose of 3 x 10^9 CFU (1 x 10^9 /strain). The same procedure was followed for the negative control in this experiment, the cocktail comprising three *Bacillus* isolates that showed no *in vitro* activity against *C. difficile.* For use of the supernatant an aliquot was filter sterilised (0.45 µm). For spores SG277 was grown on DSM agar for 72 hours at 37ºC. Spore crops were harvested from the plate using a cell scraper, washed three-times in dH_2O and then heat-treated to kill residual vegetative cells. Spores were suspended in dH_2O to give a concentration of 2.5 x 10^{10} spores/ml, with a 200 µl dose providing 5 x 10^9 spores. For lyophilised SG277, a single colony was grown in 25 ml BHIB (18 hours, 37ºC). Cell pellets were frozen at -20°C O/N. The next day tubes containing frozen pellets from 25 ml culture were covered with perforated parafilm and lyophilised O/N. When ready, they were stored at -20°C. On the day of use, pellets from four flasks were harvested and resuspended in 2 ml of PBS (approximate 200 µl dose of 5 x 10^9). For preparation of the SG277 preparative SEC fraction for dosing to mice, culture was precipitated using 20% (w/v) ammonium sulphate ($AmSO_4$) and resuspended at a 30x concentration in PBS (pH= 7.4). 1 ml of the $AmSO_4$ precipitate was separated on a gel filtration, size exclusion chromatography (SEC) column (Superdex 200) using PBS as an isocratic buffer and fractions tested for activity against CD630 in a microdilution assay. Active fractions were combined, washed using a 10 kDa molecular weight cut off centrifugal filter (MWCO, Vivaspin) and concentrated to a final volume of 1 ml in dH_2O. Mice were dosed with 200 µl of this SEC fraction.

2.30.2 Mouse colonisation experiments

Animals (C57BL/6, female, aged 6-7 weeks) were dosed i.g. with clindamycin (30 mg/kg) and 24 hours later challenged with 10^2 spores of CD630. Animal groups were dosed (0.2 ml, i.g.) with prophylactic treatments before and after challenge with CD630 using the regimen shown in Figure 4.12. Caeca were removed 24 hours post-infection for analysis of colonisation.

2.30.3 Hamster experiments

Golden Syrian Hamsters (female) were 16-18 weeks old. For the hamster challenge, animal groups (n = 6) were dosed (i.g.) with clindamycin (30mg/kg body weight) and then challenged three days later with 10^2 spores of CD630. Before and after CD630 challenge animals were dosed (i.g.; 2 ml/dose) with the treatments described above. The treatment regimen was six doses before CD630 challenge (-48, -36, -24, -12, -4 and -1 hours) and then three-times/day post-challenge for twelve days. Animals were monitored for symptoms of disease progression and culled upon reaching the clinical endpoint. The symptoms of CDI were scored as severe/clinical end point (wet tail >2 cm, high lethargy), mild (wet tail <2 cm) or healthy. Caeca were removed and analysed for CD630 ethanol resistant CFU and toxins A and B. Figure 4.15 is a diagrammatic representation of the regimen.

2.31 Extraction and purification of the active molecule/s from bacterial culture (Chapter 4 and 5)

For purification, SG277, amongst the most active of the *B. amyloliquefaciens* strains was selected. For figures comparing various *Bacillus* strains (SG297, SG83, SG140), the same purification method was used (Figure 5.12). After cultivating the cells overnight in BHIB broth at 37^{o}C bacterial cells were removed from the supernatant by centrifugation at 8000 g for 10 min. and the supernatant was filter-sterilised through a 0.45 µm membrane. The sterile supernatant (54 ml) was then precipitated with $AmSO_4$ overnight at 4^{o}C using saturated $AmSO_4$ solution (6 ml) giving a final concentration of 20% (w/v). Following centrifugation at 8000 g for 15 min. the supernatant was removed, and the precipitate resuspended in 1.8 ml PBS. To remove excess $AmSO_4$ the filtrate was dialysed overnight at 4^{o}C. After dialysis, 1.5 ml of 0.1% (w/v) SDS in PBS was added to the 1 ml of the resulting filtrate and 2.5 ml applied to a Superdex 200 column (L × I.D. 30 cm × 10 mm) and fractionated by SEC under denaturing conditions using PBS (0.1% w/v SDS) as the running buffer. Fractions were tested for activity against CD630 using a microdilution assay and positive fractions were combined and dialysed overnight in PBS at 4^{o}C to remove remaining SDS. The solution was concentrated using a 10

kDa molecular weight cut off (MWCO) (Vivaspin) to a final volume of 1 ml of dH_2O. This material will henceforth be referred to as SG277 preparative SEC fraction. The sample (ordinarily 30 µl) was loaded on a uBondapack Phenyl, 125 µm 30 cm x 3.9 mm (Waters) column and separated by RP-HPLC using a Waters 600E system controller with a 600 pump and an ABI Kratos 757 absorbance detector. The mobile phase components were (A) 0.5% acetic acid in 60% MeOH and (B) 0.5% acetic acid in 95% MeOH. The fractions were injected in buffer A and the products were eluted at a flow rate of 0.5 ml/min. with a linear gradient of solvent B, developed from 0% to 100% (60 min). The elution pattern was monitored by determining absorbance at 220 nm, and resultant fractions were concentrated using EZ-2 Genevac centrifugal evaporator and then either tested for activity against CD630 using a microdilution assay (2.24.2) or identified by mass spectrometry. For experiments involving combinations of components, specific fractions were combined and then vacuum evaporated. Fractions were resuspended in dH_2O and tested for activity against CD630 in a microdilution assay. MALDI-TOF-MS analysis was performed on a Bruker Reflex III system equipped with a sealed nitrogen pulsed laser (337 nm, line width 0.1 nm, 4 ns pulse duration, 300 microjoules rated pulse energy, average power 5 mW at 20 Hz). A working solution of matrix was prepared by mixing 60 µl of the alpha-cyano-4-hydroxycinnamic acid matrix stock solution in MeOH (MALDI-QUALITY ^{TM}, Agilent Technologies), 30 µl of acetone and 1 µL of 1% (v/v) TFA in a glass vessel. Individual RP-HPLC or SEC-HPLC fractions were mixed with the matrix solution and deposited onto a stainless steel MALDI plate. Mass spectra were acquired using the reflection mode. The instrument was calibrated to the mass accuracy of at least 30 ppm. HPLC and MALDI-TOF analysis performed in close collaboration with Dr Mikhail Soloviev, University of London.

2.32 SEC-HPLC separation of SG277 (Chapter 5)

SG277 preparative SEC fraction were fractionated under denaturing conditions in organic solvent on a SEC-HPLC and TSK Gel G2500PW XL column (7.6 x 300 mm) with isocratic elution at a flow rate of 1 ml/min using a Waters 600E system controller with a 600 pump and an ABI Kratos 757 absorbance detector. The mobile

phase was 65% acetonitrile (ACN) and 0.1% Acetic acid. The eluted material was detected by absorbance at 220 nm. Fractions were collected and dried using a vacuum evaporator prior to further analysis by microdilution assay against CD630 (2.24.2), and MALDI-TOF-MS (described above in 2.31).

2.33 Cumulative activity of HPLC purified fractions (Chapter 5)

SG277 SEC fraction was separated using RP-HPLC (as described above); chlorotetaine, iturin, fengycin and surfactin were collected as individual peaks. On a replicate separation all individually collected peaks were collected into a single tube. All fractions were vacuum evaporated for 5 hours until only a pellet remained. Samples were resuspended in PBS and tested for activity against CD630 in a microdilution assay as a proportion of the initial injection volume. The mathematical sum of the activity was calculated by addition of all activities demonstrated by individual peaks while total activity was determined by testing the individual peaks collected together in a single tube. SG277 SEC fraction pre-separation was used as a control. Commercially produced lipopeptides iturin, fengycin and surfactin (Sigma) were dissolved in 50% MeOH at a concentration of 2 mg/ml. To test commercially produced lipopeptides together, equal volumes of each lipopeptide was combined, vacuum evaporated and resuspended in 50% MeOH at a third of the original volume so that each lipopeptide would be at a final concentration of 2 mg/ml. These solutions were then tested against CD630 in a microdilution assay. 50% MeOH was used in isolation as a control.

2.34 PEG precipitation and caesium chloride centrifugation (Chapter 5)

1 L of a SG277 culture in BHIB was grown from a single colony O/N at 37^{o}C and supernatant collected after centrifugation at 8,000 g for 10 min. The supernatant was then filter-sterilised through a 0.20 µm membrane. Saturated polyethylene glycol (PEG) solution (Sigma) was added to the sterile supernatant to a final concentration of 8% and incubated for 4 hours at 4^{o}C. After incubation, the solution

was centrifuged (10,000 g for 30 min. at 4ºC) and the pellet suspended in 10 ml of SM buffer (per litre; 5.8 g NaCl, 2 g $MgSO_4.7H_2O$, 50 ml 1 M Tris-HCl pH 7.5). 4 ml of the filtrate was then layered on top of a caesium chloride (CsCl) gradient consisting of: 4 ml of 1.3 g/cm^3 CsCl, 4 ml of 1.4 g/cm^3 CsCl and 4 ml of 1.5 g/cm^3 CsCl in that order. This gradient was centrifuged in an Optima XPN-90 Ultracentrifuge (Beckman Coulter) with a SW32 rotor (150,000 g, 18 hours, 4ºC). Following centrifugation bands were carefully removed using a pipette, tested for activity and analysed by transmission electron microscopy (TEM).

2.35 SDS-PAGE and soft-agar overlay (Chapter 4 and 5)

Samples of supernatants were fractionated by 20% SDS-PAGE at 120 V for 1 hour. The gel was fixed for 1 hour with isopropanol (20%) and acetic acid (10%) and SDS was removed by soaking overnight at 37ºC in dH_2O. Gel lanes were carefully excised and placed on BHIS agar supplemented with CD supplement to inhibit contamination but not *C. difficile*. Strips were overlaid with 10 ml soft BHIS agar (CD supplement) (0.7%) containing 1 ml of a logarithmic culture (OD_{600} of 0.4-0.5) of CD630. Plates were incubated anaerobically overnight at 37ºC and observed for zones of inhibition.

2.36 Stability of SG277 filtrate and lyophilised supernatant (Chapter 5)

SG277 was produced by growing a culture for 16 hours in BHIB at 37ºC from a single colony, after which the bacterial cells were removed from the supernatant by centrifugation at 8000 g for 10 min. and the supernatant was sterile-filtered through a 0.45 µm membrane and aliquoted (1 ml) into sterile Eppendorf tubes. For lyophilised samples, 1 ml aliquots of the same filtered supernatant were freeze dried for 24 hours. Aliquots were stored at either -20ºC, 4ºC, RT or at 37ºC for (up to 8 weeks) and one aliquot from each storage regimen was frozen at -20°C every week. A sample stored at -20°C was tested weekly to control for activity loss of already collected samples. For testing of inhibitory activity against CD630 using the microdilution assay, fresh supernatants were thawed and lyophilised supernatants

were dissolved in 1 ml of dH_2O on the day of use. 200 µl was removed from each sample and half of this volume was pre-heated for 15 min. at 65°C prior to the assay. Activity was then calculated as a percentage of the original sample activity.

2.37 Characterisation of SG277 and SG297 extracellular activity (Chapter 5)

The no treatment control was diluted 1:1 in dH_2O (v/v) prior to activity testing, and all samples diluted by treatments were further diluted if necessary, with dH_2O so that filtrate was at a final concentration of 1:1 (v/v). For heat treatments, filtrates (0.5ml) were incubated in an oven at the selected temperature (60^oC, 70 ^oC, 80 ^oC, 90 ^oC and 100 ^oC) for 30 min. and allowed to cool to RT before the assay. Autoclaving of filtrate was performed at 121^oC, 20 psi for 20 min. For simulated gastric fluid (SGF; 0.2% w/v NaCl, 3.5 mg/ml pepsin), three solutions were used at pH, 2, 3 and 4 by adjusting with HCl. Filtrates (0.5 ml) were incubated with an equal volume of SGF for 1 hour at 37^oC before assay. Supernatants (0.5 ml) were incubated with the following enzymes (all from Sigma) at 1 mg/ml final concentration for 1 hour at 37^oC before assay; lysozyme, lipase, amylase. For the proteases, pronase, trypsin and proteinase K (Thermo Scientific) were used under the same conditions as the prior mentioned enzymes. For solvent treatments, 0.5 ml of filtrate was vortexed for 1 min. with 0.5 ml of 100% toluene, chloroform or acetone and incubated for 1 hour. A final concentration of 0.1% SDS (w/v) was mixed with 0.5 ml of filtrate and incubated at RT for 3 hours. Various concentrations of glutaraldehyde (0.1, 0.25, 0.5 and 1%) were incubated with 0.5 ml filtrate at 37°C for 2 hours. Glutaraldehyde was neutralised by glycine at a molar ratio of 1:10. All samples were tested against CD630 in a microdilution assay. The highest dilution factor that showed inhibitory activity was recorded. Treatment only controls (no filtrate) was used for all tests.

2.38 Determination of surface associated lytic activity (Chapter 5)

A single colony of SG277 was grown in BHIB (25 ml) for 8 hours at 37^oC. Cells were pelleted, the supernatant retained, and the pellet washed three-times with

sterile PBS. The pellet was suspended in pre-reduced BHIS broth (25 ml). Aliquots of washed cells and the supernatant were also, separately, heat-treated (80ºC, 30 min.) to kill any residual cells. 0.5 ml of a tenfold dilution of the suspensions (washed cells, and heat-treated suspensions), or 0.5 ml of the retained supernatant (no heat or heat-treated), were individually mixed with 0.5 ml of CD630 that had been grown (anaerobically) in BHIS medium (6 hours, 37ºC) and left for 15 min. in an anaerobic chamber. 0.5 ml of the mixture was added to 4.5 ml of soft (0.6%) micropropagation agar (Apollo Science) and poured onto a pre-reduced BHIS agar plate. Plates were incubated overnight at 37ºC anaerobically. Activity was shown by complete lysis of the bacterial lawn. SG277 cell suspensions were mixed with soft agar and incubated overnight as a control and as aerobes, exhibited no growth.

2.39 Determination of approximate molecular weight using molecular weight cut-offs (Chapter 5)

Using filter sterilised (0.45 μm) supernatant of SG277 grown O/N in BHIB, supernatant was centrifuged (8000 g, 30 min.) through a succession of MWCOs (100 kDa, 30 kDa and 10 kDa). Each time an aliquot of filtrate was removed for testing and the remainder was passed through the proceeding MWCO. After each centrifugation the retentate was removed and normalised using BHIB to the original volume placed into the MWCO. All samples were tested against CD630 in a microdilution assay.

2.40 Effect of MeOH on the molecular weight of the active complex (Chapter 5)

SG277 SEC fraction in dH_2O was centrifuged (8,000 g, 15 min.) through a 30 kDa MWCO filter to ensure activity measured was above 30 kDa. The retentate containing the high MW micelles was collected and MeOH added to a final concentration of 50% (v/v) and incubated for 1 hour at RT. The resulting mixture was centrifuged under the same conditions as described above using a 5 kDa MWCO filter and the filtrate containing the low MW, MeOH disrupted

biosurfactant molecules were collected, aliquoted in equal volumes into 2 separate Eppendorfs and vacuum evaporated. The remaining pellets were resuspended in either dH_2O or 50% MeOH (v/v) and centrifuged using 30 kDa MWCO filters or 5 kDa MWCO filters, respectively. Retentates and filtrates were collected from every step. All fractions were normalised by volume and tested for activity against CD630 using a microdilution assay in order to trace the path of the activity.

2.41 Dynamic light scattering (DLS) analysis (Chapter 5)

Samples were diluted or reconstituted from dried in 150 mM sodium phosphate buffer (pH 7.2) and cleared by centrifugation at 17,000 g for 1 hour prior to analysis. 100 µl was transferred to a micro-cuvette, equilibrated at 25°C and measured in triplicate using Zetasizer Nano ZS (Malvern). The diameter was estimated from the Z-average size. Polydispersity index and intercept were obtained from the cumulants fit using the Zetasizer software v7.11 (Malvern). Individual RP-HPLC peaks used in this experiment; iturin, fengycin and surfactin (1-3) can be viewed in Figure 4.9. This experiment was performed in close collaboration with Dr Enrico Ferrari, University of Lincoln and Dr Mikhail Soloviev, Royal Holloway.

2.42 Protein analysis of SG277 preparative SEC separation (Chapter 5)

As previously described, 1 ml of $AmSO_4$ precipitated SG277 in 1.5 ml PBS (0.1% SDS) was run through a Superdex 200 gel filtration column, and the resulting fractions eluted tested for activity against CD630 in a microdilution assay and for protein concentration using a NanoDrop 2000 at A_{280}nm. In order to ascertain the approximate molecular weight of the eluted active complex, bovine serum albumin (BSA) and lysozyme were dissolved in 2.5 ml PBS and run through the column as standards (2 mg per injection). The presence of these eluted proteins was detected using a NanoDrop at A_{280}nm. For MW approximation under non-denaturing conditions, separation of $AmSO_4$ ppt. SG277 was performed using PBS without the presence of SDS. Four internal standards were used, all of which were dissolved in 2.5 ml PBS prior to injection; IgG (150 kDa), BSA (66.5 kDa), lysozyme (14.3

kDa) and insulin (5.8 kDa) (2 mg per injection). The presence of these eluted proteins was detected using a NanoDrop at A_{280}nm and activity against CD630 in a microdilution assay was tested for. The retention time (min.) was plotted as a function of the log(MW) and used to plot a trendline. The straight-line equation (y = -0.05561 x x + 6.260) was solved for y (SG277 retention time: 26 min.) to determine the molecular weight of the complex.

2.43 Statistical analysis

Statistical analysis was calculated, and significance determined (p <0.05) using Welch's t-test for unequal variance unless stated otherwise. All statistical analysis was performed using Graphpad Prism software.

CHAPTER 3

Characterisation of *C. difficile* spore coat protein CotE

3.1 Introduction

C. difficile is a Gram positive, spore-forming human and animal pathogen and one of the leading causes of nosocomial antibiotic-associated diarrhoea in developed countries (Smits *et al.*, 2016). Symptoms of *C. difficile* infection (CDI) range from mild, self-limiting diarrhoea to a more severe pseudomembranous colitis and toxic megacolon that may result in death. The disease results from antibiotic-induced dysbiosis of the gut microflora that allows spores of *C. difficile* to germinate, proliferate and produce at least two inflammatory cytotoxins (TcdA and TcdB) resulting in tissue damage (Rupnik *et al.*, 2009; Shen, 2012). Toxins A and B are the main virulence factors for CDI. It has been demonstrated that vegetative cell adherence is important and a number of adhesins have been found, including SlpA, an S-layer protein involved in adhesion (Merrigan *et al.*, 2013), flagellin, that could enable cells to penetrate the mucus (Baban *et al.*, 2013), a binary toxin that may be involved in adhesion and Cwp84, a protease that can break down matrix proteins such as fibronectin and laminin (Pechine *et al.*, 2005).

As with many bacterial diseases CDI involves both host colonisation and toxin production. Colonisation comprises a number of distinct phases starting with acquisition of *C. difficile* spores, spore germination, outgrowth and proliferation. Two aspects of CDI are notable. The first is its fulminance with large numbers of spores being produced concurrent with toxin production. In animals, an infective dose of 100 spores can lead to intestinal populations of *C. difficile* 6-logs higher in as little as 24 hours (Lawley *et al.*, 2009). The second is self-limitation, and once antibiotics are withdrawn, infection rapidly subsides (Smits *et al.*, 2016). Studies on *C. difficile* colonisation have mostly focused on the role of the vegetative cell since it had been assumed that the primary role for the spore is in transmission and

dissemination of this strict anaerobe. Whether it is necessary for the spore to attach to the intestine prior to germination is unknown.

The mortality linked to CDI has increased due to the emergence of epidemic and hypervirulent *C. difficile* strains such as the 027/B1/NAP1 strains (Kansau *et al.*, 2016). Over the past decade a multitude of studies have been carried out to try to discern the mechanism behind the severity of 027/B1/NAP1 strains, but the precise causative mechanisms are yet to be found. There are, however, links between a high sporulation rate and hypervirulence, and recently Kansau *et al.* (2016) found that in a mouse model, sporulation of R20291 initiated earlier than in other strains.

Attention has understandably focused on the acute stage of infection, the role of the toxins and the ability of this bacterium to colonise the GI tract. However, a hallmark of CDI is the transient production of enormous numbers of spores ($>10^7$ spores/g of faeces) in the intestine that are ultimately shed to the environs (Lawley *et al.*, 2009). These spores are refractive to antibiotic treatment and the 'supershedder' state resulting from antibiotic therapy would occur in infected patients (and presumably livestock also), but their role during CDI is unknown (Lawley *et al.*, 2009). A number of clues suggest that the spore may contribute to CDI. For example, hamsters challenged with spores of a non-toxigenic strain of *C. difficile* (CD1342) experienced cell damage in the caecum as well as an inflammatory response (Buckley *et al.*, 2013). In a murine model, a *C. difficile spo0A* mutant is unable to persist within and effectively transmit between mice but no conclusion can be made regarding the spore's effect on disease severity due to possible regulatory mechanisms of virulence factors including toxins A and B (Deakin *et al.*, 2012). Interestingly, pre-treatment of rodents with non-toxigenic strains of *C. difficile* have demonstrated that the strain colonises the GI tract yet, following challenge ~40-60 days later with a toxin-producing *C. difficile* strain, these animals were fully protected against CDI (Nagaro *et al.*, 2013).

Other than being an agent of transmission an intriguing question that has not yet been addressed is whether the spore *per se* plays a direct role in the acute stages of infection. The outermost layer of *C. difficile* spore is distinguished by hair-like projections superficially resembling the exosporial layers of *B. anthracis* and *B. cereus* spores (Henriques and Moran, 2007). In recent studies, this outermost layer has been re-defined as a 'shroud' that is thought to consist partly of biomass originating from ruptured vegetative cells (Semenyuk *et al.*, 2014). Beneath the shroud, the electron-dense spore coat comprises thirty or more different proteins (Paredes-Sabja *et al.*, 2014). Of note is the number of antioxidant enzymes including three manganese catalases and a superoxide dismutase (Permpoonpattana *et al.*, 2011, 2013). Finally, and the subject of this chapter is CotE, an 81 kDa bifunctional protein that carries two distinct domains, an N-terminal peroxiredoxin domain and a C-terminal chitinase domain (Permpoonpattana *et al.*, 2011). The peroxiredoxin domain resembles a 1-Cys peroxiredoxin suggesting it may be involved in reducing hydrogen peroxide (H_2O_2) arising as a by-product of SodA-mediated cross-linking of spore coat proteins (Permpoonpattana *et al.*, 2011). The chitinase domain, belonging to the glycohydrolase family 18, might *prima facie* play a nutritional role in the turnover of macromolecules such as chitin.

In this chapter, the possibility of CotE having a more sophisticated function, and potentially a role in pathogenesis will be investigated. This hypothesis is based on two observations. First, some chitinases have been shown to bind mucin and carry mucolytic activity (Kirn *et al.*, 2005; DebRoy *et al.*, 2006; Sanders *et al.*, 2007; Chaudhuri *et al.*, 2010; Low *et al.*, 2013) thus providing a mechanism for colonisation via spore interaction with mucus and potentially targeting toxins to the underlying mucosal epithelium. Second, both peroxiredoxins and chitinases have been shown to be inflammatory or to be associated with inflammatory conditions (Kawada *et al.*, 2007; Sanders *et al.*, 2007; Lee *et al.*, 2011; Salzano *et al.*, 2014; Knoops *et al.*, 2016). Since symptoms of CDI closely resemble other inflammatory enteric diseases such as Inflammatory Bowel Disease (IBD) (Goudarzi *et al.*, 2014), is it perhaps possible that CotE could contribute to symptoms of disease?

This chapter focuses on the chitinase domain of CotE, and its role in the infection process. *In vitro* and *ex vivo* experiments are described that examine whether CotE can bind mucus via interaction with the glycoprotein N-acetylglucosamine (GlcNAc), a component of mucin, and whether this affects colonisation in mice and hamsters. *In vivo,* using hamster and murine models it has been examined whether isogenic mutant spores lacking CotE have differing rates of colonisation, and whether any effect is seen upon symptoms and morbidity within infected animals. The potential of CotE in eliciting an immunogenic response has also been investigated *in vitro*.

3.2 Results

3.2.1 Confirmation of the phenotype of CotE mutants

The two mutants of *cotE* and their complements were created previously and have been described by Permpoonpattana *et al.* (2013). *cotE::CT220s* carries an insertion at the N-terminus preceding both the peroxiredoxin and chitinase domains, while the insertion in *cotE::CT1203s* is centrally positioned and precedes the C-terminal chitinase domain. For simplicity these mutants will be described here as CotE$^-$ (*cotE::CT220s)* and CotEC$^-$ *(cotE::CT1203s*). The schematic below visualises the insertions for both the mutants (Figure 3.1). The crystal structure of CotE has been determined by Tony Wilkinson (University of York, UK) and is shown in Figure 3.2.

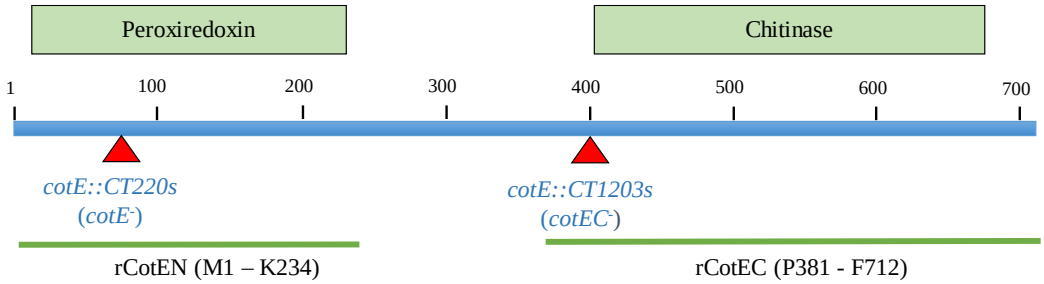


Figure 3.1. Insertional mutation of *cotE*. Shown is a schematic of the CotE polypeptide with showing the peroxiredoxin and chitinase domains together with the ClosTron insertions within the *cotE::CT220s (cotE-)* and *cotE::CT1203s (cotEC-)* mutants. The segments of *cotE* expressed in pET28b expression vectors for expression of rCotEN and rCotEC are also shown.

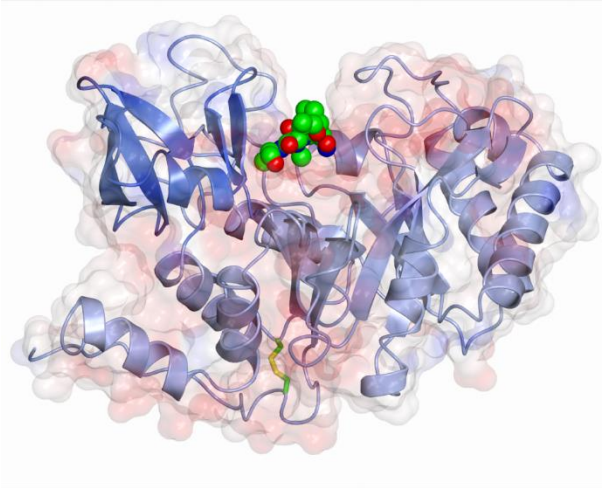


Figure 3.2. CotE structure. Ribbon rendering of CotE (348-718) with the subdomain comprising residues 605-655 in darker blue, the peptide ligand shown as spheres (red - oxygen, green - carbon) and the disulphide bonds as cylinders (yellow). A transparent electrostatic surface emphasises the active site groove. Structural analysis courtesy of Professor Anthony Wilkinson, University of York.

To demonstrate the integrity of both mutants, it was shown that each carried two copies of the *erm* gene, whereas the isogenic 630Δ*erm* parent strain carried one (Figure 3.3). This confirmed the presence of one ClosTron insertion on the chromosome, which was further verified using DNA sequencing. Therefore, the phenotypes observed for the two *cotE* mutants can be directly assigned to the absence of CotE or, alternatively, a pleiotropic effect (e.g., disruption or misassembly of the spore coat resulting from the absence of an intact CotE).

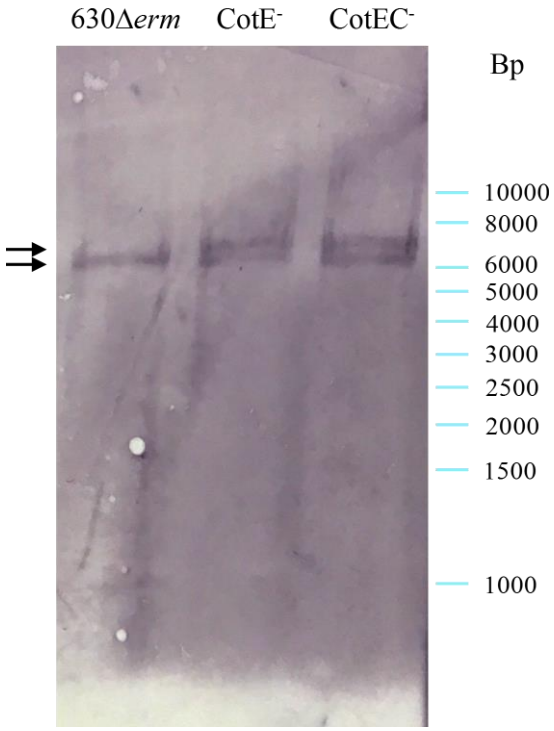


Figure 3.3. Southern blot hybridisation analysis of wild type and CotE mutants. HindIII digested chromosomal DNA from wild type and mutants probed with a labelled probe recognizing the *erm* gene. 630Δ*erm* carries one inactivated *erm* gene accounting for one band while both isogenic mutants carried an additional band indicating the presence of a Clostron intron.

Using polyclonal antisera raised in mice to the N-terminal (residues 1–234) and C-terminal (residues 381–712) domains of CotE, it was confirmed that neither mutant produced CotE (Figure 3.4). This had been shown previously, whereby an antibody recognizing the N-terminus of CotE was unable to detect CotE on spores of both mutants (Permpoonpattana *et al.*, 2013). Similarly, the study showed that antibodies that recognised the C-terminal domain were unable to detect CotE in either mutant. Theoretically, CotEC$^-$ could encode a 133-residue polypeptide carrying the entire peroxiredoxin domain that might be functional whether assembled onto the spore or not; however, it seems that this protein species is unstable and therefore degraded. By contrast, in wild type spores a number of bands were recognised including an 81 kDa species corresponding to the full-length protein and a band of approximately 40 kDa which most likely was a breakdown product. As noted previously CotE is labile and the breakdown products could result from the extraction procedure (Permpoonpattana *et al.*, 2011).

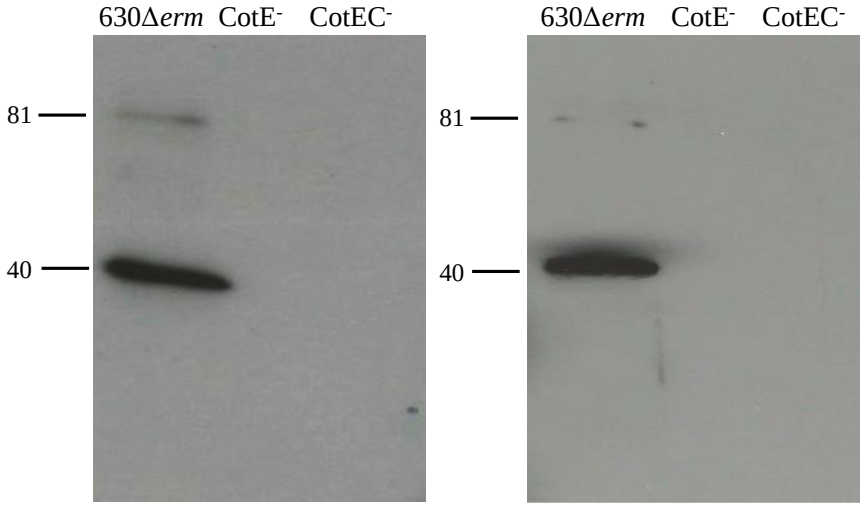


Figure 3.4. Western blot of spore coat extracts from wild type and CotE mutants. Spore coat proteins extracted from 630Δ*erm*, *cotE*$^{-}$ and *cotEC*$^{-}$ Histodenz-purified spores were fractionated on a 12% SDS-PAGE gel. The blot was probed with a polyclonal antibody (1/4000 dilution.) recognizing the C-terminus and N-terminus. Mwts (kDa.) of CotE species are indicated.

In previous work, a pRPF185-CotE complementation plasmid that carried full-length *cotE* was reported (Permpoonpattana *et al.*, 2013). The *cotE* mutant cells carrying this plasmid produced spores that were confirmed to express CotE using Western blotting (Figure 3.5). Functional complementation of the CotE mutant phenotype was demonstrated in this work and will be described here.

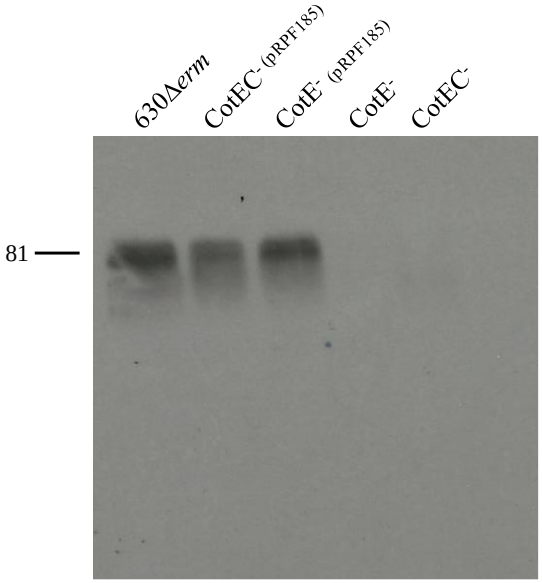


Figure 3.5. Western blot of spore coat extracts from wild type, CotE mutants and complements. Proteins extracted from the Histodenz-purified spore coats of 630Δ*erm*, and the two *cotE* mutants together with each *cotE* mutant carrying pRPF185-CotE, fractionated on a 12% SDS-PAGE gel. The blot was probed with a polyclonal antibody (1/4000 dilution.) recognizing the C-terminus. Mwt (kDa.) of full-length CotE species is indicated.

For *in vivo* assessment of the role of CotE in pathogenesis it was necessary to establish that the mutants had normal levels of toxins since toxin production is linked to stationary phase events such as spore formation and biofilm development (Semenyuk *et al.*, 2014). A cell cytotoxicity assay was performed to confirm that *in vitro* production of TcdA and TcdB was indistinguishable from that of the isogenic wild type (630Δ*erm*) strain (Figure 3.6). No significant difference was observed between 630Δ*erm* and either the CotE mutants or the *sigK*$^{-}$ mutant.

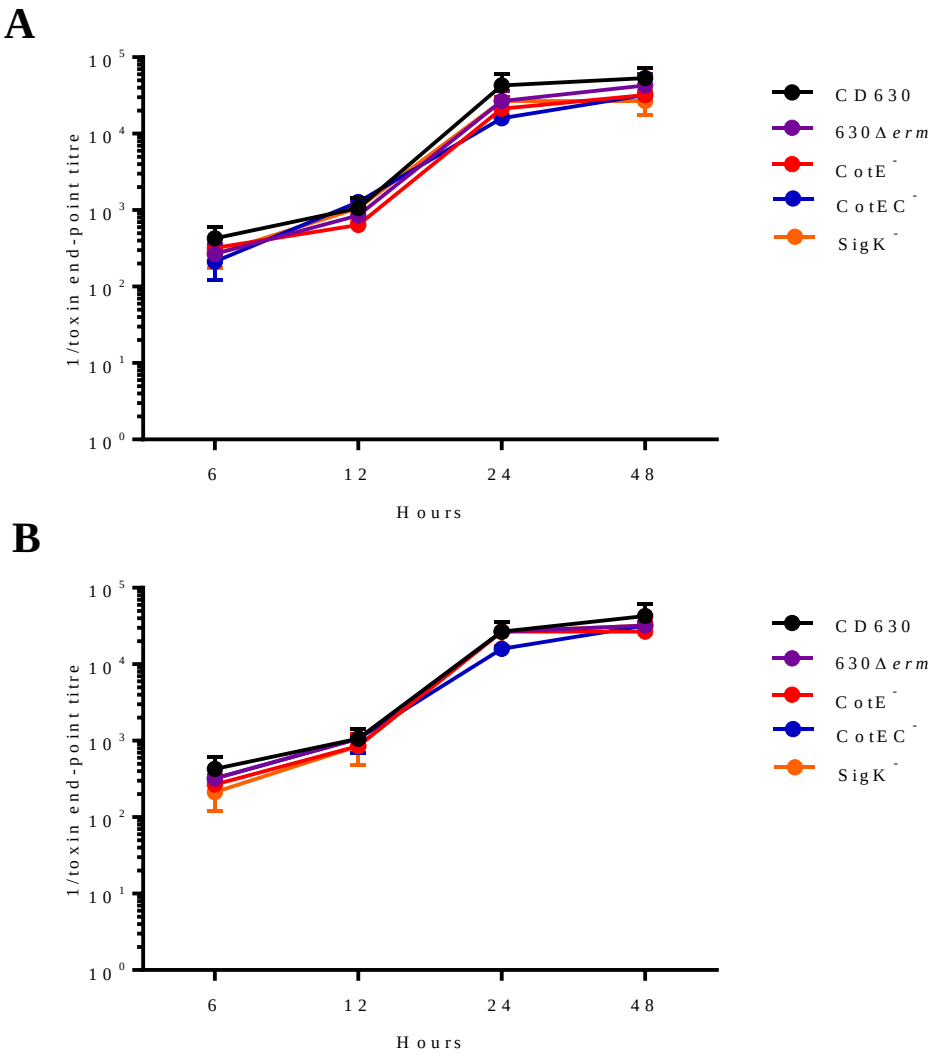


Figure 3.6. Cytotoxicity levels of toxins A and B in wild type, CotE mutants and the *sigK*$^{-}$ mutant. Cultures of 630Δ*erm*, the CotE mutants or *sigK*$^{-}$ were grown in TY media. Culture supernatants were prepared at various time points. Samples were normalised for optical density of the culture and analysed for the presence of toxins A and B by cytotoxicity on HT29 cells (**A**) or Vero cells (**B**) respectively. The experiment was independently performed three times. Error bars represent standard deviations.

A second factor that might affect the behavior of spores during infection is their ability to germinate. Since CotE is present on the spore surface, its absence might pleiotropically affect germination. The ability of mutant and wild type spores to germinate was examined, but no differences were observed between wild type and mutant spores, by the measurement of both heat-resistant and ethanol-resistant CFU counts (Figure 3.7).

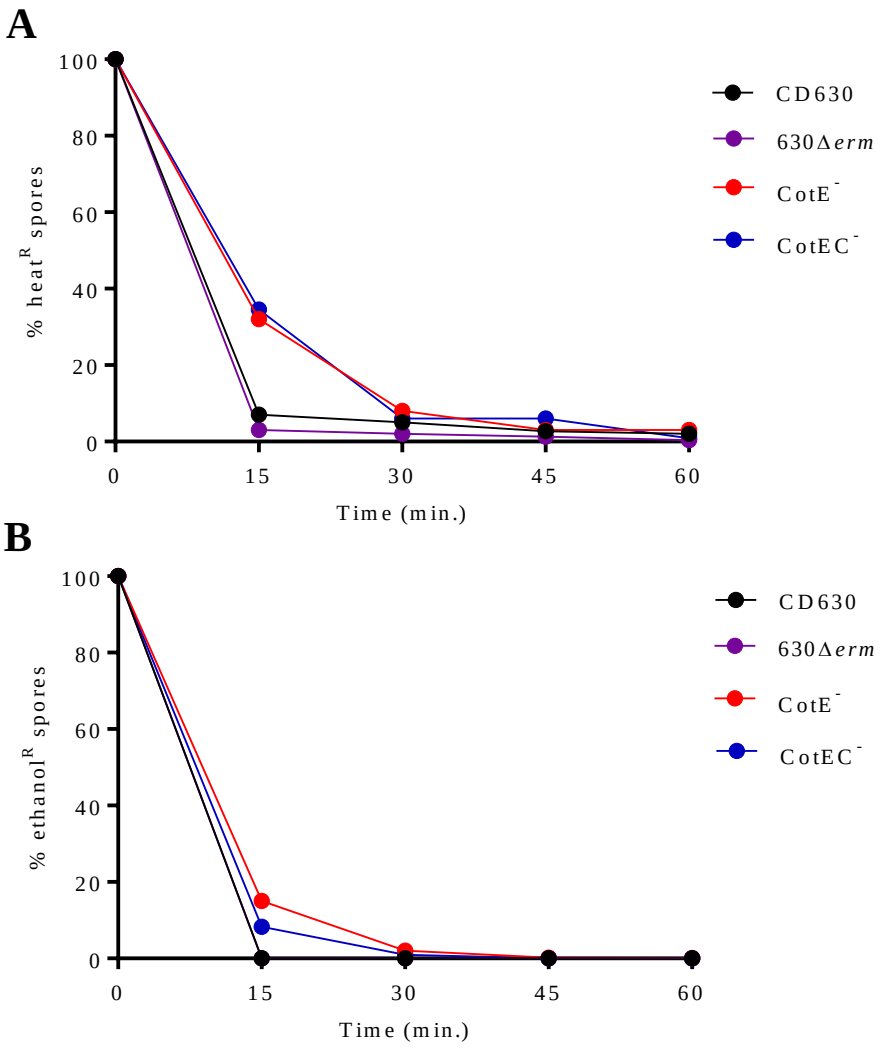


Figure 3.7. Germination of 630 and *cotE* mutants. Spore germination was determined using preparations of Histodenz-purified spores of wild type and mutants. Spores (10^6) were resuspended in BHIS medium containing 0.1% sodium taurocholate, incubated at 37ºC and at the specified time points (0-60 min.) sampled for the number of heat-resistant (60ºC, 20 min.) (**A**) or ethanol-resistant (30 min. exposure) CFU (**B**). Percentage of initial counts are plotted.

Finally, using the SATH assay the hydrophobicity of wild type and the CotE mutants was determined. Differences in hydrophobicity between the strains may contribute to adhesive properties and therefore be a factor in adhesion assays and *in vivo* experiments. The SATH method measures the hydrophobicity of spores and has been used elsewhere as a reliable indicator of spore hydrophobicity (Seale *et al.*, 2008; Huang *et al.*, 2010). However, it was observed (Figure 3.8) that the hydrophobicity of wild type and *cotE* mutant spores was equivalent (630Δ*erm* 75%, CotE$^-$ 77% and CotEC$^-$ 79%).

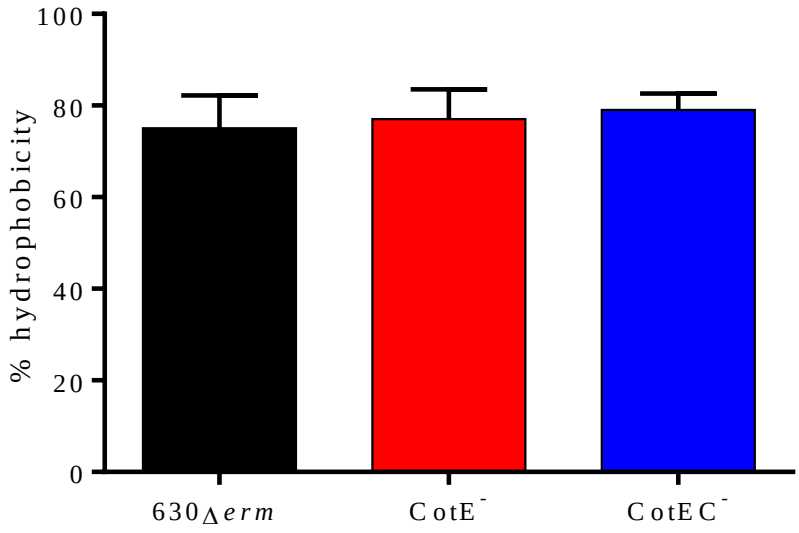


Figure 3.8. Surface hydrophobicity of 630Δ*erm* and CotE mutant spores. The SATH assay was used to calculate % hydrophobicity of Histodenz-purified spores. The experiment was independently performed three times. Error bars represent standard deviations.

To rule out the possibility that a difference in CDI pathology could be due to a difference in toxin production, in addition to checking toxin production *in vitro*, toxin production *in vivo* was determined by measuring toxin A and B levels in collected faecal samples from C57BL/6 mice over 72 hours and confirmed that these were of essentially equivalent levels between all three strains (Figure 3.9).

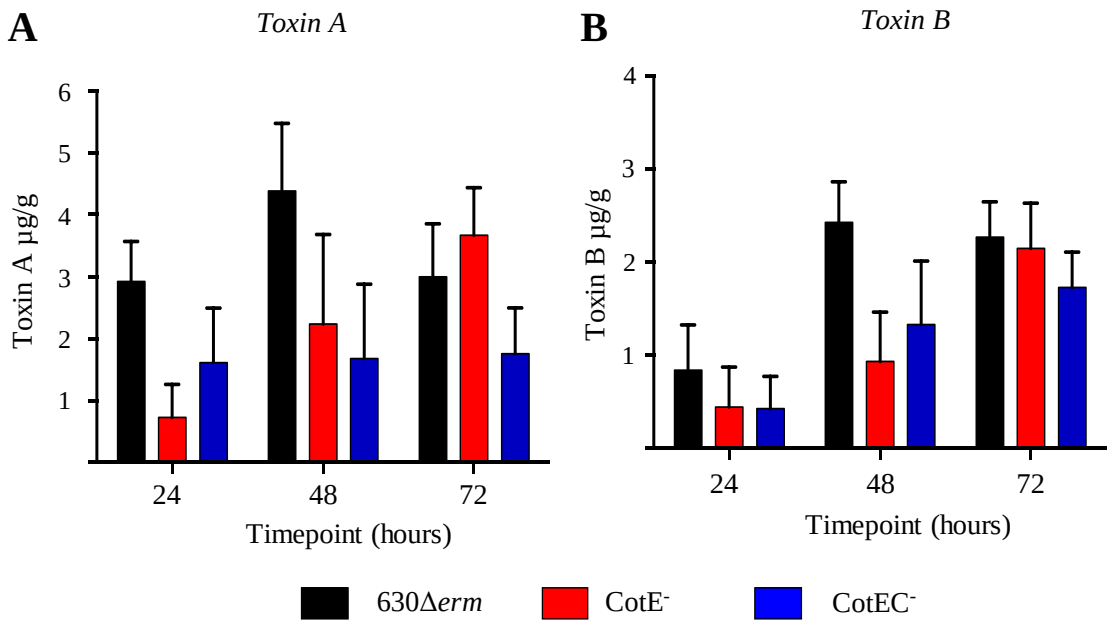


Figure 3.9. *In vivo* toxin production using a mouse model. Clindamycin-treated mice (n = 6/gp) were dosed (i.g.) with 10^3 spores of wild type CD630 or the *cotE* mutants. Levels of toxin A (**A**) and toxin B (**B**) were determined by ELISA in faeces sampled at 24, 48- and 72-hours post-infection. Error bars represent standard deviations.

3.2.2 CotE mediated spore adhesion to mucus

Adhesion of mutant and wild type spores to mucus was evaluated by using the human intestinal cell line HT29-MTX. HT29-MTX is a mucus-secreting cell line that exhibits differentiated goblet cells that secrete low levels of MUC2 mucins typically found in the small and large intestine. Studies using *Salmonella* have shown that HT29-MTX is more physiologically relevant than HT-29 cells for studies of pathogen interactions with the mucus layer (Gagnon *et al.*, 2013). Cells were grown for 14 days to allow for maximal levels of mucus in HT29-MTX and then inoculated with spores and counts were determined after 2 hours of incubation (Figure 3.10). The presence of mucus was confirmed in HT29-MTX cells using alcian blue staining **(**data not shown). Wild type 630Δ*erm* spores exhibited higher levels of adhesion (15-20%) to HT29-MTX cells compared to both *cotE* mutants, which showed significantly ($p \leq 0.001$) lower levels of adhesion. In *cotE* mutant strains carrying the pRPF185-CotE plasmid, levels of adhesion were equivalent to wild type demonstrating functional, *in trans*, complementation of the CotE phenotype. In liquid cell culture medium, it was confirmed that no germination of

spores took place over a 2-hour incubation period (data not shown). Image analysis of antibody-labelled spores adhering to HT29-MTX cell lines (day 14) was also used to confirm that wild type spores were more abundant on mucus-producing HT29-MTX monolayers (Appendix A, Figure 1).

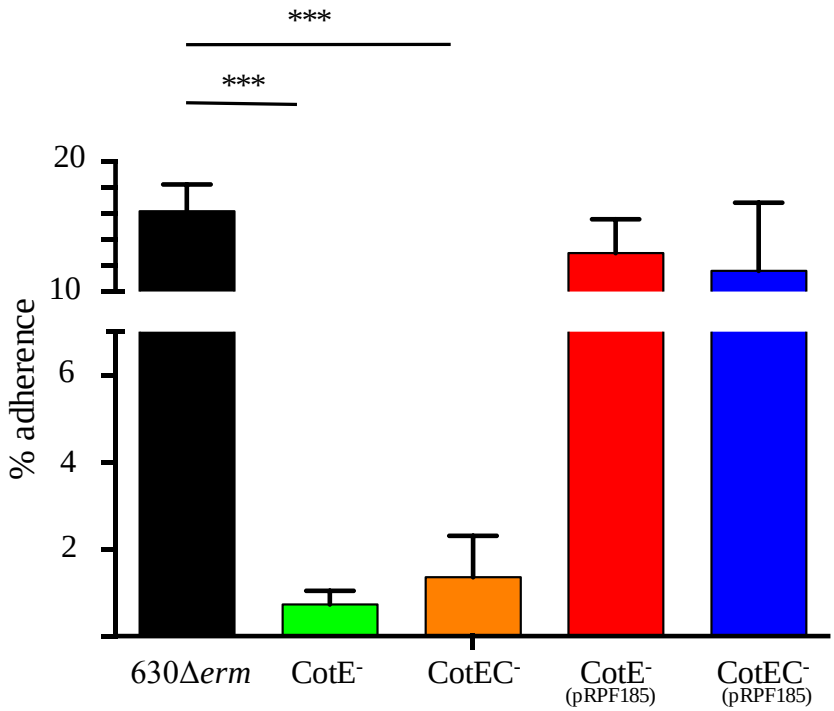


Figure 3.10. HT29-MTX adhesion assay. Spores of 630Δ*erm*, (*cotE*$^{+}$), *cotE*$^{-}$, and *cotEC*$^{-}$ isogenic strains or *cotE* mutants carrying the pRPF185-CotE plasmid were tested for their adherent properties to HT29-MTX cell monolayers (MOI 100:1). The experiment was independently performed three times. Error bars represent standard deviations. ***$p \leq .001$ by the Welch unequal variance t-test.

In a second, *ex vivo* approach the adhesion of wild type and mutant spores to ileum and caecum biopsies obtained from a piglet was measured. These regions of the GI tract are most frequently colonised by *C. difficile* (Goulding *et al.*, 2009; Buckley *et al.*, 2011) and exhibit the highest levels of epithelial cell damage. Samples carried a thick mucus layer allowing direct application of spores to the mucus matrix. Histodenz-purified spores of 630Δ*erm*, CotE^{-} and CotEC^{-} were incubated with explants for 1 and 3 hours. The mucus layer was then washed, and the number of adhering spores determined by counting on BHISS agar plates. This analysis revealed that for both *cotE* mutants spore adhesion to ileum (Figure 3.11A and B) and caecum (Figure 3.11C and D) explants was significantly reduced (~2-fold) compared to adhesion of wild type spores at both incubation times.

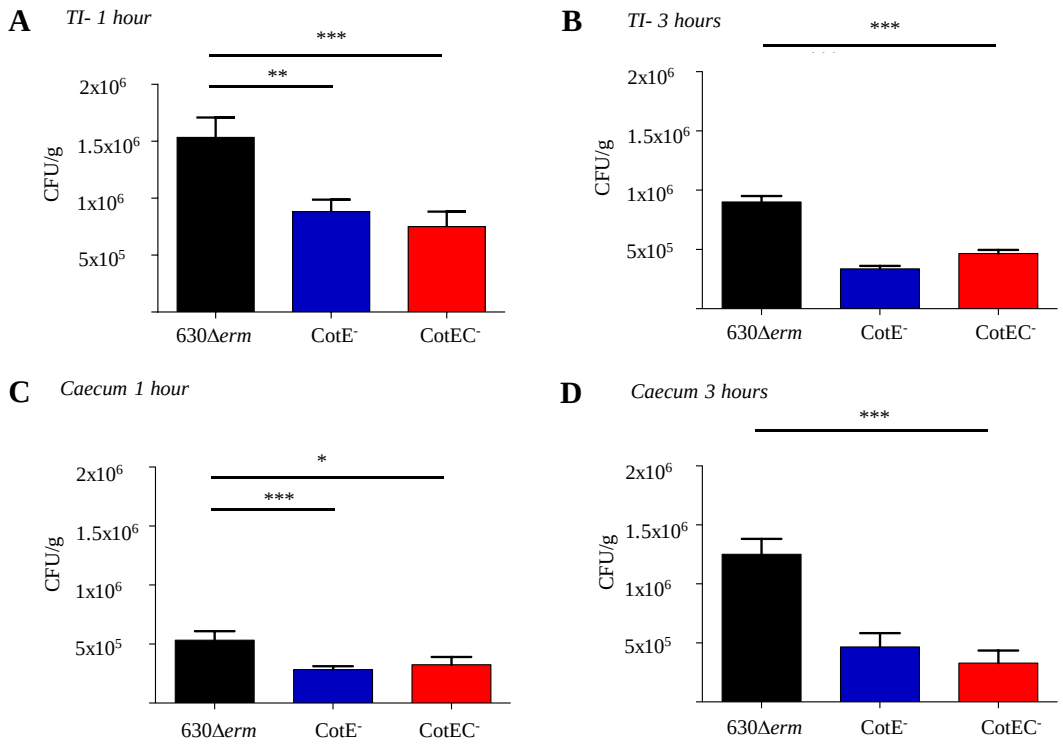


Figure 3.11. Adhesion to pig mucus *ex vivo*. Adhesion of spores of 630Δ*erm*, CotE$^-$ and CotEC$^-$ to pig mucus isolated from tissues *ex vivo*. Tissues were obtained from the terminal ileum (TI, **A** and **B**) and caecum (**C** and **D**). Spores were incubated with tissues for 1 hour (**A** and **C**) and 3 hours (**B** and **D**). Results, expressed in CFU per gram of tissue, are from experiments conducted in triplicate. *$p < .05$, **$p \leq .01$, *** $p \leq .001$ by the Welch unequal variance t-test.

A further experiment, confirming that *cotE* is involved in binding of spores to mucus, was performed using wild type spores preincubated with antibodies recognizing the C-terminal domain of *cotE* before being added to HT29-MTX cells (Figure 3.12). The data showed that adhesion of wild type spores could be significantly reduced by preadsorption with CotE antibodies.

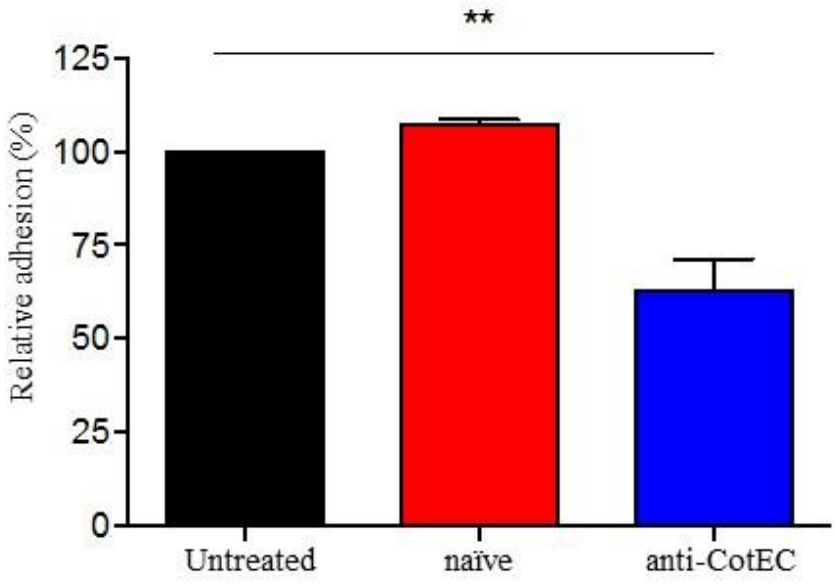


Figure 3.12. Antibody blocking of CotE. Spores of 630Δ*erm* (CotE+) were preincubated with anti-CotEC antibody or naive serum before adding to HT29-MTX cells. Data for untreated spores and spore incubated with naive of anti-CotEC serum are shown. The experiment was independently repeated three-times. Error bars represent standard deviations. $^{**}p \leq .01$ by the Welch unequal variance t-test.

The *in vitro* studies with cell lines and *ex vivo* studies using explants demonstrate that *cotE* mutants devoid of either the chitinase domain or both the chitinase and peroxiredoxin domains have a reduced ability to adhere to cells and that this binding is to the mucus layer.

3.2.3 CotE mediates spore binding to mucin and enhances colonisation

To dissect CotE's affinity for mucus, an ELISA-based method was performed to examine binding of recombinant CotE protein fragments to mucin, the main component of mucus, as well as GlcNAc and GalNAc. GlcNAc is the repeating unit in chitin and is also present in mucin glycoproteins (McGuckin *et al.*, 2011) and glycosylated proteins present on intestinal epithelial cells (Finne *et al.*, 1989). GalNAc is the C4 epimer of GlcNAc and is found (together with GlcNAc) in mucin. These sugars are also components of the glycosaminoglycan side chains present on chondroitin sulfate proteoglycan 4, which is a potential human receptor for *C. difficile* toxin B (Yuan *et al.*, 2015). Significantly more binding of rCotEC, comprising the C-terminal chitinase domain, to mucin, GlcNAc, and GalNAc was

observed compared to rCotEN that carried the N-terminal peroxiredoxin domain (Figure 3.13).

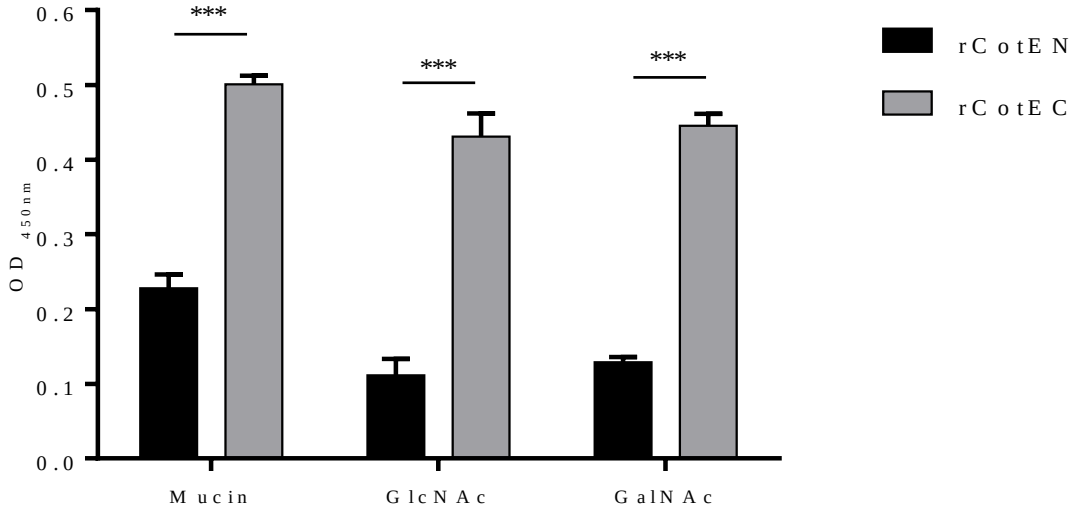


Figure 3.13. Binding of CotE to mucin, GlcNAc and GalNAc. The binding of proteins corresponding to the N-terminal (rCotEN) and C-terminal (rCotEC) domains of CotE to mucin or ligands was measured by enzyme-linked immunosorbent assay (ELISA) at optical density 450 nm. The bars represent the OD_{450} of recombinant proteins at a concentration of 500 Nm. The experiment was performed independently three times. Error bars represent standard deviations. ***$p \leq .001$ by the Welch unequal variance t-test.

In many vegetative pathogenic bacteria, binding to mucus and mucin is a mechanism for attachment to the intestinal epithelium and host colonisation (Kirn *et al.*, 2005; McGuckin *et al.*, 2011). It could be speculated then that CotE enables C. *difficile* spores to interact with mucus. To address this, the ID_{50} of mutants in a murine model was determined (Table 3.1), as previously described by Phetcharaburanin *et al.* (2014). In this model, animals do not show symptoms of disease, and colonisation is measured by the presence of toxin and CFU (2–3 logs higher CFU than initial dose) in the caecum. Wild type 630 spores had an ID_{50} of 10^1 whereas values for the *cotE*$^-$ (10^3) and *cotEC*$^-$ ($10^{2.3}$) mutants were 1–2 logs higher, demonstrating that CotE had a role in the ability of spores to infect and colonise the host.

Table 3.1. The 50% infectious dose of *C. difficile* strain 630 and mutant spores in a murine colonisation model[a]

Strain	ID_{50}
630 (CotE+)	10^{1}
CotE^{-}	10^{3}
CotEC^{-}	$10^{2.3}$

[a] Mice are considered positive for CDI when caecum tests positive for toxin and CFU were 2-logs higher than the initial dose. The ID_{50} calculation was based on the Reed and Muench method (Reed and Muench, 1938).

3.2.4 CotE facilitates the degradation of mucin

Chitin-binding proteins in bacteria can also function as mucolytic enzymes and so CotE was investigated for this trait (Sanders *et al.*, 2007; Mondal *et al.*, 2014). The ChiA2 chitinase from *Vibrio cholerae* utilises intestinal mucin as a source of nutrients enabling proliferation of this pathogen and contributing to virulence (Mondal *et al.*, 2014). Using a modified mucin degradation assay (Zhou *et al.*, 2001), it was shown that CotE facilitated the breakdown of mucin. After 48 hours of incubation, a zone or halo of mucin degradation was observed in amido black–stained mucin agarose wells containing wild type spores (Figure 3.14). By contrast, very limited degradation was apparent in wells containing an identical number of spores for either of the *cotE* mutants. This phenotype, however, was restored in *cotE* mutant spores carrying pRPF185-CotE. Chitinases have been reported to have mucolytic activity and it has been suggested that low levels of proteases present in chitinase preparations may account for mucin degradation (Sanders *et al.*, 2007). Vegetative cells of *C. difficile* have been shown previously to be unable to degrade mucin (Wilson and Perini, 1988) and this was confirmed with the wild type and mutant strains (data not shown).

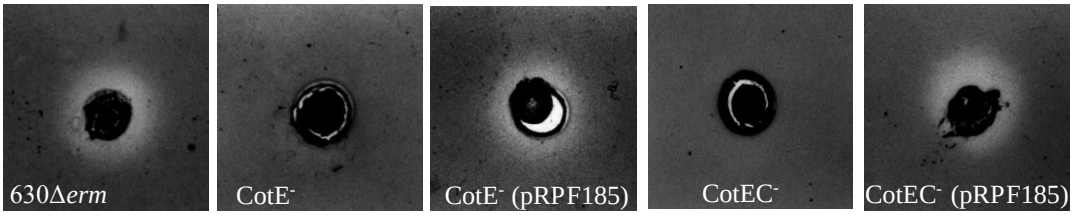


Figure 3.14. Spore degradation of mucin using amido black staining. Mucin degradation assays for 630Δ*erm*, *cotE* mutants, and complemented strains (carrying pRPF185-CotE). Spores were applied to wells cut in agarose containing mucin and were incubated aerobically for 48 hours at 37°C before staining with amido black.

Degradation of mucin by CotE was confirmed using fluorescently labelled mucin and reversed phase high-performance liquid chromatography analysis. Incubation of mucins with wild type *C. difficile* spores resulted in the appearance of a distinct peak, which elutes from the mildly hydrophobic stationary phase by the gradually increasing concentration of organic eluent (Figure 3.15). Although the original mucin preparation is polydisperse and does appear to have two to three distinct fractions under the current separation conditions, the new peak was unique to the wild type spores. This component is positioned ahead of the main mucin elution peak (under the RP mode conditions used), which may indicate reduced overall hydrophobicity of that component compared to the main mucin elution peak. Such a change could be caused by limited proteolysis or cleavage of a small part of the mucin glycoprotein, such as, for example less hydrophilic N or C terminal vWF or Cytseine-rich domains. The latter might be a mechanism used by *C. difficile* to breach the defensive mucus barrier in the gut or simply to generate a more soluble and digestible nutrient source. Both of these are consistent with the need to generate more soluble mucins and to change viscoelastic properties of mucus and are consistent with the presence of mucolytic activity in wild type *C. difficile* spores. The inability of the CotE$^-$ mutant to produce an equivalent increase of the less hydrophobic mucins indicates the absence of either proteolytic or chitinolytic activity on the spore surface.

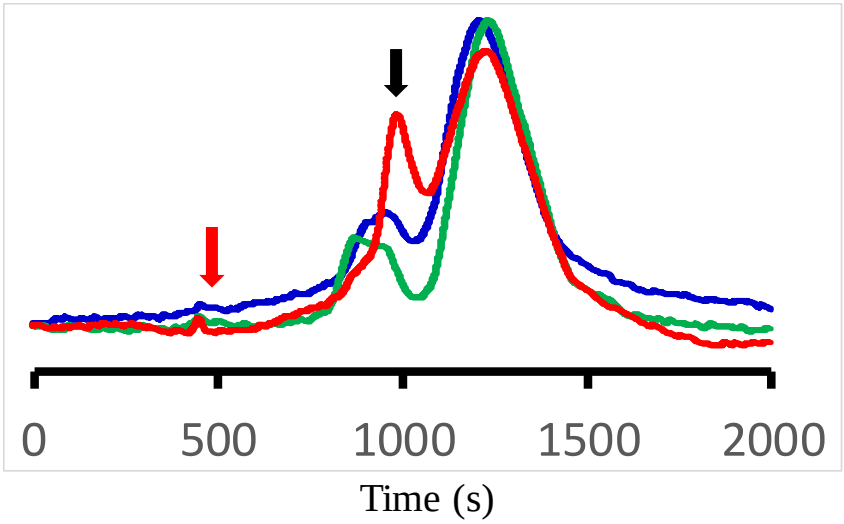


Figure 3.15. Reversed phase HPLC analysis of mucin degradation. The distinct peak (black arrow) indicates the appearance of more polar components and increased solubility of mucins after incubation with wild type (630Δ*erm*) spores only (red line). Treatment of mucin with pure CotE$^-$ spores had no such effect (green line). Untreated mucin (blue line). The red arrow represents the start of the gradient elution. Material detected at 220 nm. Analysis performed in collaboration with Dr Mikhail Soloviev, Royal Holloway University.

3.2.5 The inflammatory properties of CotE

Inflammation is a primary outcome of CDI and is assumed to result from the action of the toxins TcdA and TcdB. Interestingly, as mentioned earlier peroxiredoxins and chitinases are known to be inflammatory and might contribute to symptoms. This is particularly salient if one considers the rapid and exponential increase in spore numbers that typify CDI (Lawley *et al.*, 2009). *In vivo* assessment of the contribution of CotE to inflammation is complicated because as noted already, TcdA and TcdB secreted by vegetative *C. difficile* cells are also inflammatory (Madan and Petri, 2012). Accordingly, qRT-PCR was performed to assess expression of the inflammatory cytokines IL-6, MIP-2α and TNF-α (Figure 3.16) in J774A.1 macrophages that had been incubated with rCotEN and rCotEC proteins. rCotEN was clearly able to induce all three cytokines at significant levels equivalent to lipopolysaccharide (LPS) used as a control. By contrast rCotEC showed no induction of IL-6 and MIP-2α and a low level of TNF-α induction.

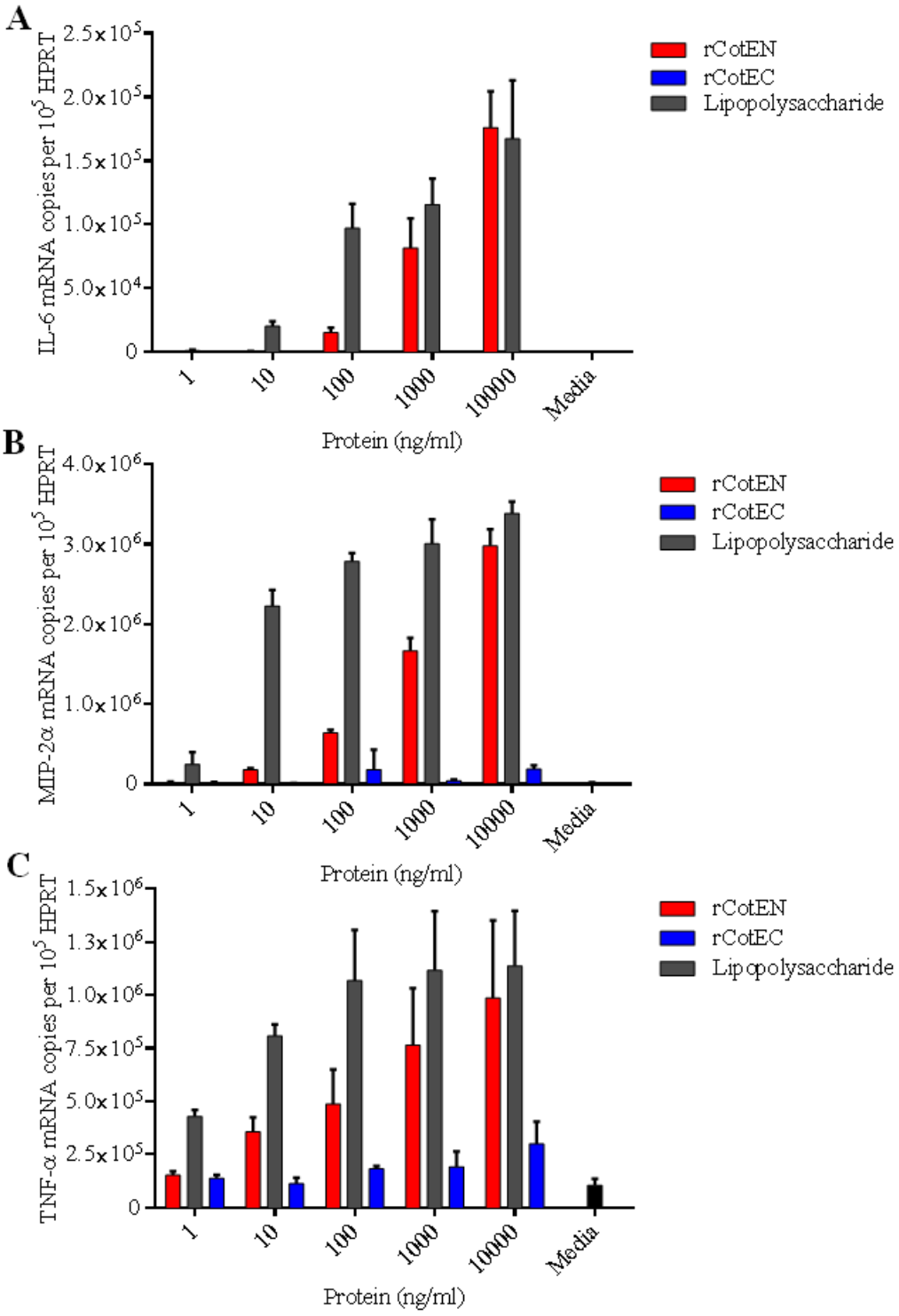


Figure 3.16. ***In vitro*** **cytokine induction using recombinant proteins.** J774A.1 macrophages (1 x 10^6) were incubated (5 hours) with different amounts of rCotEN, rCotEC and LPS. qPCR was used to determine levels of expression of IL-6 (**A**), MIP-2α (**B**) and TNF-α (**C**) compared to endogenous expression of the housekeeping gene HPRT. The experiment was independently performed three times. Error bars represent standard deviations.

In addition, cytokine induction in macrophages incubated (3 hours) with 630Δ*erm*, CotE$^-$ or CotEC$^-$ spores was measured. Under these conditions the time period was too short to allow spores to germinate, grow and produce toxins. The data showed that spores of wild type and both mutants induced expression of all three cytokines but for the *cotE* mutants, expression was significantly reduced in all cases except for TNF-α expression in the CotEC$^-$ mutant (Figure 3.17). The greatest reduction was observed for the N-terminal *cotE* mutant (CotE$^-$) that lacks both peroxiredoxin and chitinase domains. Interestingly, there were also significant differences in expression of MIP-2α and IL-6 between the *cotE* mutants with the CotE$^-$ mutant being less stimulatory. In these experiments, Histodenz-purified spores were used to ensure that there was no contamination with residual vegetative cells, and it was also confirmed that no spore germination occurred during the 3-hour incubation period in the culture medium alone (data not shown). This *in vitro* data suggests that *C. difficile* spores themselves have the potential to induce inflammatory responses and this can be partially attributed to the CotE peroxiredoxin domain.

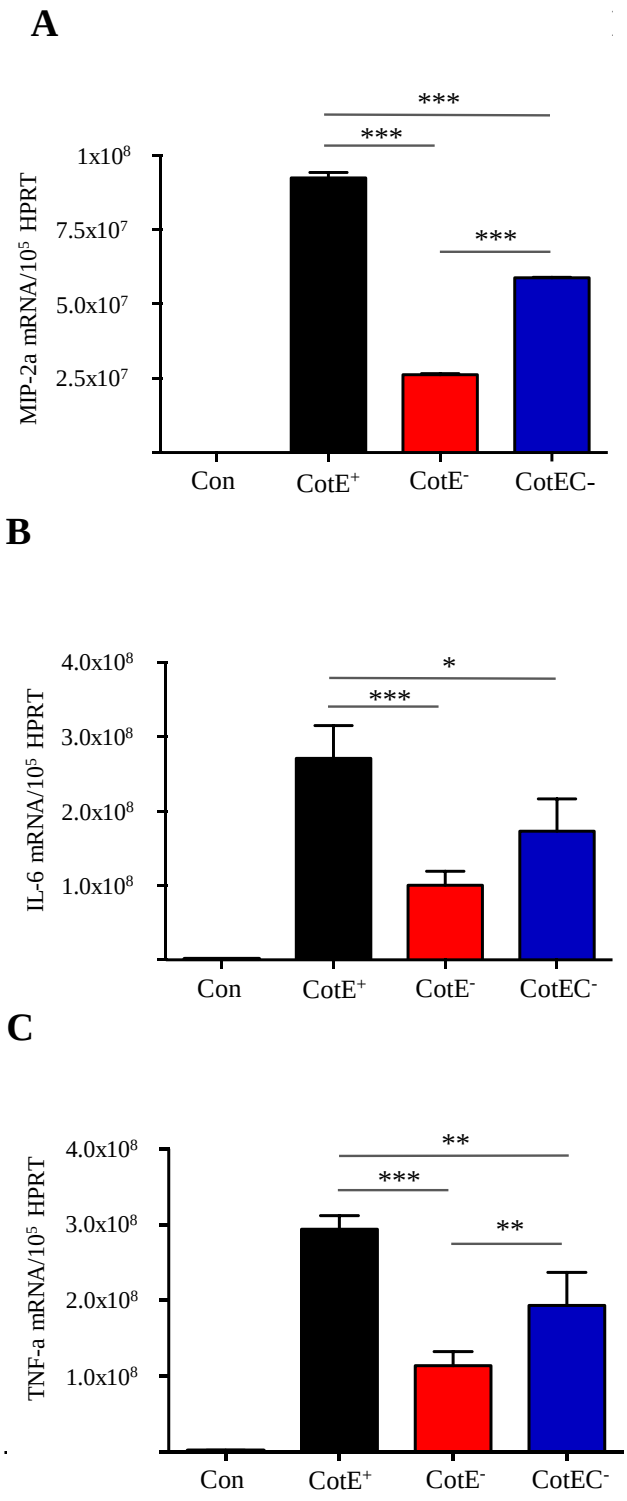


Figure 3.17. *In vitro* cytokine induction using spores. J774A.1 macrophages (1 x 10^6) were incubated (3 hours) with spores of CotE+ *(630Δerm), cotE*$^-$ and *cotEC*$^-$ at a MOI of 10. qPCR was used to determine levels of expression of MIP-2α (**A**), IL-6 (**B**) and TNF-α (**C**) compared to endogenous expression of the housekeeping gene HPRT. The experiment was independently performed three times. Error bars represent standard deviations. *$p < .05$, **$p \leq .01$, *** $p \leq .001$ by the Welch unequal variance t-test.

3.2.6 The effect of CotE on virulence in hamsters

Degradation of mucin by intestinal pathogenic bacteria has been shown to contribute to virulence (McGuckin *et al.*, 2011). Accordingly, the effect of CotE on virulence using the hamster model of infection was investigated (Goulding *et al.*, 2009). In this model, the colonisation of the host can be observed from specific symptoms, or the presence of toxins and/or spore CFU in the caecum or faeces. Virulence is reflected by survival rate and/or by the time from colonisation to clinical endpoint (severe symptoms). In both mutants, 17% of hamsters were not colonised and survived until the end of the experiment (ten days) whereas with wild type, 100% of hamsters were colonised and eventually succumbed to infection (Figure 3.18A). While the time from colonisation to clinical endpoint for 630 was approximately six hours, with CotE^{-} and CotEC^{-} the time was 28 and 27 hours, respectively (Figure 3.18B).

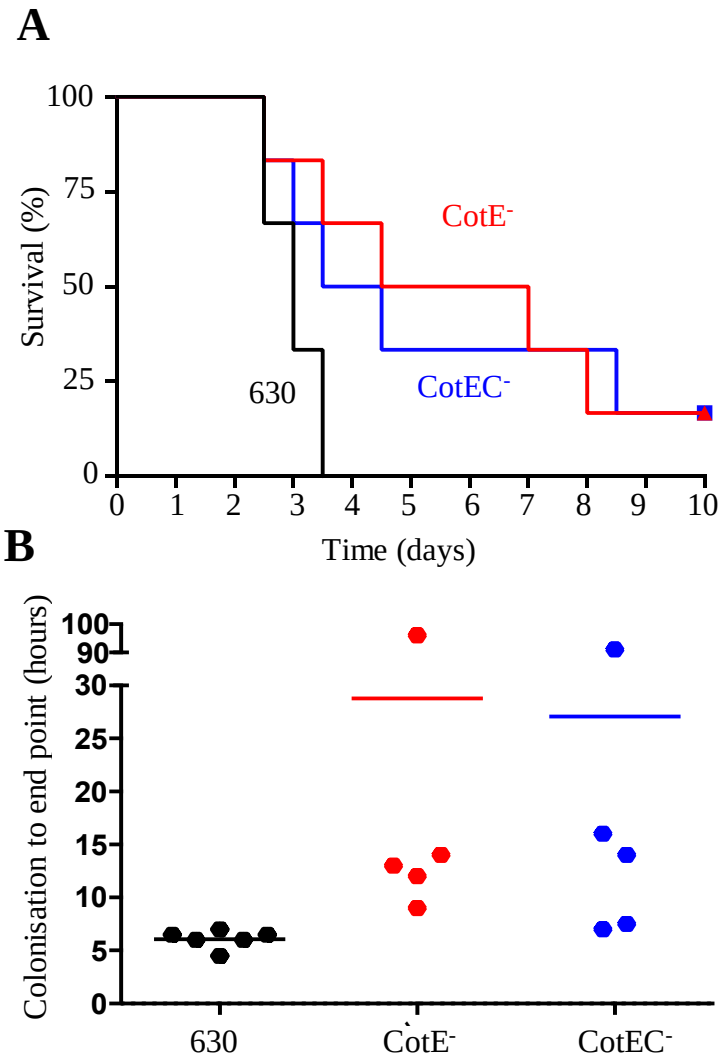


Figure 3.18. Virulence of wild type and *cotE* mutant spores in hamsters. Groups of hamsters (n = 6) were administered clindamycin and, 16 hours later, dosed by intragastric gavage with spores (10^2) of 630, *cotE*⁻, and *cotEC*⁻. Kaplan–Meier survival (**A**). Time from initial symptoms (wet tail and/or presence of faecal toxins) to clinical endpoint (virulent phase) are shown (**B**). Note that one hamster from each mutant group was not colonised and did not show symptoms.

Interestingly, among colonised hamsters (5/6), there was one hamster from each of the mutant groups which was positive for toxin in faeces but developed no symptoms and only reached clinical endpoint after approximately 90 hours (Figure 3.18B). At 82 hours post-inoculation, 100% of hamsters infected with strain 630 spores had reached clinical endpoint, whereas with *cotE*⁻ and *cotEC*⁻ only 33% and 50%, respectively, had become moribund (Figure 3.19A–C).

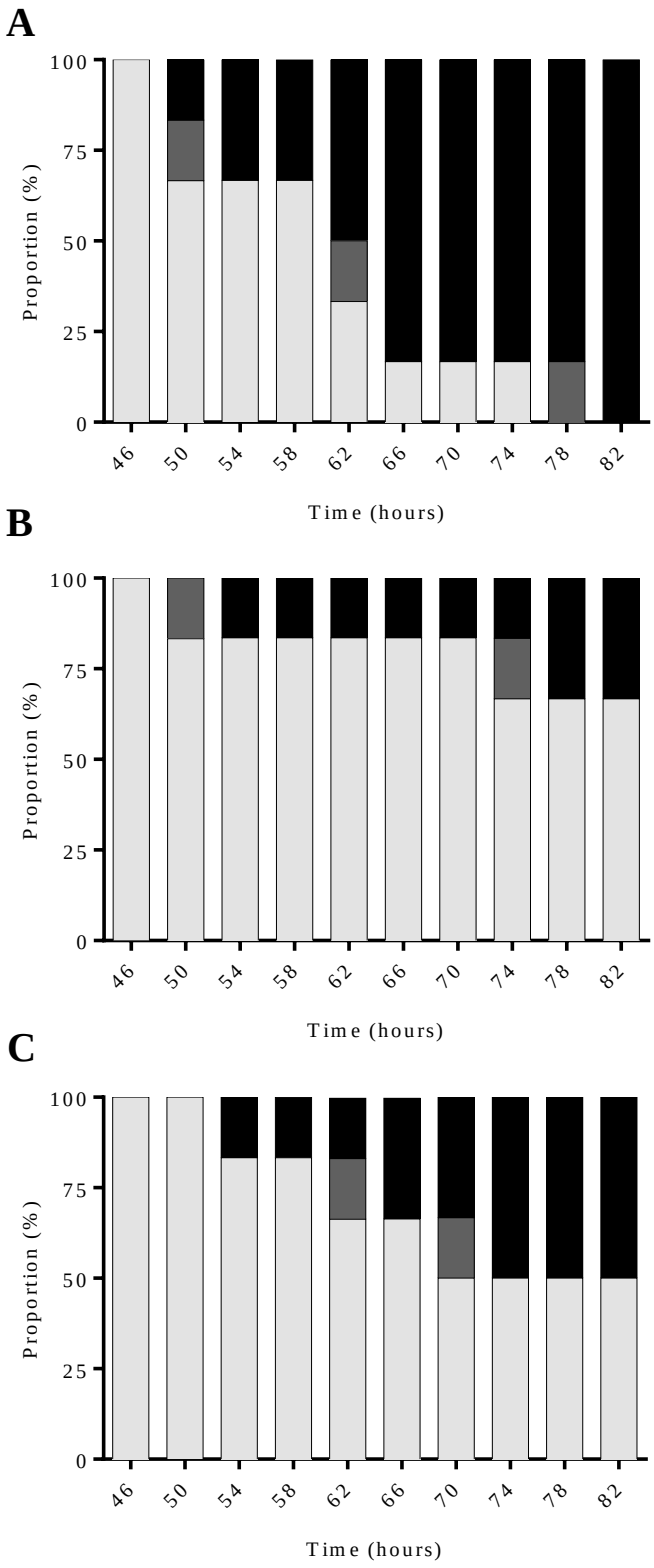


Figure 3.19. Symptom score for 630 and *cotE* mutants challenge in hamsters. Symptoms in the hamster study of Figure 3.18 displayed according to severity. Animals dosed with wild type 630 spores (**A**). CotE^{-} spores (**B**). CotEC^{-} spores (**C**). Light grey – healthy, dark grey – moderate symptoms, black – moribund.

Once hamsters reached clinical endpoint, caeca were checked to confirm the presence of toxins and *C. difficile* spores at roughly equivalent levels between groups (Figure 3.20). To rule out the possibility that the delay in symptoms and morbidity of hamsters could be due to a difference in toxin production, the kinetics of toxin production both *in vitro* (Figure 3.6) and *in vivo* were determined (Figure 3.9) and confirmed that they were of essentially equivalent levels between all three strains. The germination rate of the mutant and wild type spores was also checked and confirmed to be the same (Figure 3.7).

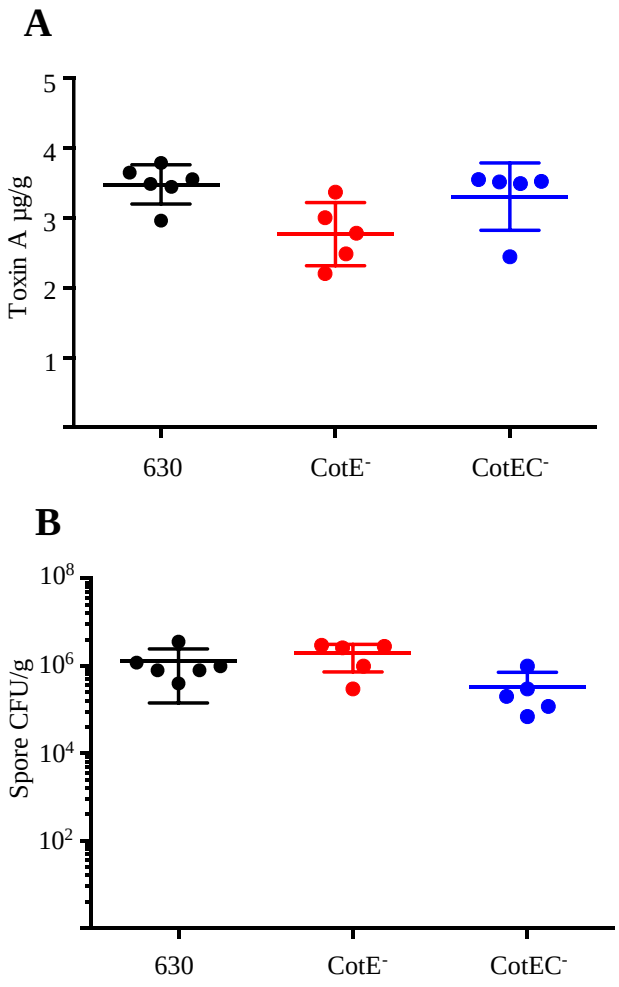


Figure 3.20. Levels of toxin A and spore CFU counts in hamster caecal samples. Hamsters were dosed (i.g.) with 10^2 spores of wild type *C. difficile* 630 or *cotE* mutants. Levels of toxin A (determined by ELISA) (**A**) and counts of ethanol resistant spores (CFU/g) (**B**) in caecum samples of infected animals were determined. The presence of functional toxins A and toxin B was also verified using cytotoxicity assays using HT29 (toxin A) and VERO (toxin B) cells (data not shown).

To investigate whether sporulation affects virulence, vegetative cells of wild type and a sporulation defective *sigK*⁻ mutant were used to infect hamsters following the same dosing regimen as that used above with spores. Mutation of *sigK* (encoding a sporulation-specific sigma factor controlling spore coat biosynthesis (Haraldsen and Sonenshein, 2003) will affect the terminal stages of sporulation-specific gene expression in the mother cell chamber and, unlike *spoOA*, is less likely to exert pleiotropic effects on expression of additional virulence genes. While it took 72 hours for all strain 630–infected hamsters to reach clinical endpoint, 168 hours was required for all *sigK*⁻ infected animals to succumb (Figure 3.21).

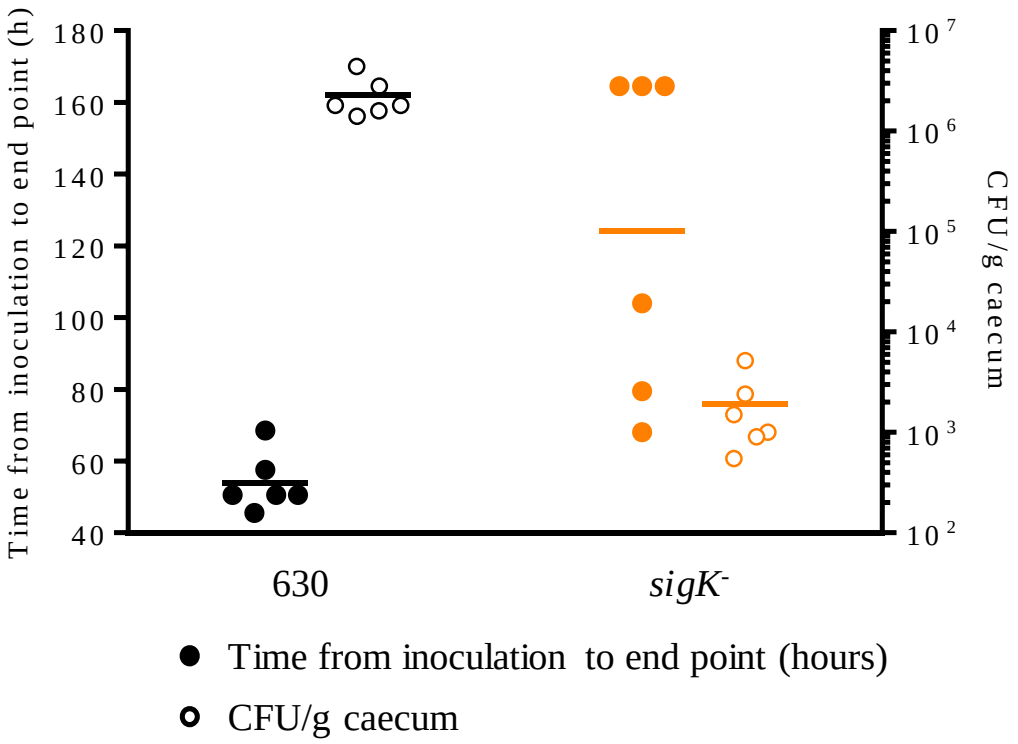


Figure 3.21. *sigK*⁻ mutants display lower virulence in hamsters. Groups of hamsters were administered clindamycin and then, 16 hours later, dosed by intragastric gavage with vegetative cells (10^6) of 630 (*cotE*⁺) or JP051 (*sigK*⁻). Data represented as time from inoculation of *C. difficile* vegetative cells until time of clinical endpoint combined with ethanol-resistant spore counts from caecum.

Since hamsters dosed with *sigK*⁻ cells exhibited delayed mortality, the possibility of differential toxin production *in vivo* was ruled out by measuring toxin kinetics in mice (Figure 3.22). 10^6 vegetative cells of either the *sigK*⁻ mutant or CD630 were dosed to mice (4/gp), faeces collected daily and tested for quantity of toxins A and

B. No significant differences were observed between wild type CD630 and the *sigK*⁻ mutant.

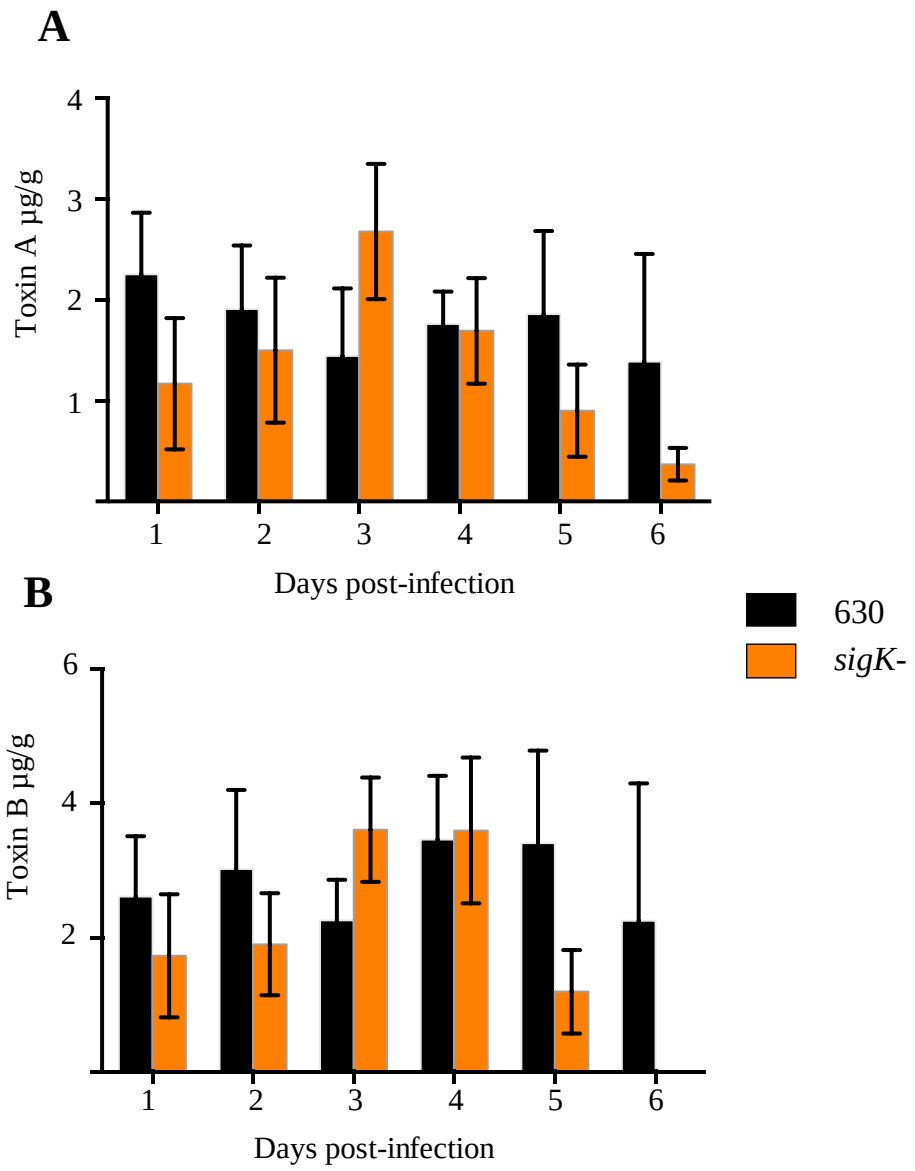


Figure 3.22. Kinetics of *sigK*⁻ toxin production in mice. Using the non-fatal colonisation model (Holloway model) mice (4/gp) were dosed with clindamycin and then 24 hours later dosed (i.g.) with 10^6 vegetative cells of wild type 630 or an isogenic mutant (*sigK*⁻) unable to produce spores. Levels of toxins A (**A**) and B (**B**) were determined by ELISA from faecal samples at the times indicated. The presence of functional toxins A and toxin B was also verified using cytotoxicity assays using HT29 (toxin A) and VERO (toxin B) cells (data not shown). Error bars represent standard deviations.

Note that in caecum samples, a low spore CFU count was found since the *sigK*⁻ mutant being oligosporogenous was presumably able to produce a very low level (approximately 4-logs less spores than 630 per gram of faeces) of functional spores (Figure 3.21) (Pereira *et al.*, 2013). These results suggest that (i) spores contribute

to the virulence of CDI, and (ii) CotE, and potentially additional spore-associated proteins, are involved in colonisation.

3.2.7 Evaluation of GlcNAc and glucosamine in a murine model of CDI

To further test the importance of CotE in the colonisation of the host by *C. difficile*, GlcNAc or glucosamine hydrochloride was orally administered to mice in drinking water (ad libitum) to determine whether they were able to prevent colonisation by CD630. By administering GlcNAc in drinking water, competitive inhibition of the CotE chitinase domain to the GlcNAc present in mucin was attempted, thereby preventing initial binding of the spore and subsequent colonisation. It has been previously shown that GlcNAc can be used to competitively inhibit the binding of *Vibrio cholerae* vegetative cells to chitin (Tarsi and Pruzzo, 1999) and of chitin binding protein GbpA to mucin (Bhowmick *et al.*, 2008). Glucosamine hydrochloride is a supplement often used in the treatment of osteoarthritis (Gregory *et al.*, 2008) of which GlcNAc is a derivative of (Tomonaga *et al.*, 2016).

From day -2 until the end of the experiment mice received sterile drinking water with solubilised GlcNAc or Glucosamine at concentrations of 20, 50 or 100 mg/ml ad libitum. Animals in the naive group received sterile drinking water only. Clindamycin (30 mg/kg) was dosed to mice on day -1 and they were challenged with CD630 (10^2 spores) on day 0 (Figure 3.23).

Throughout the experiment the quantity of GlcNAc and glucosamine consumed was considerably high. All animals in the GlcNAc 50 mg/ml and 100 mg/ml group looked healthy throughout the experiment, but when the caecum was removed it was observed to be slightly swollen and of a yellow colour.

At the end of the experiment the caecum was removed and analysed for Toxin A and B content and for ethanol resistant CFU (Figure 3.24).

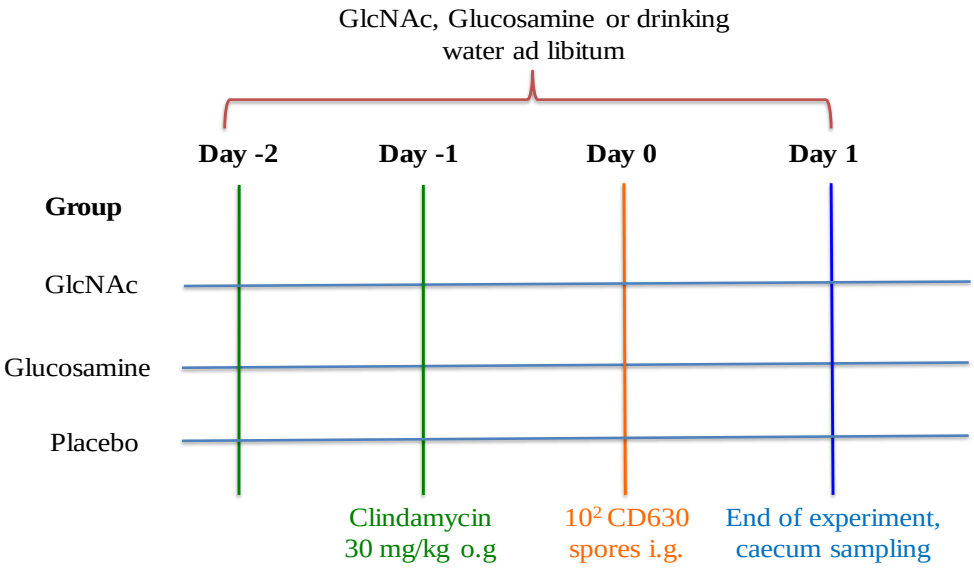


Figure 3.23. Regimen for GlcNAc/glucosamine evaluation in a murine model. GlcNAc and glucosamine given at concentrations of 100, 50 and 20 mg/ml in drinking water. The caecum was analysed for toxins A and B and ethanol resistant CFU.

Throughout the experiment the amount of water consumed by the mice was measured so as to estimate the actual amount consumed. Table 3.2 displays the quantities consumed and the equivalent amount for a 60 kg human.

Table 3.2. Quantities of GlcNAc and glucosamine consumed during the study

Group	Daily dose administered to mouse (mg)	Daily dosage per kg (mg)	Daily equivalent for 60 kg human (mg)
GlcNAc 20 mg/ml	60	2400	144,000
GlcNAc 50 mg/ml	140	5600	336,000
GlcNAc 100 mg/ml	250	10000	600,000
Glucosamine 20 mg/ml	84	3360	201,600
Glucosamine 50 mg/ml	140	5600	336,000
Glucosamine 100 mg/ml	100	4000	240,000

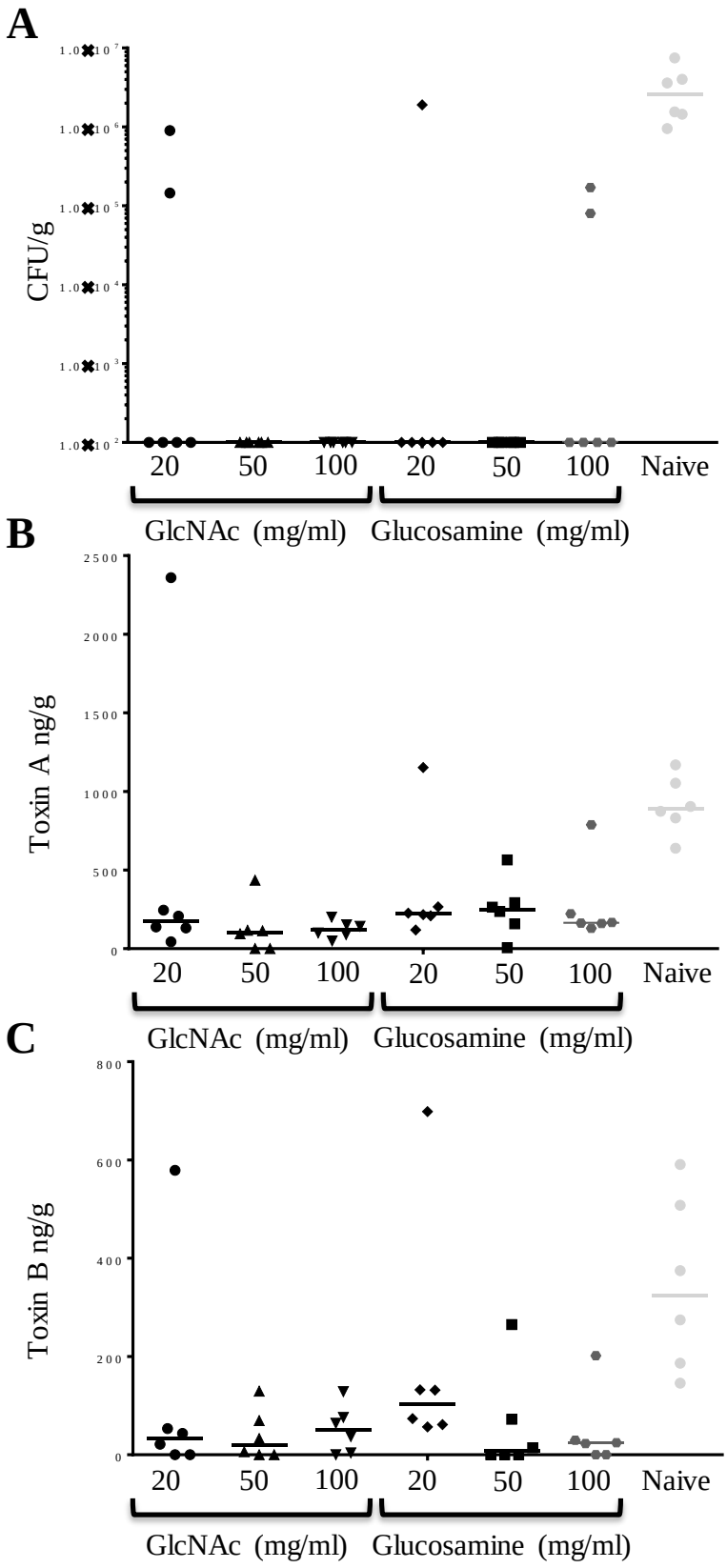


Figure 3.24. Effect of GlcNAc and glucosamine administration on CDI. At 24 hours post CD630 challenge mice were culled, and caecum was removed. Ethanol resistant CFU (**A**), levels of toxin A (**B**) and toxin B (**C**) were determined.

Interestingly, all concentrations of GlcNAc and of glucosamine showed efficacy at preventing CDI. Both toxin levels and CFU counts were diminished when mice were administered either of the two monosaccharides. The lack of CFU would indicate that prevention of colonisation, rather than mere toxin neutralisation had occurred, therefore lending support to the hypothesis that GlcNAc can act as a competitive inhibitor to CotE and prevent interaction with mucin and subsequent colonisation and infection. The concentrations and quantity consumed by the mice was very high and so further experiments will aim to reduce the amount required whilst still preventing CDI.

3.3 Discussion

This chapter provides important insights into the role of the spore in CDI: first, that the spore is responsible for initial colonisation by targeting and binding mucus, enabling the establishment of infection and, second, that the spore is an active agent in the acute stage of infection. Although an interaction of the spore with mucus might not be unexpected, this can now be attributed to at least one spore-associated protein, CotE, a bifunctional peroxiredoxin/chitinase that is embedded in the outermost layers of the spore. What is surprising is that this spore enzyme is functionally active and able to bind directly to mucin monomers (the glycoproteins GlcNAc and GalNAc) and directly or indirectly facilitates mucin degradation. Some chitinases are thought to degrade mucus (Sanders *et al.*, 2007) and chitin binding proteins are considered colonisation factors that have been found in several other important pathogens including *Listeria monocytogenes* (Chaudhuri *et al.*, 2010), *Legionella pneumophila* (DebRoy *et al.*, 2006), and *Escherichia coli* (Low *et al.*, 2013). In each case, the proteins are secreted and have a direct role in virulence, colonisation, and infection. *Vibrio cholerae* is a well-studied example where two proteins, GbpA and ChiA2 (1 of 2 chitinases in *V. cholerae*), act synergistically to bind and degrade both chitin and mucin. GbpA is a chitin-binding protein that is able to recognise GlcNAc present in chitin, as well as on the surface of epithelial cells, enabling colonisation of host cells by *V. cholerae*. Meanwhile, Chia2 has been demonstrated to deglycosylase mucin and release nutritionally

available sugars such as GlcNAc, aiding in the growth and survival of *V. cholerae* vegetative cells (Kirn *et al.*, 2005).

The adhesion of intestinal pathogenic bacteria to the gastrointestinal tract is a crucial aspect of host colonisation as it prevents them being mechanically cleared (Ribet and Cossart, 2015). In the case of *C. difficile*, the spore is the transmissible entity and it has been shown here that for the successful establishment of infection, the spore must also adhere to the host. Interestingly, Theriot *et al.* (2016) demonstrated that, within the small intestine, spores are able to germinate regardless of antibiotic perturbation. It is therefore possible that spores are able to initiate colonisation in the small bowel in the absence of microbiota disruption and that an intact microbiota is required to suppress vegetative *C. difficile* outgrowth to prevent clinical disease. It was recently shown that mucosal antibodies against the C-terminal of toxin A also recognise the spore and vegetative cells of *C. difficile* and prevents CDI in hamsters by blocking colonisation (Hong *et al.*, 2017). Here it has been shown that the spore binds to the gastrointestinal tract prior to germination to initiate CDI via interactions mediated by CotE. In the study shown here, animals (both mice and hamsters) infected with spores lacking CotE show a marked reduction in colonisation. It is important to consider that several adhesins may be responsible for initial spore attachment. In support of this, Phetcharaburanin *et al.* (2014) identified hair-like appendages comprised of the BclA1 protein that make "first contact" with the host mucosa. However, this study observed no differences between the severity of CDI caused by mutant BclA1 and wild type 630 in hamsters.

Spores therefore appear able to enhance the severity of disease once initial colonisation has been established. This is clear from hamster studies showing that the time required to develop symptoms in colonised animals was markedly delayed in animals dosed with spores devoid of CotE. To explain this, several scenarios can be envisaged. One possible explanation could be that by degrading mucus, CotE facilitates transit of toxins to the underlying epithelial cells and so mucin degradation is an integral step in pathogenesis. Another possibility is that the severity of disease is due to the combined action of toxins and spores interacting

with the large number of macrophages and neutrophils produced during the acute stages of infection leading to the production of inflammatory cytokines (Rupnik *et al.*, 2009). In support of this it has been shown, *in vitro*, that spores are inflammatory, and that this response is significantly reduced in the absence of CotE. Also demonstrated is that recombinant CotEN induces high levels of expression of the inflammatory cytokines IL-6, MIP-2α and TNF-α. The spore acting as a virulence factor has been shown in *Bacillus anthracis* where a spore surface superoxide dismutase enhances virulence by as much as 40-fold in a mouse model of anthrax (Cybulski *et al.*, 2009).

Over the past decade, a multitude of studies have been conducted to understand the severity of epidemic and hypervirulent 027/B1/NAP1 strains, but precise causative mechanisms have yet to be found and links between high sporulation rates and hypervirulence remain inconclusive (Akerlund *et al.*, 2006; Burns *et al.*, 2011). A direct role for the spore in acute infection has implications for the concept of hypervirulence, a phenomenon that so far lacks clarity. Most compelling are recent *in vivo* studies showing (i) increased persistence of hypervirulent 027 strains (R20291) in the gastrointestinal tract (Phetcharaburanin *et al.*, 2014) and (ii) increased levels of intestinal sporulation that correlated with increased severity of disease (Kansau *et al.*, 2016). Using isogenic strains, administration of cells of a *sigK*$^{-}$ mutant that exhibits very low levels of spores resulted in an extended period of infection, post-colonisation. One straightforward explanation is that increased spore numbers together with greater intestinal persistence exacerbate symptoms of CDI by CotE mediated mucin degradation and/ or inflammation.

CHAPTER 4

The Role of Allochthonous *Bacillus* in the Suppression of CDI

4.1 Introduction

Although CDI is primarily a nosocomial infection mainly affecting the elderly, studies have shown an increasing rate of CDI in young people and healthy individuals without a history of antibiotic use (Depestel and Aronoff, 2013). Clindamycin resistance has historically been one of the largest contributing factors in the development of CDI in humans and animals (Gerding, 2004). CDI rates are higher in developed countries such as the US and UK, although antibiotics are more heavily used in developing countries (Lessa *et al.*, 2012, 2015; Gupta *et al.*, 2014). Perhaps, the distribution of hypervirulent strains and variations in diets and hygiene contribute to this difference in rates of CDI.

Faecal microbiota transplantation (FMT) has been shown highly effective in the treatment of recurrent CDI. A single treatment has reliably been shown to resolve 85-90% of cases while two treatments, up to 100% (Gough *et al.*, 2011; Costello *et al.*, 2015; Drekonja *et al.*, 2015; Khoruts and Sadowsky, 2016). The scientific rationale for FMT is based on two assumptions, either that patients with dysbiosis have lost their healthy microbiota or that the microbiota is unable to retain its normal functionality. Traditional FMT techniques aim to transfer a stable, viable and diverse microbial community contained in stool preparations from healthy donors. The therapeutically active agent(s) could comprise bacteria, components of faecal water (e.g., the virome) or potentially products of the donor's human cells (Ott *et al.*, 2017).

Due to the potential long-term safety concerns of FMT (Smits *et al.*, 2013; Petrof and Khoruts, 2014) there has been a shift to the use of defined mixtures of bacteria (bacteriotherapy) that have been derived from donor faeces. In a seminal study

using mice, cocktails of intestinal bacteria were isolated from the faeces of healthy animals that prevented the relapse of CDI in mice (Lawley *et al.*, 2012). Interestingly, the intestinal microbiota of treated animals was shown to shift towards that of healthy animals with an increased bacterial diversity. The six species included obligate and facultative anaerobic species representing three of the four predominantly intestinal microbiota phyla (*Staphylococcus warneri*, *Enterococcus hirae*, *Lactobacillus reuteri*, *Anaerostipes* sp. Nov., *Bacteroidetes* sp. Nov, and *Enterorhabdus* sp.nov.). In the Lawley *et al.* (2012) study autoclaved faeces, faecal filtrates and individual strains failed to suppress infection leading to the conclusion that these bacteria displaced the *C. difficile* population by competition. However, FMT has not yet been shown to be effective when used as a primary CDI treatment without the use of antibiotics in conjunction.

The study described above together with others using defined or mixed populations of faecal bacteria (Orenstein *et al.*, 2016) suggest competitive exclusion as the mechanism for suppression of CDI and that the active agents emanate from the bacterial fraction. Therefore, it is possible that the activity might arise from one or more components derived from the bacteria. For example, antimicrobial compounds or metabolites produced by the transplanted bacteria or bacteriophages integrated into the bacterial genome (prophages), which are subsequently activated and released following transfer to the patient. With FMT, stool water together with bacteria is transferred to the patient and this fraction is itself rich in bacterial debris, proteins, antimicrobial compounds, metabolic products and oligonucleotides/DNA. Using just the faecal filtrate, symptoms of CDI were abolished in five patients exhibiting chronic-relapsing infection following nasojejunal transfer of the sterile filtrate (Ott *et al.*, 2017). Symptoms were eliminated for six months post-transfer confirming that the sterile stool water could abolish relapsing CDI. The active agent present in faecal water has remained elusive and no protein candidate was identified. Two explanations were proposed, first, fragments of the bacterial cell wall or DNA that might stimulate (via pattern recognition receptors) the host immune system to facilitate growth of beneficial bacteria. Second, through the

transfer of bacteriophage virions or induction of temperate bacteriophages, that directly or indirectly, suppress growth of *C. difficile*.

The human microbiota contains anywhere from 100-1000 bacterial species with considerable diversity between individuals (Qin *et al.*, 2010). This population is dominated by strict anaerobes, so an important question has been how they transfer between individuals. This can now be partly explained by the remarkable finding that about 60% of genera found in the human GI tract are spore-forming bacteria representing 30% of the total intestinal microbiota (Browne *et al.*, 2016). In this study spore-forming bacteria isolated from human faeces were cultured anaerobically then identified using analysis of the 16S rRNA. Clearly, spores (endospores) have the ability to survive outside of the host indefinitely so are well suited for transfer to other humans.

Culture under anoxic conditions would preclude the isolation and identification of many aerobic spore formers such as members of the *Bacillus* genus. Standard metagenomics is unable to routinely detect bacteria at concentrations below 10^5 bacteria/g (Lagier *et al.*, 2012) but using a culture-based approach coupled with MALDI-TOF and 16S rRNA sequencing the presence of the *Firmicutes* and particularly *Bacillus* species has been illuminated (Lagier *et al.*, 2012). Species of *Bacillus* are not normally considered part of the intestinal flora yet they are readily isolated from human samples (Fakhry *et al.*, 2008; Hong *et al.*, 2009; Alou *et al.*, 2016). Moreover, if present in the GI tract as spores they will be refractory to DNA extraction methods routinely in use, so the presence of aerobic spore formers is likely to be underestimated. Although generally considered as soil microorganisms a number of *Bacillus* species are probably allochthonous members of the intestinal microbiota that are acquired from food and the environment and are thought to be present in human faeces at levels of ~10^4/g (Hong *et al.*, 2009). In the environment vegetative cells of *Bacillus* species are commonly found associated with decomposing plants and the rhizosphere but in the soil they mostly exist in the dormant spore form (Nicholson, 2002). When ingested *Bacillus* spores have been shown able to germinate in the small intestine, proliferate and re-sporulate followed

by shedding in the faeces (Casula and Cutting, 2002; Tam *et al.*, 2006; Cartman *et al.*, 2008; Ghelardi *et al.*, 2015). Although under defined nutrient conditions some *Bacilli* can grow anaerobically (Nakano and Zuber, 1998) they are mostly considered as aerobes. The lower intestinal tract is typically deprived of oxygen but sufficient levels are present in the small intestine to permit growth of *Bacillus* species (Marteyn *et al.*, 2011). The *Bacillus* genus carries a number of species that are thought to have beneficial properties and indeed some are used as probiotics (Hong *et al.*, 2005). *Bacilli* are well known for the diversity of compounds they secrete, notably, enzymes (amylases, proteases, lipases, cellulases), short chain fatty acids that result from fermentation of dietary carbohydrates and antimicrobials and all of these can produce beneficial effects (Ilinskaya *et al.*, 2017). Of particular relevance are the antimicrobials comprising peptide and lipopeptide antibiotics, bacteriocins and bacteriocin-like substances, some of which are non-ribosomally produced (Stein, 2005; Abriouel *et al.*, 2011; Zhao *et al.*, 2016; Ilinskaya *et al.*, 2017). These *Bacillus* antimicrobials have broad-spectrum activity against Gram-positives, Gram-negatives as well as fungi (Abriouel *et al.*, 2011; Ayed *et al.*, 2015).

The efficacy shown by these allochthonous bacteria in preventing infection by pathogens has recently been reported, where intestinal *Bacillus* produced fengycin was able to abolish colonisation in mice by S*taphylococcus aureus* by inhibiting quorum sensing (Piewngam *et al.*, 2018). Previous studies have demonstrated that spores of *B. subtilis* administered to mice or hamsters reduce symptoms and the onset of *C. difficile* infection (CDI) (Permpoonpattana *et al.*, 2011; Colenutt and Cutting, 2014; Hong, Hitri, *et al.*, 2017). Similarly, *Bacillus amyloliquefaciens* has been shown to suppress CDI in a mouse model of infection (Geeraerts *et al.*, 2015). Recent studies have focussed on the undoubtedly important role of secondary bile acids play in the inhibition of *C. difficile* spore germination and outgrowth in non-antibiotic treated, CDI resistant organisms (Buffie *et al.*, 2015; Koenigsknecht *et al.*, 2015; Winston and Theriot, 2016). However, *C. scindens*, a bacterium heavily implicated in the possible prevention of CDI via enzymatic synthesis of *C. difficile* inhibitory deoxycholate is unable to confer full protection against CDI (Buffie *et al.*, 2015). This has recently been supported by the observation that *C. scindens* is

often present in the GI tract of CDI patients (Amrane *et al.*, 2018). These observations therefore predict the involvement of other factors, in addition to bile salt regulatory mechanisms, to underlie the resistance displayed by health individuals against CDI.

A hypothesis was therefore proposed, whereby *Bacillus* species acquired from the environment play a role in helping to control the proliferation of *C. difficile* and this chapter will investigate the mechanism underlying this phenomenon. This hypothesis is based on several observations. The high rate of asymptomatically colonised *C. difficile* in infants (reaching 75%), followed by declining levels (to 0% at 2 years old) with increasing age is counterintuitive, and suggests that exposure to the environment and therefore more *C. difficile* does not result in increasing levels in the GI tract (Al-Jumaili *et al.*, 1984; Yamamoto-Osaki *et al.*, 1994; Jangi and Lamont, 2010; Rousseau *et al.*, 2012). Although this could be explained by the development of a mature microbiota, another contributing factor could be increased environmental exposure allowing the introduction of *Bacillus* and other probiotics to the GI tract which are able to inhibit *C. difficile* colonisation. In piglets the same trend is observed; *C. difficile* colonisation is correlated with animal age, 73-100% of neonatal piglets were found to be positive with *C. difficile* but levels decline to 0% just 2 weeks after birth (Schneeberg *et al.*, 2013; Grześkowiak *et al.*, 2018).

We posit that a soluble substance is present in the small intestine which suppresses the growth of *C. difficile* and is produced by *Bacillus*. This substance has been implicated previously in hamsters whereby soluble factors produced by the microbiota suppress the growth and toxin production of *C. difficile* (Miezeiewski *et al.*, 2015). This soluble factor may also help to explain the successful treatment of CDI by sterile filtrates from donor stool (Ott *et al.*, 2017). These studies suggest that soluble excretions from indigenous microbiota may contribute to resistance against CDI. Due to the complexity of *C. difficile* resistance there are likely to be many contributing factors. Secondary bile acids have been strongly implicated in the control of *C. difficile* germination and outgrowth in the gastrointestinal tract (Sorg and Sonenshein, 2008; Koenigsknecht *et al.*, 2015; Theriot *et al.*, 2016;

Winston and Theriot, 2016). The small intestine is purported to be the site for *C. difficile* germination and the large intestine the site of secondary bile acid mediated growth inhibition (Koenigsknecht *et al.*, 2015). Recently it has been demonstrated that a diet low in microbiota-accessible carbohydrates may contribute to *C. difficile* outgrowth (Hryckowian *et al.*, 2018). In this chapter, data will be shown suggesting that allochthonous bacteria are able to secrete soluble antimicrobials which are able to inhibit the growth of germinating *C. difficile* spores within the small intestine and the protective effects these bacteria have against CDI *in vivo* will be demonstrated.

4.2 Results

4.2.1 Isolation of bacteria from the GI tract with activity against *C. difficile*

As 50-60% of the bacterial genera from the intestinal microbiota are able to produce resilient spores (Browne *et al.*, 2016), both aerobic and anaerobic spore formers were isolated and tested for activity against *C. difficile*. Approximately 1000 colonies of heat resistant aerobic and anaerobic bacteria in faeces of mice and hamsters were isolated prior to screening for activity against *C. difficile*. Manual isolation of colonies was considered to be the most accurate method of assessing the intestinal microbiota, as standard metagenomics is generally considered not able to detect bacteria at concentrations lower than 10^5 bacteria/g (Lagier *et al.*, 2012)

Ten days before the start of the experiment, C57BL/6 mice were kept in IVC cages with autoclaved water, UV-treated food and sterile bedding. Fresh faecal samples were taken before and after treatment with clindamycin. Clindamycin, administered by i.g. gavage, was used at a concentration (30 mg/kg) sufficient to induce CDI but here, the animals were not challenged with *C. difficile*. Homogenised faeces were heat-treated to kill vegetative cells and serial dilutions made on agar plates, which were incubated aerobically or anaerobically. Resultant colonies would arise from heat-resistant spores that had germinated and a total of 500 colonies from the aerobic or anaerobic plates were colony purified for further analysis. First, the number of colonies that inhibited growth of *C. difficile* strain 630 (CD630) were

determined using a microdilution assay that measured activity from a cell-free supernatant of the cultured bacterium (Table 4.1).

Table 4.1. Intestinal spore formers pre- and post-clindamycin treatment.

Source	Spores[a]	Pre-clindamycin			Post-clindamycin		
		No. isolated	No. anti-CD activity[b]	CFU/g faeces	No. isolated	No. anti-CD activity[b]	CFU/g faeces
Mouse faeces	Aerobic spore formers	500	40	1×10^3	500	4	4.1×10^1
	Anaerobic spore formers	500	0	0	500	0	0
Hamster faeces	Aerobic spore formers	100	26	3.25×10^3	100	11	1.38×10^2
	Anaerobic spore formers	100	0	0	0	0	0

[a] spores isolated by aerobic or anaerobic incubation of heat-treated faeces.
[b] activity against CD630 using a well-diffusion assay of sterile cell free supernatants from colony cultures.

Surprisingly, no anaerobic isolates either before or after clindamycin treatment were found to be able to inhibit CD630. Similarly, no *Lactobacillus* (100 colonies picked from MRS selective agar) had activity (data not shown). On the other hand, 1×10^3/g faeces of the aerobic, spore-forming, isolates (n = 40) carried anti-*C. difficile* activity in the extracellular material but after clindamycin treatment the number of isolates carrying anti-*C. difficile* activity had reduced to 4.1×10^1/g faeces (n = 4) (Table 4.1). Interestingly, *Bacillus* species could not be detected in mouse faecal samples using 16S metagenomic sequencing (data not shown). This could be in part due to the presence of *Bacillus* spores rather than vegetative cells or the total number of *Bacillus* being below the detection limit for metagenomic analysis (Lagier *et al.*, 2012).

Figure 4.1 demonstrates the significant differences observed between number of colonies with activity before compared to after clindamycin treatment. Prior to antibiotic treatment, mice had over 1 x 10^3 colonies with activity against *C. difficile* per gram of faecal sample, but this number was significantly diminished after mice were treated with clindamycin.

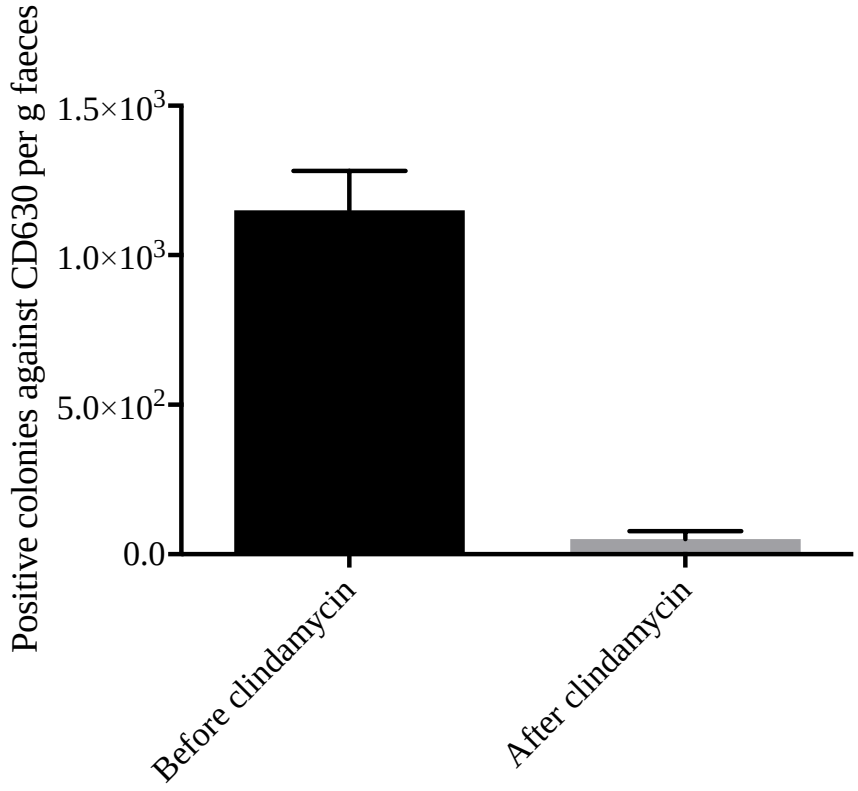


Figure 4.1. Anti-*C. difficile* colonies in faeces of mice before and after antibiotics. Data taken from Table 4.1 with one independent repeat. Error bars represent standard deviations.

A similar study using hamsters was done and a similar phenomenon observed with 3.25 x 10^3/g faeces of aerobic spore formers carrying activity against *C. difficile* and this being reduced to 1.38 x 10^2/g faeces post-clindamycin treatment (Table 4.1). Figure 4.2 highlights the discrepancy between the number of colonies with activity against *C. difficile* prior to clindamycin treatment, compared with after antibiotic therapy.

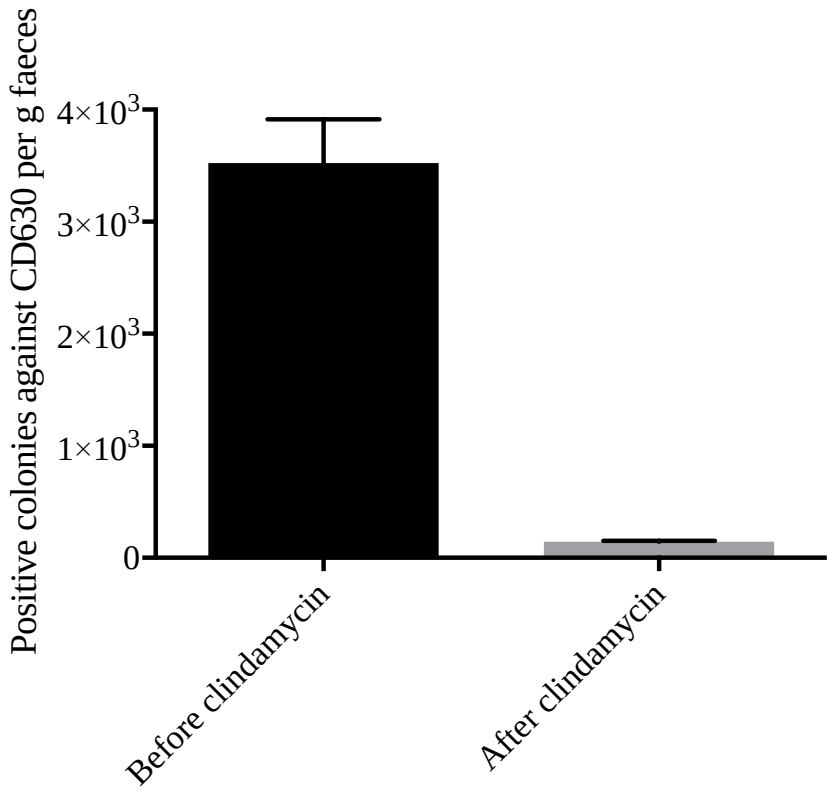


Figure 4.2. Anti-*C. difficile* colonies in faeces of hamsters before and after antibiotics. Data taken from Table 4.1 with one independent repeat. Error bars represent standard deviations.

Subsequently, the aerobic isolates that carried anti-*C. difficile* activity from mouse faecal samples were characterised. Using *gyrA* sequencing, which is more informative than 16S rRNA sequencing (Chun and Bae, 2000), it was demonstrated that only three *Bacillus* species were present, *B. amyloliquefaciens* (n = 22), *B. subtilis* (n = 10) and *B. licheniformis* (n = 12*)* (Table 4.2 and Figure 4.3A). Using an assay for biosurfactant activity, it was demonstrated that the *B. amyloliquefaciens* (n = 22) and *B. subtilis* (n = 10) isolates all carried biosurfactant activity in their cell-free supernatants (Table 4.2 and Figure 4.3B). It was also observed that they all formed mucoid colonies and were sensitive to clindamycin. However, the *B. licheniformis* isolates (n = 12) were all resistant to clindamycin, did not carry biosurfactant activity nor produce mucoid colonies.

Table 4.2. Phenotype of aerobic spore formers with activity against *C. difficile* isolated from mouse faeces[a]

Bacillus species	Phenotype[b]	No. Pre-Clindamycin	No. Post-Clindamycin
B. amyloliquefaciens	$Clin^R$ BS^+	0	0
	$Clin^R$ BS^-	0	0
	$Clin^S$ BS^+	19	3
	$Clin^S$ BS^-	0	0
B. subtilis	$Clin^R$ BS^+	0	0
	$Clin^R$ BS^-	0	0
	$Clin^S$ BS^+	10	0
	$Clin^S$ BS^-	0	0
B. licheniformis	$Clin^R$ BS^+	0	0
	$Clin^R$ BS^-	11	1
	$Clin^S$ BS^+	0	0
	$Clin^S$ BS^-	0	0

[a] from Table 4.1

[b] $Clin^R$ = clindamycin resistant, $Clin^S$, clindamycin sensitive, BS^+, biosurfactant activity, BS^-, no biosurfactant activity. Resistance to clindamycin was determined using a microdilution assay with resistance defined as an MIC of > 4 mg/L (EFSA, 2012).

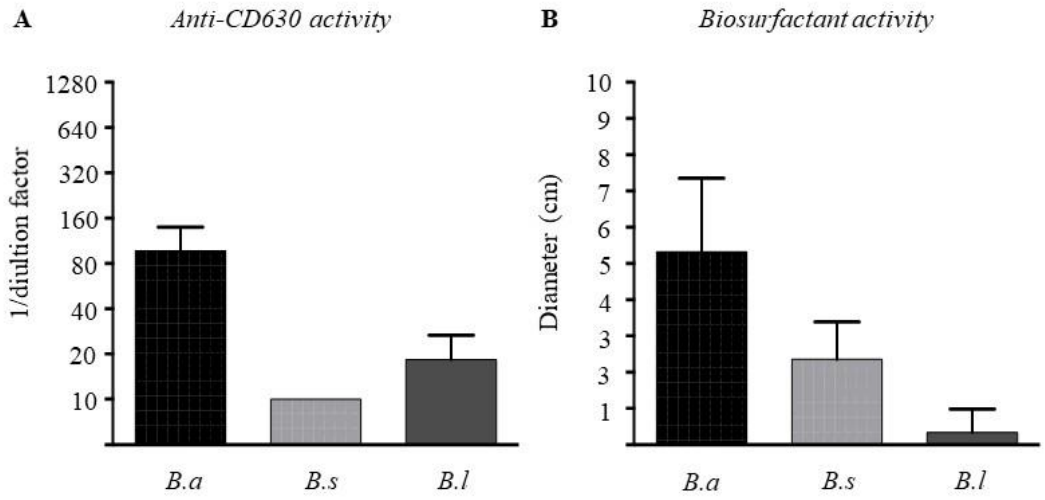


Figure 4.3. Anti-*C. difficile* and biosurfactant activity of isolated colonies. The cell-free supernatants from cultures of *all B. amyloliquefaciens* (B.a.), *B. subtilis* (B.s.) and *B. licheniformis* (B.l.) strains that were isolated from mouse faeces (Table 4.1). Endpoint titres (average) against CD630 were determined using a microdilution assay (**A**) and biosurfactant activity (average) was determined using an oil-dispersion assay (**B**). Error bars represent standard deviations.

Finally, SDS-PAGE analysis of the cell free supernatants produced revealed that *B. amyloliquefaciens* and *B. subtilis* carried a low molecular weight species able to inhibit *C. difficile* when overlaid on the SDS-eluted acrylamide gel (Figure 4.4).

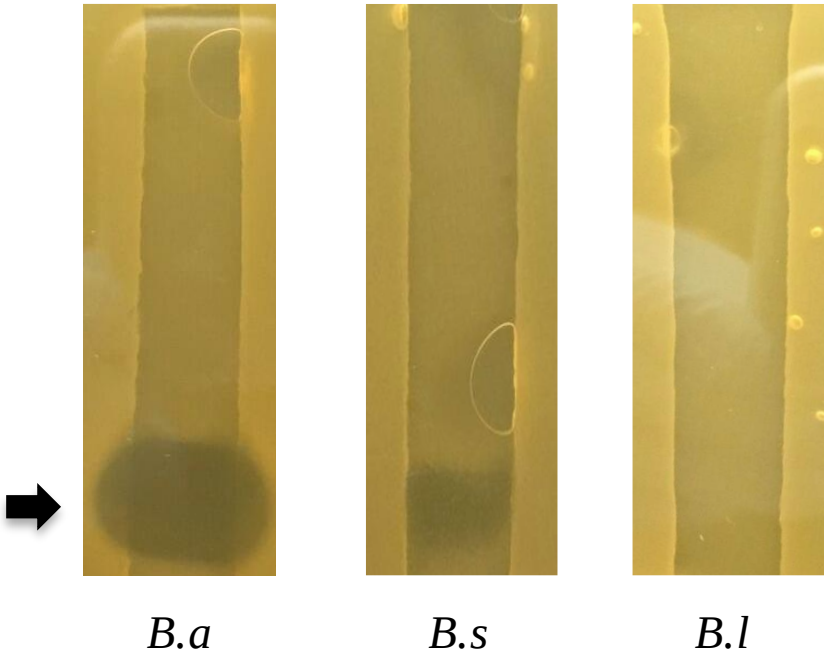


Figure 4.4. Molecular weight of anti-*C. difficile* activity of *Bacillus* species. Samples of supernatants were fractionated by 20% SDS-PAGE. Strips were overlaid with 10 ml of soft BHIS agar (0.7% w/v) containing 1 ml of a logarithmic culture of CD630. A prominent band of inhibition was present in *B. amyloliquefaciens* (*B.a*) and *B. subtilis* (*B.s*) supernatants corresponding to about 1 kDa (arrow), but not with *B. licheniformis* (*B.l*).

The resultant band of inhibition demonstrated by *B. amyloliquefaciens* and *B. subtilis* suggests that the compound responsible is approximately 1 kDa in molecular weight. The absence of a band by *B. licheniformis* may either suggest that the active compound is unstable and has therefore degraded or is present in very low quantities.

In order to show that the intestinal cohort of aerobic spore formers represents an allochthonous population, groups of mice were housed in either conventional cages or IVCs using three animals per cage. IVCs carry HEPA-filtration and prevent exposure of animals to airborne bacteria. Animals received sterile food and water together with regular changes of sterile bedding. Every ten days faeces were examined for the presence of aerobic bacteria including total and heat-resistant

(representing bacterial spores) CFU. As shown in Figure 4.5A, over time, the number of aerobic bacteria identified in faecal samples remained constant at about 10^5 - 10^6 CFU/g. The level of spores (10^3 – 10^4/g) in faeces closely matched the predicted concentration in human faeces (Tam *et al.*, 2006) (Figure 4.5B).

Remarkably, for animals housed in IVCs, spore counts showed a marked temporal reduction and after 40 days no spores could be detected (n.b., 10^2 CFU is at the limit of detection). This decline in aerobic spore counts was not observed in mice housed in conventional cages implying that the aerobic population of spore formers were allochthonous and had been acquired from the environment. Based on this data, C57BL/6 mice caged in IVCs for 60 days, or in conventional cages were challenged with 10^3 spores of CD630 without prior antibiotic treatment to gauge whether the decrease in aerobic spore formers would diminish colonisation resistance and render mice susceptible to CDI (data not shown). However no signs of infection were observed in faecal or caecal samples (toxins or CFU) indicating that other factors are also most likely involved in preventing CDI in healthy mice, such as microbiota linked bile acid regulation (Winston and Theriot, 2016). Another possibility is the remaining presence of *Bacillus* species, but either as vegetative cells in the small intestine or at a level below the limits of detection.

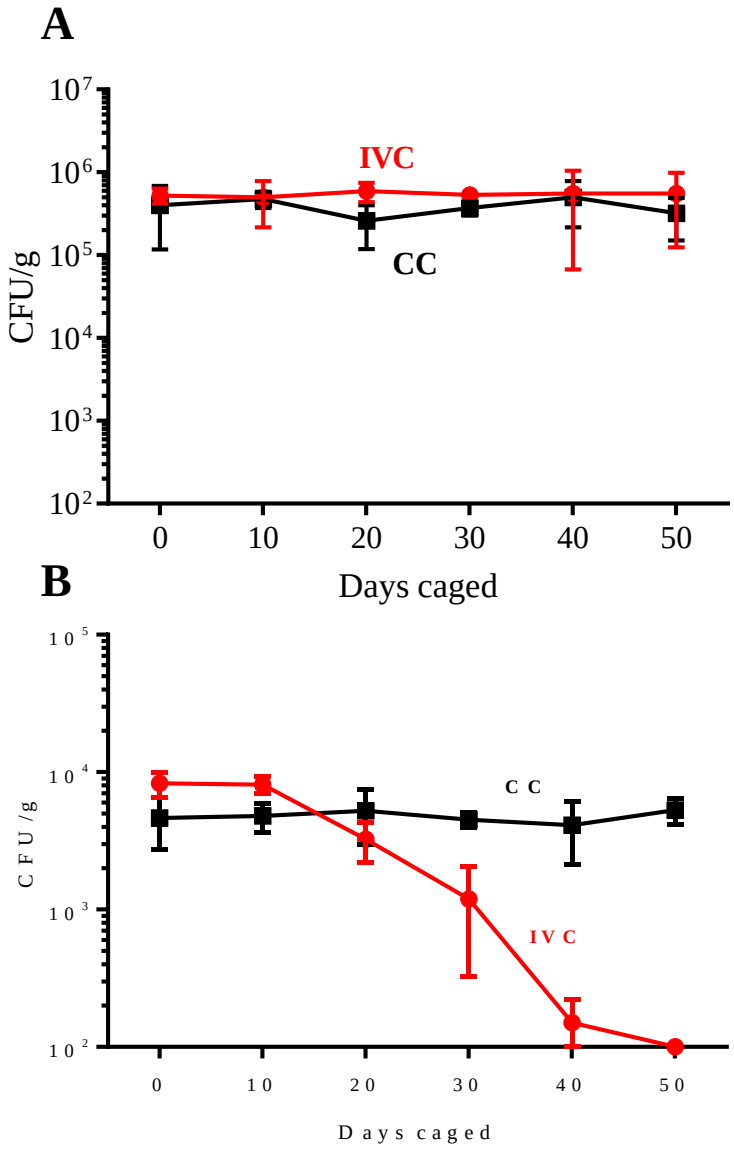


Figure 4.5. Intestinal *Bacilli* represent an allochthonous population. Mice (n = 6) were housed in either conventional cages (CC) or IVCs with three animals per cage. Faeces was sampled every ten days and the total counts (**A**) or heat-resistant, spore counts (**B**), determined following aerobic incubation. The experiment was performed twice. Error bars represent standard deviations.

4.2.2 Dynamics of *C. difficile* germination and growth in the murine gastrointestinal tract

It has recently been demonstrated that upon ingestion, *C. difficile* spores first initially adhere to the mucin within the small intestine via chitinase activity present on the spore surface (Hong, Ferreira, *et al.*, 2017). To colonise the GI tract these spores must first germinate within the small intestine. Studies have shown that

germination and outgrowth of *C. difficile* is dependent on specific primary bile acids, and after antibiotic perturbation of the intestinal microbiota there is an alteration of the bile acid profile within the small intestine which is conducive to both germination and growth (Sorg and Sonenshein, 2009; Giel *et al.*, 2010). However more recent studies have demonstrated *C. difficile* spores' ability to germinate in the GI tract regardless of antibiotic treatment (Koenigsknecht *et al.*, 2015; Theriot *et al*., 2016). An early study looking at the population dynamics of ingested *C. difficile* spores in hamsters also noted the occurrence of germination in non-antibiotic treated animals (Wilson *et al.*, 1985). So, to confirm that germination occurs both in clindamycin and non-clindamycin treated mice *ex vivo* and *in vivo* germination experiments were conducted.

Figure 4.6 shows an *ex vivo* germination assay performed by incubating Histodenz purified CD630 spores with small intestinal contents from clindamycin treated and non-treated mice. Samples were taken at 0, 1- and 5-hours post addition of CD630 spores to the intestinal extracts. Germination of spores was measured by heat resistant CFU counts on BHISS agar plates.

The data demonstrates a similar trend as reported by Koenigsknecht *et al.* (2015) and Theriot *et al*. (2016), with small intestine from both clindamycin treated and untreated mice displaying a similar germination rate. At the 5-hour time point, 100% of the added spores had germinated in both clindamycin treated and untreated small intestine samples. At 1-hour post addition of spores, although approximately 1×10^3 heat resistant spores remained, the germination was significant with a 3-log drop in CFU. Interestingly, it can be observed that in the small intestine of non-antibiotic treated mice there is a significant reduction in viable, vegetative CFU at both 1- and 5-hours post addition of spores. This loss of viability is absent in clindamycin treated small intestinal contents, suggesting that a soluble compound within the small intestine of untreated mice is toxic to *C. difficile* vegetative cells.

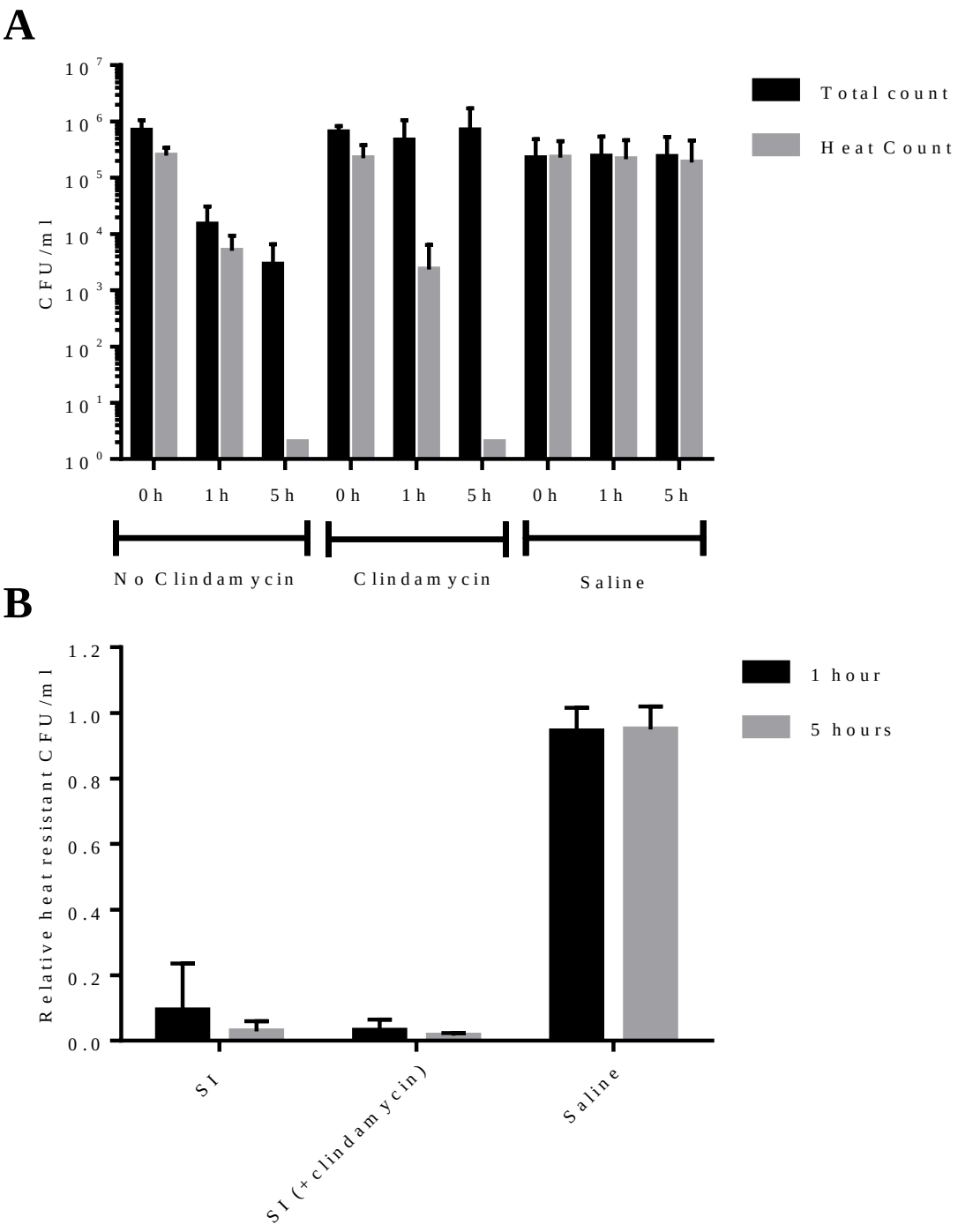


Figure 4.6. *C. difficile* spores germinate in small intestine *ex vivo* regardless of antibiotic treatment. *Ex vivo* germination of CD630 spores (10^6 CFU/ml) in small intestinal contents (SI) of clindamycin and non-clindamycin treated mice measured by comparing heat resistant CFU at 1 hour and 5 hours incubation to the 0-hour time point. A comparison between total and heat counts (**A**) and the relative heat resistant CFU/ml, calculated in relation to the heat count at 0 hour are shown (**B**). The experiment was performed three times. Errors bars represent standard deviation.

To support the conclusions from the *ex vivo* germination experiment (Figure 4.6) and to validate that the same phenomenon occurs within the gastrointestinal tract *in vivo*, a germination experiment was designed using C57BL/6 mice (Figure 4.7), modified from Wilson *et al*. (1985).

The results from this experiment demonstrate that ingested spores rapidly germinate within the small bowel of mice with and without antibiotic treatment (Figure 4.7). At 2 hours post challenge, the proportion of spores within the total count in the stomach of clindamycin treated and untreated mice remains high, 66% and 68% respectively. This indicates that CD630 has a relatively low rate of germination in the stomach of treated/untreated mice, with only 34% and 32% of spores undergoing germination by 2 hours post challenge. However, in the small intestine of clindamycin treated mice only 0.77% of the total count is comprised of spores, indicating a germination rate of 99%. The numbers are similar in the case of untreated mice, with 0.5% of spores comprising the total count and a germination rate of almost 100%. Like the small intestine, the large intestine has a very small proportion of spores comprising the total count, which can be explained by the fact that the spores have already germinated in the small intestine and it is principally the newly germinated vegetative cells which have passed down the GI tract into the large intestine. This has been demonstrated previously by Koenigsknecht *et al.* (2015) who added *C. difficile* spores to small intestinal *ex vivo* extracts and determined within 6 hours the majority of *C. difficile* spores germinate regardless of antibiotic administration.

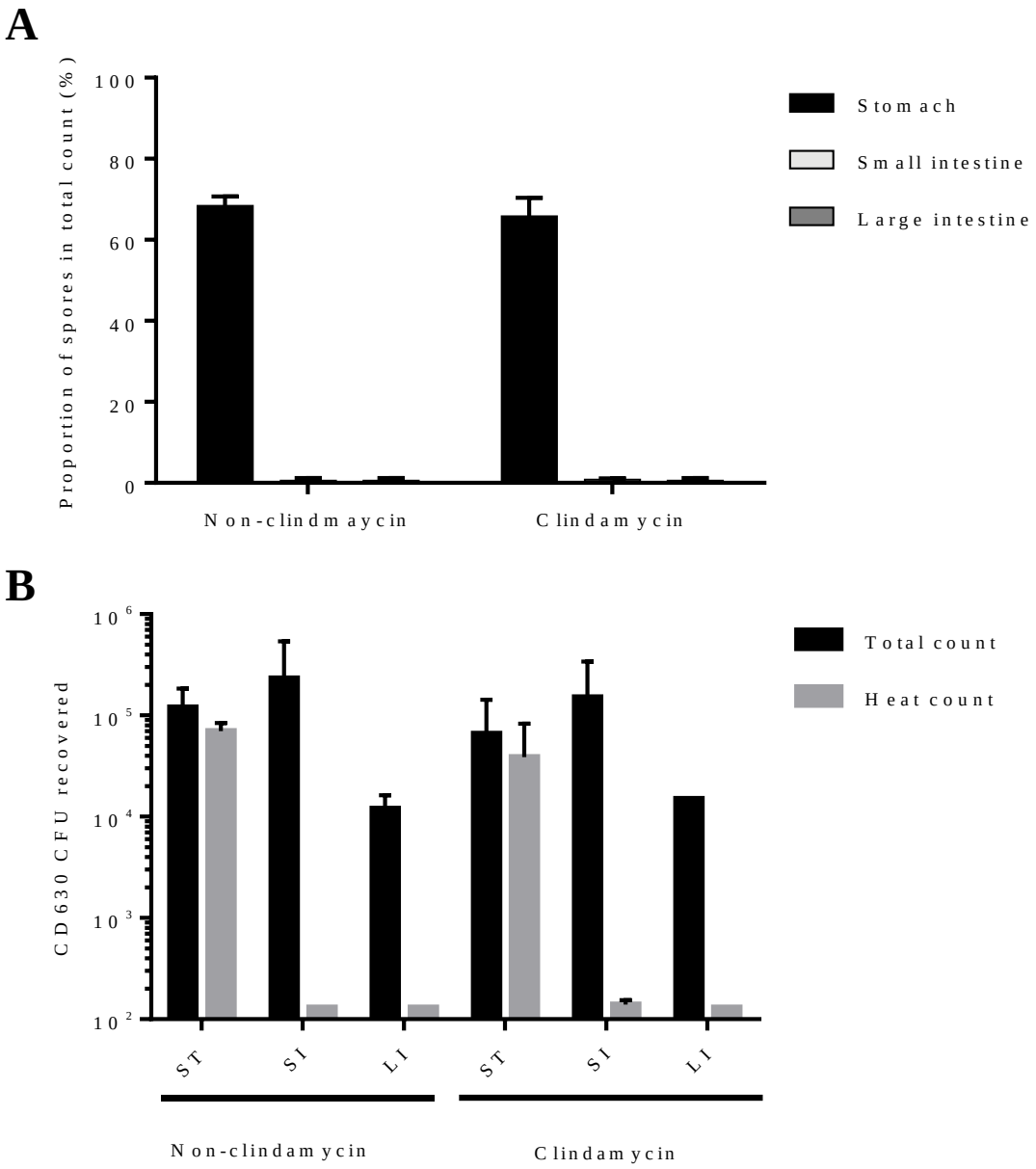


Figure 4.7. *C. difficile* spores germinate in small intestine *in vivo* regardless of antibiotic treatment. *In vivo* germination of CD630 spores at 2 hours post challenge from stomach (ST), small intestine (SI) and large intestine (LI) contents. Mice were dosed with 10^7 spores, culled at selected time points and had intestinal contents removed and plated for CFU counts. The proportion of heat resistant spores (60ºC, 20 min.) as a % of the total count is displayed (**A**), as well as the recovered total, and heat resistant CFU (**B**). The experiment was performed twice. Error bars represent standard deviations.

So, the question would be why, if germination occurs without antibiotic use, does infection not occur? Studies have demonstrated that secondary bile acids play a key role in preventing outgrowth (Shen, 2015; Theriot *et al*., 2016; Winston and Theriot, 2016) but we posit that allochthonous *Bacillus* also have a role to play in the colonisation resistance displayed by healthy organisms against CDI. We

hypothesise that *Bacillus* secrete inhibitory compounds in the GI tract of mice which are able to inhibit the outgrowth of vegetative *C. difficile* and so help to prevent the onset of infection. Antibiotic therapy alters the profile of the microbiota, decimating the population of *Bacillus* (Figure 4.1 and 4.2), thereby ceasing the production of anti-*C. difficile* antimicrobials and the corresponding drop in colonisation resistance that can lead to the development of CDI.

In order to confirm whether the production of soluble microbials by *Bacillus* may protect against CDI *in vivo*, first the purification, isolation and identification of the active substance had to be performed *in vitro*. This was done using a human isolate of *B. amyloliquefaciens* named SG277, which had previously been demonstrated to have high inhibitory activity against CD630 (*see* Chapter 5). The process of characterisation, purification, isolation and identification of this activity will be described in greater detail in Chapter 5. Eventually, active compounds were isolated using the multi-step purification method detailed in Figure 4.8.

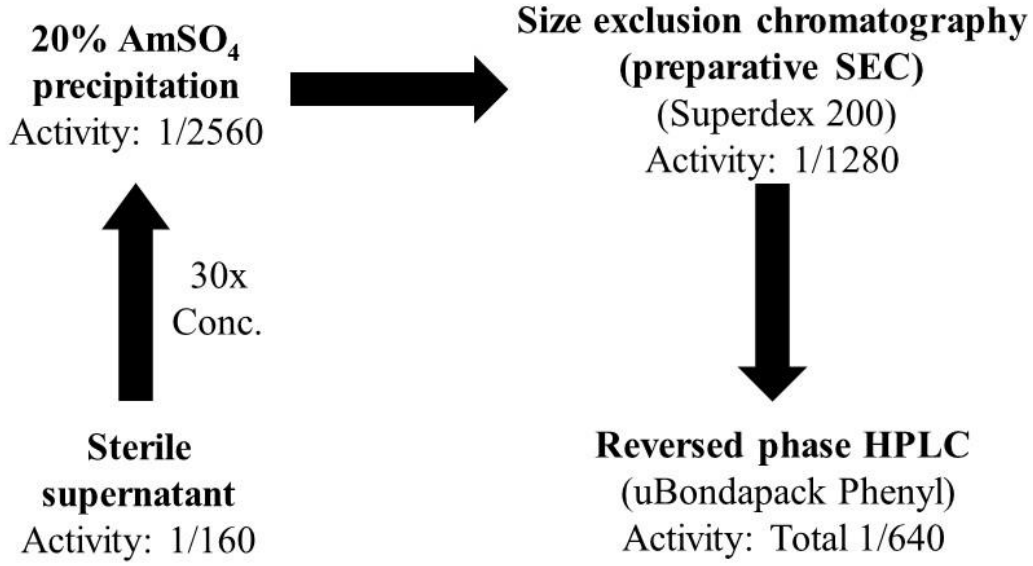


Figure 4.8. Purification procedure for active compounds within SG277 filtrate. SG277 was grown from a single colony in BHIB (37ºC, 16 hours) and the culture centrifuged, sterile filtered (0.45 µm) and the supernatant ammonium sulphate precipitated at a final concentration of 20% (w/v, 4ºC, O/N), and resuspended at a final concentration of 30x in PBS. The resulting precipitate was dialysed followed by resuspension in PBS (+ 0.1% w/v SDS) and fractionated using size-exclusion chromatography. Active fractions were recombined, washed using a 10 kDa MWCO and loaded on a uBondapack Phenyl column and separated by RP-HPLC. Activity was tested against CD630 at every stage using a microdilution assay. Activity for the RP-HPLC separated fractions were measured by combining the total separation.

Using 20% ammonium sulphate ($AmSO_4$), the SG277 precipitate was found to carry activity against *C. difficile* as well as biosurfactant activity. The apparent low molecular weight by SDS-PAGE (Figure 4.4) did not agree with the $AmSO_4$ precipitation since 20% $AmSO_4$ should only precipitate high molecular weight or possibly hydrophobic molecules. Gel filtration of the 20% $AmSO_4$ precipitate using a Superdex 200 column and the subsequent high molecular weight elution (see Figure 5.9) also confirmed that the active components are of higher molecular weight, suggesting perhaps the formation of a complex.

To characterise the active components further, size-exclusion chromatography (SEC) and a microdilution assay were utilised to identify active fractions that were then analysed further by reversed phase HPLC (RP-HPLC). To isolate active species for later confirmation by mass spec (MS) MALDI-TOF analysis the preparative SEC fraction was separated on a phenyl column using a MeOH (+ 0.1% (v/v) acetic acid) gradient; from 65% to 95%. This column separates according to hydrophobicity, as MeOH concentration increases, the greater the hydrophobicity of the species that is eluted. MeOH is a denaturing agent and interferes with hydrophobic interactions thereby potentially breaking up complexes into their constituent monomers. Fifteen distinct fractions were obtained by RP-HPLC (Figure 4.9), tested for activity against CD630 and analysed by mass spectrometry.

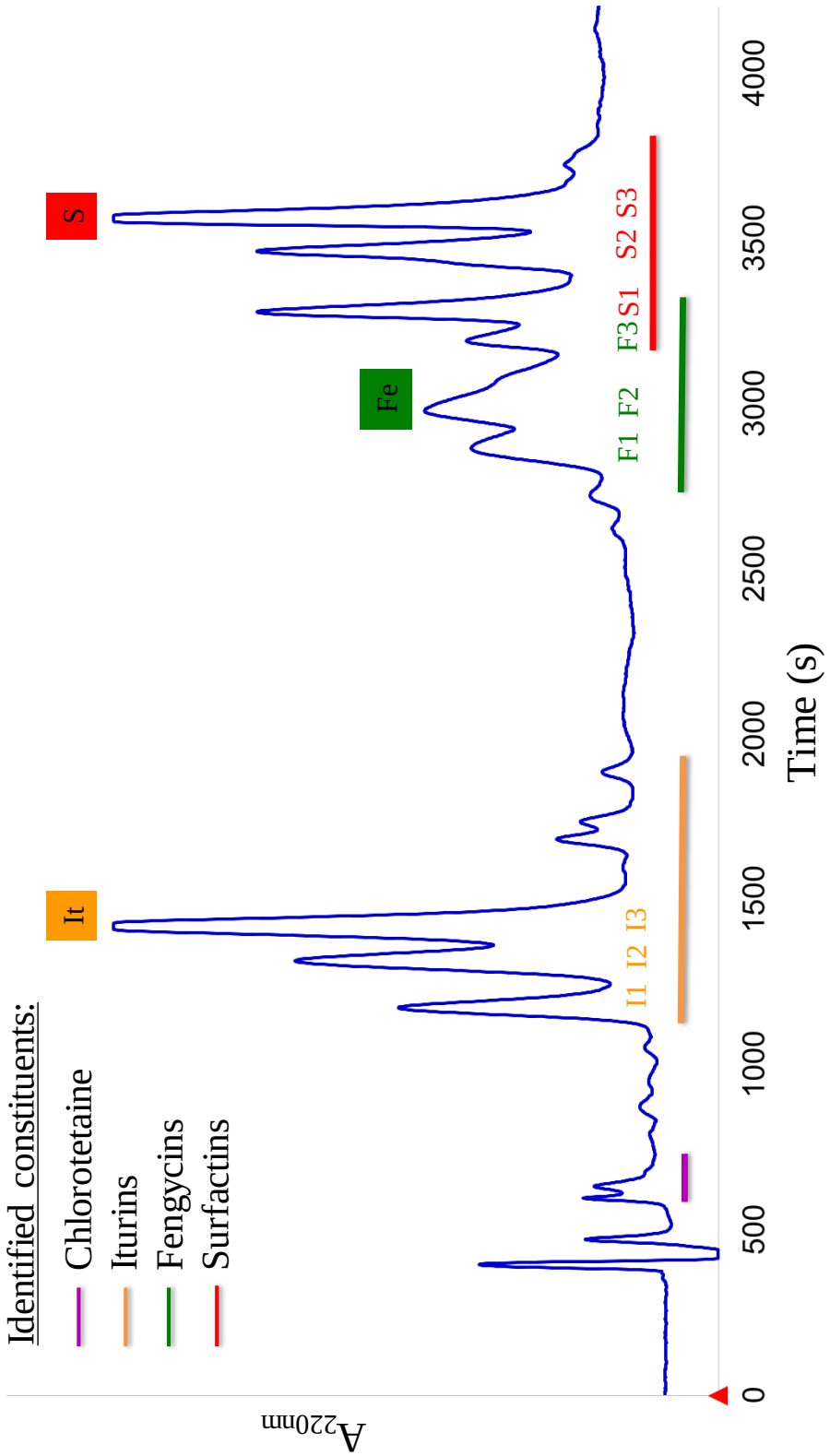


Figure 4.9. RP-HPLC of preparative SG277 SEC fraction. RP-HPLC analysis of SG277 supernatant, grown in BHIB reveals the presence of low molecular weight antibiotic chlorotetaine and three distinct families of lipopeptides - iturins, fengycins and surfactins. Red triangle denotes the injection time. Peak identification number (1-3) labelled underneath the individual peaks.

Different isoforms of the lipopeptides, iturin A, fengycin, surfactin and the low molecular weight antibiotic, chlorotetaine were identified (Figure 4.9 and Table 4.4). In addition, evidence of three additional lipopeptides, mojavensin A (Ma *et al.*, 2012), mycosubtilin and kurstakin was observed (Béchet *et al.*, 2012) (data not shown). Compounds demonstrating anti-CD630 activity are shown in Table 4.3. Fractions comprising the same molecular identity were combined and tested.

Table 4.3. Activity of SG277 RP-HPLC fractions against CD630 (from Figure 4.9)

Compound	Anti-CD activity (dilution factor)
Chlorotetaine	1/10
Iturin	1/10
Fengycin	1/20
Surfactin	1/320

Using the microdilution assay several fractions were found to carry anti-CD630 activity albeit with reduced activity, active fractions being comprised of chlorotetaine, iturin A, fengycin and surfactin. As can be seen Table 4.3, the activity demonstrated by surfactin seems to suggest that it is the principal agent of this complex. Although the activity is well below the activity shown by the SG277 preparative SEC fraction, this can perhaps be explained by loss of activity via purification and the separation of the complex resulting in monomers with less activity individually than as part of an agglomeration. It could suggest that these surfactants are forming mixed micelles of high molecular weight, explaining the low molecular weight when run on an SDS-PAGE gel as they break apart (Figure 4.4), but the high molecular weight indicated by their precipitation by 20% $AmSO_4$ (Figure 4.8) and their high molecular weight elution using SEC. This aspect of the activity will be further elaborated upon experimentally in Chapter 5.

RP-HPLC analysis of SG277 grown in BHIB medium (Figure 4.8) revealed the presence of the antibiotic chlorotetaine and three distinct groups of lipopeptides - iturins (C12 to C17), fengycins (C15 to C19) and surfactins (C12 to C17). The molecular composition of all individual fractions was confirmed by MALDI-TOF-

MS (data not shown) and the identities from each subset of molecules were analysed by MALDI-TOF (Table 4.4).

Table 4.4. MALDI-TOF analysis of active SG277 RP-HPLC peaks (from Figure 4.9) [a]

Constituents	m/z	Ions detected
Chlorotetaine	288.088	[M+Na], [M+K], [M-H+2xNa]
Chlorotetaine (^{35}Cl)	290.085	[M+Na], [M+K], [M-H+2xNa]
Chlorotetaine (^{37}Cl)	304.083	[M+Na], [M+K]
Hydroxychlorotetaine (^{35}Cl)	306.080	[M+Na], [M+K]
Hydroxychlorotetaine (^{37}Cl)		
Iturins		
C_{12}IturinA/C_{12}Mycosubtilin/C_{11}BacillomycinF	1014.512	[M+Na]
C_{14}BacillomycinL	1020.511	[M+Na], [M-H+2xNa]
C_{13}IturinA/C_{13}Mycosubtilin/C_{12}BacillomycinF	1028.527	[M+Na]
C_{14}BacillomycinD	1030.532	[M+K]
C_{15}BacillomycinL	1034.527	[M+H], [M+Na], [M+K]
C_{14}IturinA/C_{14}Mycosubtilin/C_{13}BacillomycinF	1042.543	[M+H], [M+Na], [M+K]
C_{15}BacillomycinD	1044.547	[M+K]
C_{16}BacillomycinL	1048.542	[M+H], [M-H+2xNa]
C_{15}IturinA/C_{15}Mycosubtilin/C_{14}BacillomycinF	1056.559	[M+H], [M+Na], [M+K]
C_{15}IturinB	1057.543	[M+H], [M+Na], [M+K]
C_{16}BacillomycinD	1058.563	[M+K]
C_{17}BacillomycinL	1062.558	[M-H+2xNa]
C_{16}IturinA/C_{16}Mycosubtilin/C_{15}BacillomycinF	1070.574	[M+Na], [M+K]
C_{17}IturinA/C_{17}Mycosubtilin/C_{16}BacillomycinF	1084.605	[M+Na]
Fengycins		
C_{15}FengycinA/C_{13}FengycinB//Plipastatin B C_{13}	1448.778	[M+Na]
C_{16}FengycinA/C_{14}FengycinB//Plipastatin A1	1462.793	[M+H], [M+Na]
Fengycin B (C_{16}/Ala-6)	1462.900	[M+H], [M+Na]
C_{17}FengycinA/C_{15}FengycinB//Plipastatin A 2	1476.809	[M+H], [M+Na]
Fengycin C (C_{17}/Ala-6)	1476.900	[M+H], [M+Na]
C_{18}FengycinA/C_{16}FengycinB//Plipastatin B 1	1490.824	[M+H], [M+Na]
Fengycin D (C_{16}/Val-6)	1490.900	[M+H], [M+Na]
C_{19}FengycinA/C_{17}FengycinB//Plipastatin B 2	1504.840	[M+Na]
Fengycin E (C_{17}/Val-6)	1504.900	[M+Na]
Surfactins		
C_{12}SurfactinA/C_{13}SurfactinB/C_{12}SurfactinC/Pumilacidin	993.634	[M+Na], [M-H+Na+K]
C_{13}SurfactinA/C_{14}SurfactinB/C_{13}SurfactinC/Pumilacidin	1007.650	[M+Na], [M+K], [M-H+2xNa], [M-H+Na+K]
C_{12} Surfactin (linearized)	1011.600	[M+Na], [M-H+2xNa]
C_{14}SurfactinA/C_{15}SurfactinB/C_{14}SurfactinC/Pumilacidin	1021.665	[M+Na], [M+K], [M-H+2xNa], [M-H+Na+K]
C_{13} Surfactin (linearized)	1026.600	[M+K]
C_{15}SurfactinA/C_{16}SurfactinB/C_{15}SurfactinC/Pumilacidin	1035.681	[M+Na], [M+K], [M-H+2xNa], [M-H+Na+K]
C_{14} Surfactin (linearized)	1040.600	[M+K]
C_{16}SurfactinA/C_{17}SurfactinB/C_{16}SurfactinC/Pumilacidin	1049.696	[M+H], [M+Na], [M+K]
C_{17}SurfactinA/C_{18}SurfactinB/C_{17}SurfactinC/Pumilacidin	1063.712	[M+K]

[a] correlating to the identities found within the surfactin, fengycin, iturin and chlorotetaine groupings shown in Figure 4.9. MALDI analysis performed in collaboration with Dr Mikhail Soloviev, Royal Holloway University.

Bacillus endospores often integrate themselves into the human microbiota by exposure to the environment and consumption of vegetables (Tam *et al.*, 2006).

These endospores can subsequently germinate into metabolically active, vegetative cells (Casula and Cutting, 2002) within the small intestine and temporarily colonise the GI tract (Duc *et al.*, 2004). The subsistence of *Bacillus* within the small intestine may be due to the nutrient rich environment, and the higher oxygen levels as compared to further down the GI tract (Albenberg *et al.*, 2014). The small intestine is also the site of *C. difficile* germination (Wilson *et al.*, 1985; Koenigsknecht *et al.*, 2015) (Figure 4.6 and 4.7) and so it would seem likely that inhibition of growth begins there. To determine whether the small intestine of non-clindamycin treated mice contain soluble compounds able to inhibit growth of *C. difficile*, an assay was developed to test the effect that soluble compounds have on the viability of newly germinated CD630 spores in clindamycin treated and non-clindamycin treated animals (Figure 4.10).

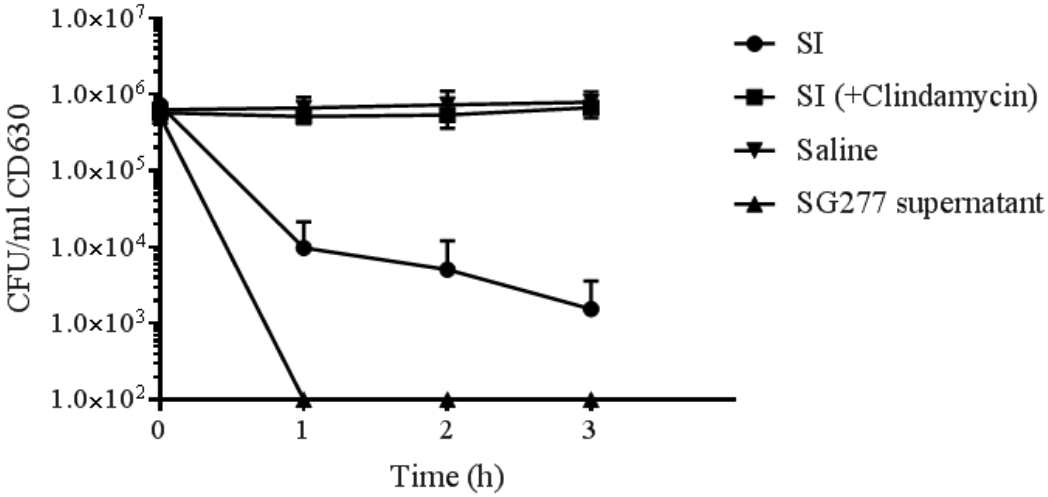


Figure 4.10. Effect of small intestinal extracts on survival of germinating *C. difficile* spores. Intestinal contents were extracted in 90% (v/v) MeOH, vacuum evaporated, resuspended in saline and diluted in 1:1 BHIS (2% sodium taurocholate). CD630 spores were added to a concentration of 1 x 10^6/ml, and aliquots taken for enumeration on BHISS agar at each time point. Heat counts (60^{o}C, 20 min.) were performed at each time point to ensure germination had taken place (data not shown). SG277 supernatant was used as a positive control for the assay. The experiment was performed twice. Error bars represent standard deviations.

Only SG277 supernatant, and the small intestinal contents from non-clindamycin treated mice showed an inhibitory effect upon *C. difficile* vegetative cells. Together these results demonstrate that ingested spores rapidly germinate within the small bowel of mice with and without antibiotic treatment but are killed only in non-

clindamycin treated intestinal samples. This may be explained by increased levels of bile salts in untreated sample, such as muricholate and its derivatives which have been shown to have an inhibitory effect upon *C. difficile* growth (Francis, Allen and Sorg, 2013) and to decrease in the ileum upon antibiotic administration (Theriot *et al*., 2016). However, was it also possible that *Bacillus* secreting anti-*C. difficile* iturin/surfactin/fengycin also contribute to this 'killing' effect?

To determine whether *Bacillus* may be contributing to the data observed in Figure 4.10, and therefore colonisation resistance against *C. difficile* an extraction procedure was performed to see if the lipopeptides could be identified within the gastrointestinal tract of mice and whether they exhibit any activity. The extraction procedure involved passing the intestinal content extraction through a Sep-Pak C18 column at a concentration of 20% MeOH to remove the most hydrophilic of molecules, reducing background and further purifying the sample so as to be able to separate it via RP-HPLC (Figure 4.11).

A single injection into the uBondapack Phenyl column consisted of extract from 0.5 g of intestinal content. Additional sample, again from 0.5 g originally was resuspended in 50 µl of saline and tested for activity against CD630 in a microdilution assay to give the 'total extract activity', shown in Figure 4.11. All individual peaks were analysed by MALDI-TOF, and in the untreated sample only, several peaks of iturin, fengycin and surfactin were identified; no lipopeptides could be detected in the intestinal extract from clindamycin treated mice. Regions corresponding to the lipopeptide locations were combined, evaporated, resuspended in 50 µl saline and tested for activity against CD630 in a microdilution assay.

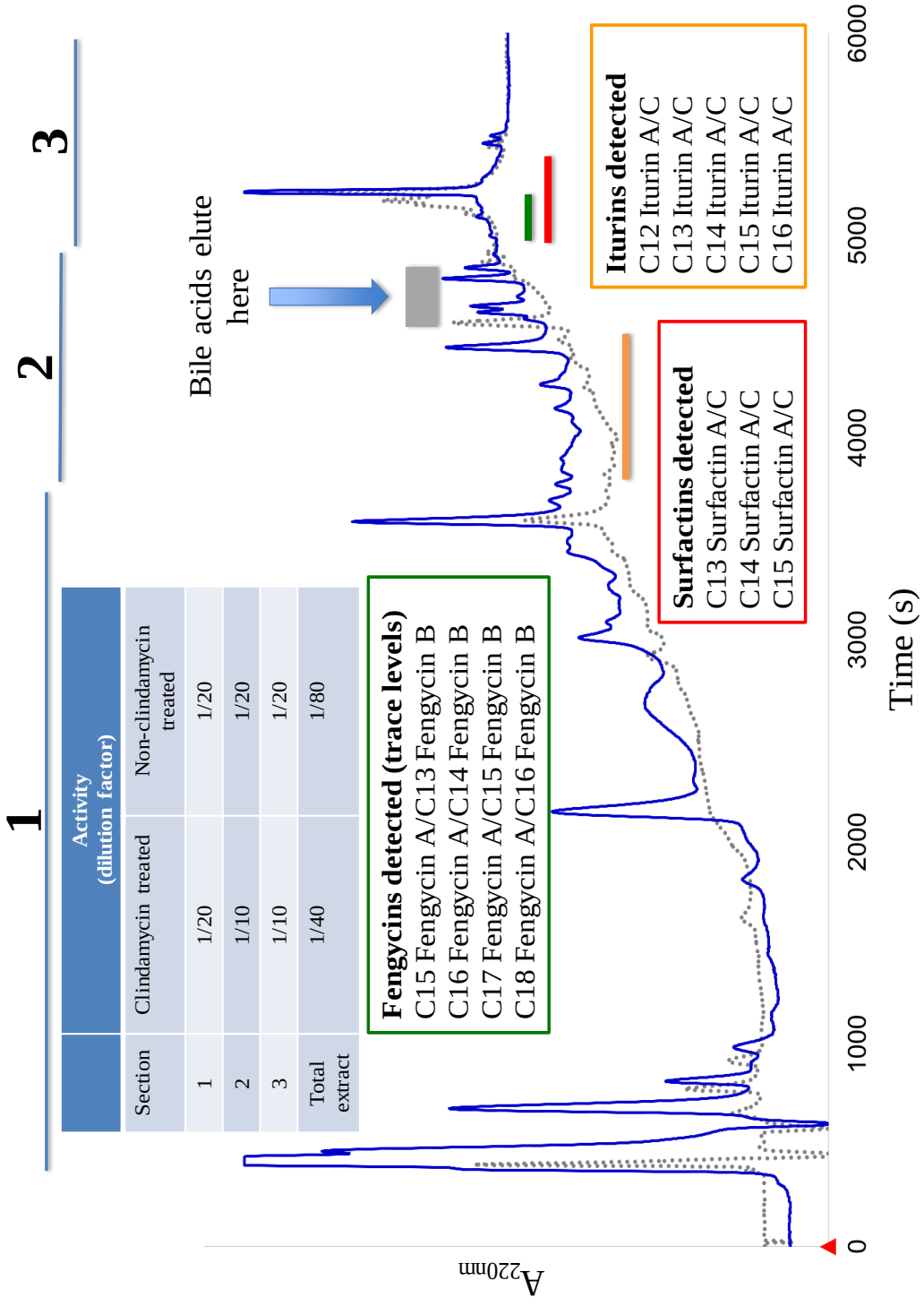


Figure 4.11. RP-HPLC of small intestine from untreated vs clindamycin treated mice. The blue trace represents small intestinal extract from untreated mice, and the grey dotted trace small intestinal extract from clindamycin treated mice. The presence of lipopeptides iturin, fengycin and surfactin were detected using MALDI-TOF analysis only in untreated mice and the locations and isoforms are highlighted. A variety of secondary bile acids have been separated using the same conditions (data not shown), and the area in which they elute is highlighted in grey. Fractions were combined within the regions designated by the 3 blue lines at the top of the diagram, named 1, 2 and 3, evaporated, resuspended in saline and tested for activity against CD630 in a microdilution assay. Total extract prior to separation was also tested.

Interestingly, the activity within region 1 is identical for both clindamycin and non-clindamycin treated intestinal content. This would represent the most hydrophilic and polar constituents of the extract. However, activity between the samples differs for both region 2 and 3, with the clindamycin sample having an endpoint titre of 1/10 for both and the non-clindamycin treated sample having an endpoint titre of 1/20. Region 2 likely includes secondary bile salts, and as can be seen in the chromatogram there are a set of peaks designated by the grey line in the non-clindamycin treated sample which may confirm the presence of bile salts, and perhaps explain the difference in activity. Four secondary bile salts, all shown to be inhibitory to *C. difficile* were found to elute in this area; ursodeoxycholate, hyodeoxycholate, lithodeoxycholate and deoxycholate (data not shown). Region 3 however, contains only fengycin and surfactin in the non-clindamycin treated sample and the activity was twice that of the treated sample, an end point titre of 1/20 and 1/10 respectively. The total activity prior to separation for non-clindamycin treated sample is also twice that of treated sample, 1/80 to 1/40 respectively. This data suggests a correlation between the presence of lipopeptides in the small intestine and an observed *ex vivo* inhibitory effect upon *C. difficile*; the difference in activity between clindamycin treated and untreated mice seemingly being in part due to the presence of *Bacillus* lipopeptides in the latter, although interference by additional factors (e.g. secondary bile salts) cannot be completely ruled out.

4.2.3 Inhibition of CDI *in vivo*

To determine whether *in vitro* activity to *C. difficile* could translate to inhibition *in vivo*, two groups of mice were dosed (intragastric, i.g.) with suspensions of bacteria isolated from the pre-clindamycin faecal samples (Table 4.1). Group 1 were dosed with a mixture of three *Bacillus* species (*B. amyloliquefaciens, B. subtilis* and *B. licheniformis*) that showed activity against *C. difficile* while Group 2 were dosed with three *Bacillus* isolates that showed no *in vitro* activity against *C. difficile*. Animals were first dosed (i.g.) with clindamycin and then, before and after challenge, they were dosed (i.g.) with the different bacterial mixtures (Figure 4.12).

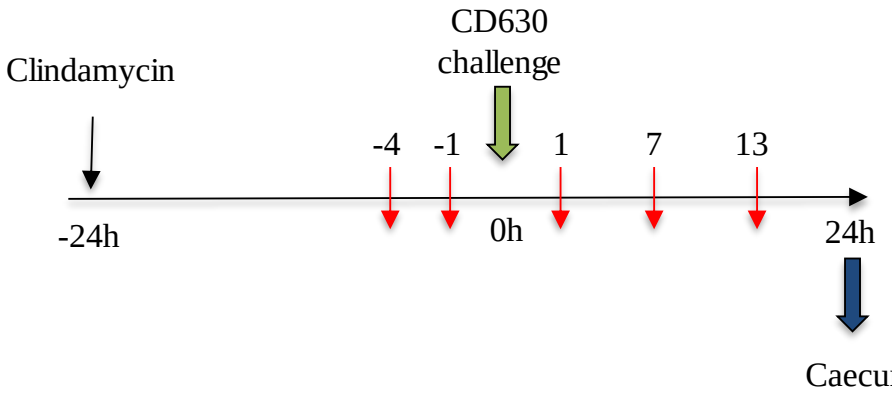


Figure 4.12. Dosing regimen for testing inhibition of CDI in C57BL/6 mice. Mice were dosed first with clindamycin (i.g.; 30 mg/kg) and then given five doses of the bacterial suspensions. After the second dose mice were challenged with 10^2 spores of *C. difficile* strain 630. Caecum was collected at 24 hours post challenge for toxin and CFU analysis.

All animals in the 'positive cocktail' group showed no evidence of CDI as demonstrated by the absence of toxin A (Figure 4.13A) and *C. difficile* CFU (Figure 4.13B) in caecum samples. By contrast, animals dosed with *Bacilli* showing no *in vitro* activity against *C. difficile* exhibited clear signs of *C. difficile* colonisation (high levels of toxin A and *C. difficile* CFU), equivalent to naïve mice.

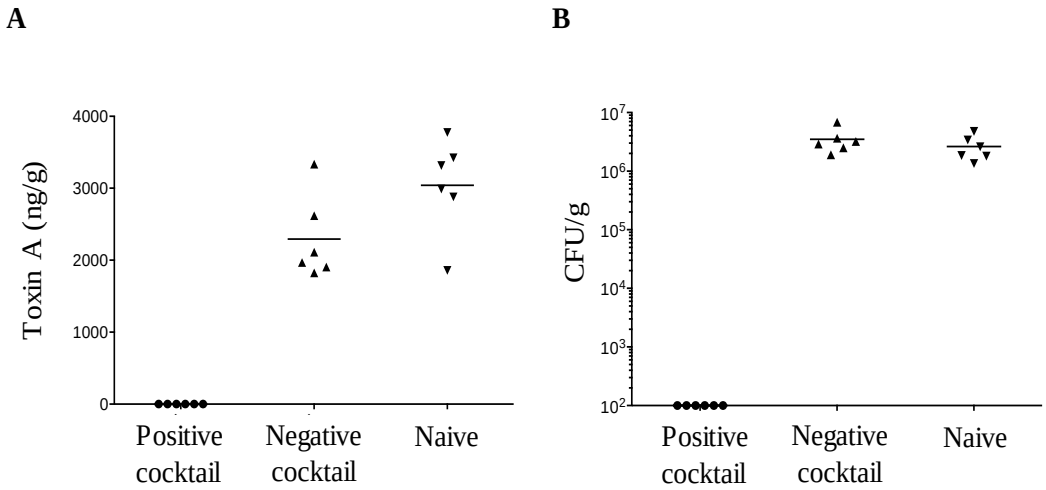


Figure 4.13. Mouse-derived *Bacillus* species prevent colonisation of *C. difficile*. Groups of mice (n = 4) were dosed (intra-gastric) with suspensions of *Bacillus* isolated from the mouse GI tract (Table 4.1). Group 1 mice comprised three isolates that had inhibitory activity against *C. difficile* while Group 2 contained three isolates that had no activity. For each group the oral dose comprised a total of 3 x 10^9 bacteria consisting of 1 x 10^9 bacteria from each isolate tested. A third group consisted of naïve animals dosed only with PBS. 24 hours post challenge all animals were sacrificed and levels of toxin A (**A**) and *C. difficile* CFU (**B**) was determined in caecum samples.

Mouse and hamster models were used to test whether a *B. amyloliquefaciens* strain on its own, SG277, could provide protection against CDI. A number of different administrations were considered, first; use of the sterile, cell-free supernatant, second, a suspension of spores, and finally a suspension of a SG277 culture washed and either suspended in PBS or suspended in the supernatant. For the latter approach, overnight cultures of SG277 were used that were found to contain a mixture of vegetative cells and spores (70-100% spores, data not shown). For evaluation in mice a model of CDI was used where two attributes are used to define colonisation, the presence of toxins and *C. difficile* (CFU) in the caecum (Hong, Ferreira, *et al.*, 2017; Hong, Hitri, *et al.*, 2017). Animals were dosed with the four different SG277 preparations using the regimen shown in Figure 4.12. As well as a naïve group, a group dosed with *B. amyloliquefaciens* SG378 cells (suspended in their supernatant) that did not show any *in vitro* inhibition to *C. difficile* was also included. The level of *C. difficile* caecum CFU and the toxins A and B (Figure 4.14) showed that five doses of SG277 supernatant or a cell suspension suspended in either PBS or supernatant prevented colonisation of CD630 as shown by the absence of bacterial CFU and toxins A and B in the caecum.

Use of the sterile supernatant achieved almost complete arrest of colonisation with only one mouse having low levels of *C. difficile* CFU and toxins in the caecum. Surprisingly, SG277 spores showed no effect on *C. difficile* colonisation and were similar to SG378 and the naïve group. Overnight culture of SG277 showed equivalent efficacy in preventing colonisation and toxin production regardless of whether the pellet was resuspended in PBS or supernatant prior to dosing.

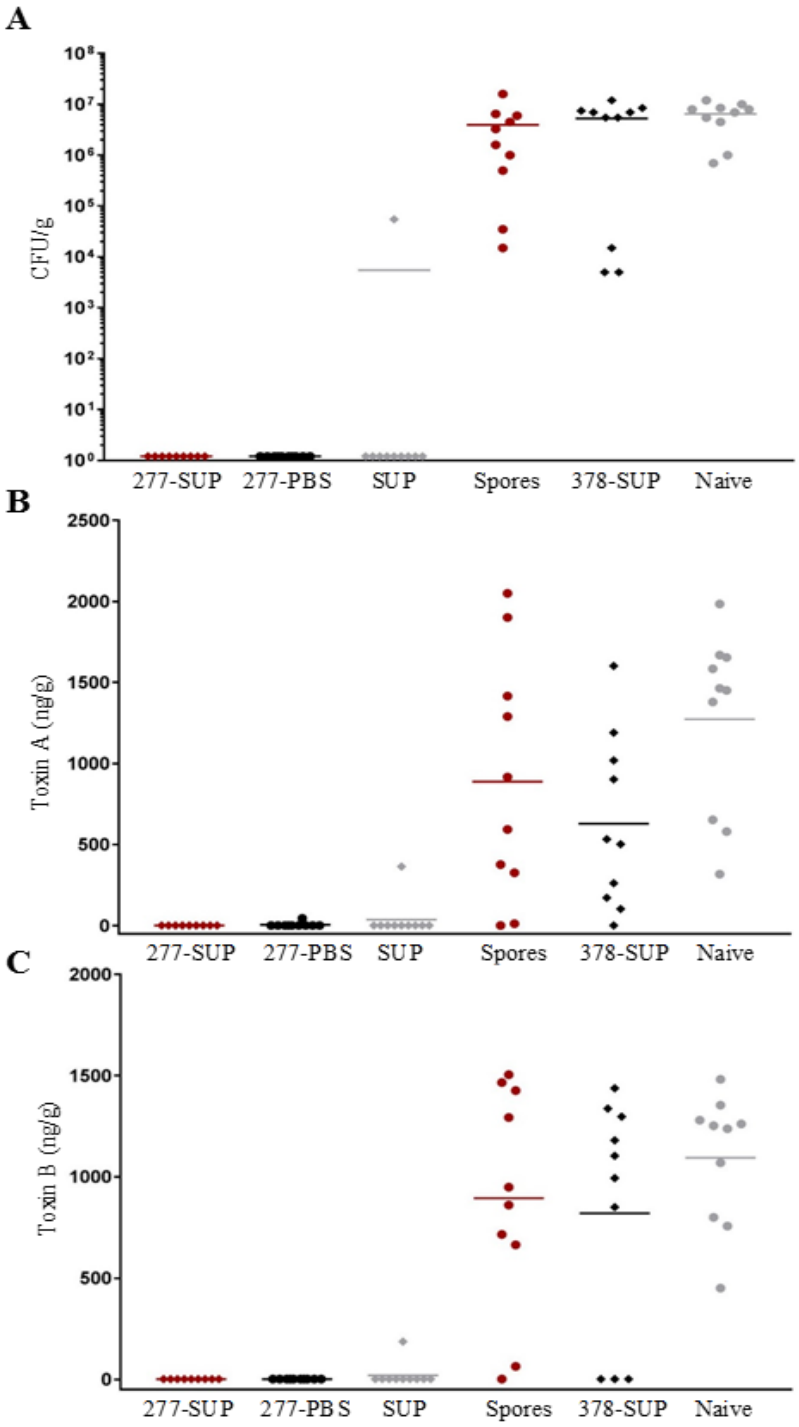


Figure 4.14. Prevention of *C. difficile* colonisation in mice. Groups of mice (n = 10) were dosed (i.g.) five times with different prophylactic treatments using a schedule as shown in Figure 4.12. 24 hours after challenge of clindamycin-treated mice with 10^2 spores of CD630, caecum samples were taken for analysis of *C. difficile* CFU (**A**) and toxins A (**B**) and B (**C**). Treatments were: 277-SUP, SG277 overnight culture suspended in supernatant; 277-PBS, SG277 overnight culture suspended in PBS, SUP, sterile cell-free supernatant of SG277 grown overnight; Spores, purified spores of SG277; 378-SUP, overnight culture of SG378 suspended in their sterile cell-free supernatant; naïve, animals received PBS buffer.

Hamsters are considered a 'gold-standard' for evaluation of CDI (Sambol *et al.*, 2001). Hamsters were used to evaluate the ability of the same SG277 treatments described above to prevent CDI in mice. The dosing strategy involved multiple doses of each treatment (Figure 4.15) using groups of six hamsters per treatment.

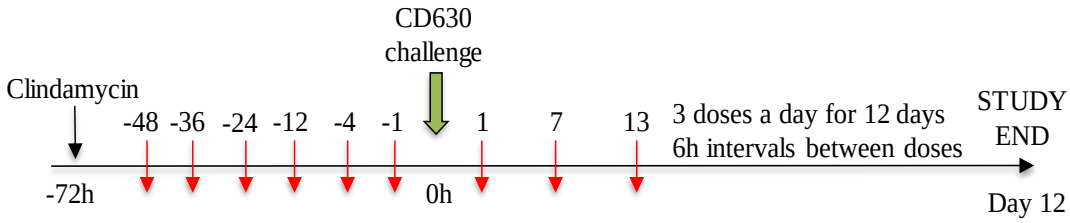


Figure 4.15. Dosing regimen for testing inhibition of CDI in Golden Syrian hamsters. Hamsters were dosed first with clindamycin (i.g.; 30mg/kg) and then were given six doses before CD630 challenge and then three-times/day post-challenge for 12 days. Animals were monitored for symptoms of disease progression and culled upon reaching the clinical endpoint. Caeca were removed and analysed for EtOH resistant CD630 CFU and toxins A and B.

SG277 cells suspended in supernatant provided 100% protection to a lethal challenge with CD630 (Figure 4.16). SG277 cells suspended in PBS produced somewhat lower levels of protection (5/6 hamsters protected) and the sterile supernatant conferred only 30% protection and within the spores group only one animal was protected. Naïve animals and animals dosed with SG378 cells showed no protection.

Together with the mouse data this demonstrates that a suspension of SG277 cells (comprising vegetative cells and spores) suspended in their supernatant or in buffer prevents *C. difficile* colonisation. Two further points can be made; first, that spores have limited efficacy for which it could be assumed that insufficient numbers of spores can germinate in the GI tract to secrete the active compounds. Second, despite the *in vitro* data the cell-free supernatant was not as effective as when combined with cells. This could be due to the active compounds remaining partially attached to the cell envelope with the cells acting as a delivery system, and it is possible that cells transiently proliferate in the GI tract, further boosting the levels of the active compounds. In data not shown it has been found that SG277 administered to mice as a single dose persists (as determined by faecal shedding) for up to 10 days post-dosing.

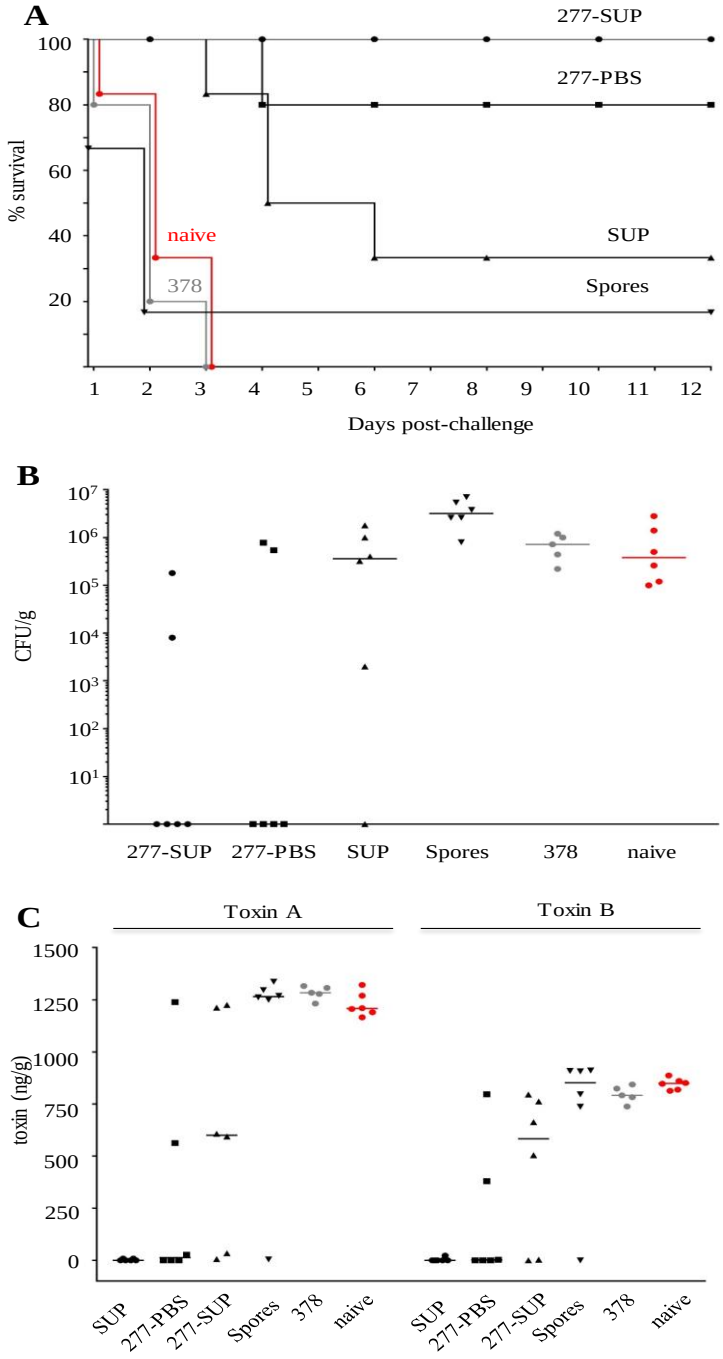


Figure 4.16. Prevention of *C. difficile* colonisation in Golden Syrian hamsters. Groups of hamsters (n = 6) were dosed (i.g.) with different prophylactic treatments before and after challenge with 10^2 spores of CD630 (Figure 4.15). Treatments were: 277-SUP, SG277 overnight culture suspended in supernatant; 277-PBS, SG277 overnight culture suspended in PBS, SUP, sterile cell-free supernatant of SG277 grown overnight; Spores, purified spores of SG277; 378-SUP, overnight culture of SG378 suspended in their sterile cell-free supernatant; naïve, animals received PBS buffer. CD630 challenge was made 3 days after clindamycin treatment (i.g.) after which animals were monitored for symptoms. Animals showing symptoms were sacrificed and Kaplan-Meier survival curves are shown (**A**). Colonisation was determined by analysis of CD630 CFU (**B**) and toxins (**C**) in caecum samples. One hamster from the SG378 was excluded form the experiment for technical reasons.

To further examine the efficacy of SG277 in the prevention of CDI, an experiment was performed using lyophilised SG277 O/N culture and purified SG277 preparative SEC fraction (Figure 4.8). The lyophilisation of treatment products is a convenient method for storage and transportation allowing for easier treatment of CDI. Meanwhile, treatment with the SEC fraction would build on the protection data gathered for the SG277 supernatant and narrow down the mode of action in the prevention of CDI to the active compounds produced by *B. amyloliquefaciens.* Using the regimen displayed in Figure 4.12, mice were administered the various SG277 preparations, sacrificed at 24 hours and caeca samples were tested for CFU and toxins (Figure 4.17).

Administration of ~5 x 10^9 CFU of freeze-dried cells/spores of *B. amyloliquefaciens* strain SG277 was efficient at suppressing CDI in a murine model. No toxin A or toxin B was detected in the caeca of mice infected by CD630 24 hours post challenge using the Holloway model of *C. difficile* colonisation. Bacteria remain viable after the lyophilisation process and they retain their antimicrobial activity (data not shown). The administration of SG277 preparative SEC fraction (as described in Section 4.2.2 and Figure 4.8) significantly decreased toxins levels in caeca when compared to the naïve group. In 3/8 samples toxins were not detected, and in the case of spore counts 2/8 mice showed no evidence of colonisation. Interestingly, SEC was not as efficacious in the prevention of CDI as the SG277 supernatant although the theoretical concentration and activity of active compounds is much higher in the SEC sample (1/1280 vs 1/160). This could possibly be explained by a difference of buffering capacity between the two solutions, supernatant being in the high protein solution BHIB while the SEC fraction was dissolved in water. Further optimisation of the dosing regime, and the type of delivery vehicle is required for SG277 preparative SEC fraction.

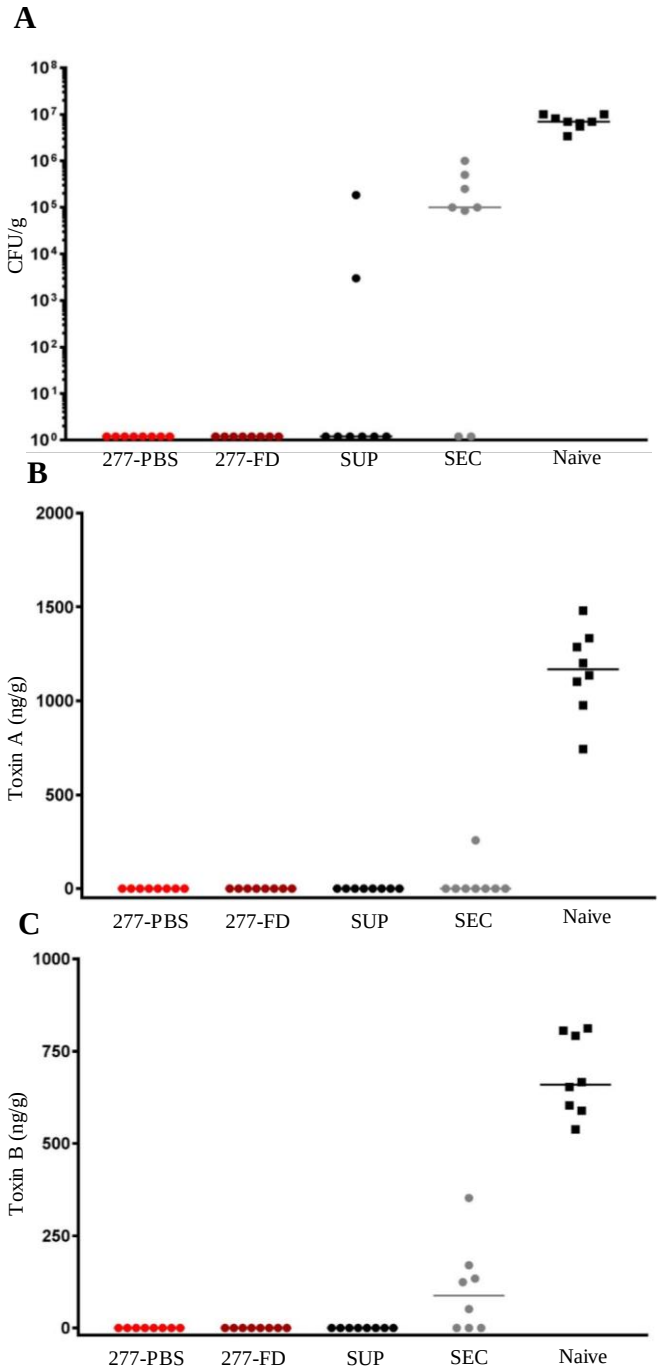


Figure 4.17. Prevention of *C. difficile* colonisation by preparative SG277 SEC fraction and lyophilised material. Groups of mice (n = 8) were dosed (i.g.) five times with different prophylactic treatments using a schedule as shown in Figure 4.12. 24 hours after challenge of clindamycin-treated mice with 10^2 spores of CD630, caecum samples were taken for analysis of *C. difficile* CFU (**A**) and toxins A (**B**) and B (**C**). Treatments were: 277-SUP, SG277 overnight culture suspended in supernatant; 277-PBS, SG277 overnight culture suspended in PBS, SUP, sterile cell-free supernatant of SG277 grown overnight; Spores, purified spores of SG277; 378-SUP, overnight culture of SG378 suspended in their sterile cell-free supernatant; naïve, animals received PBS buffer. Treatments were: 277-PBS, SG277 overnight culture suspended in PBS, 277-FD, SG277 overnight culture freeze-dried/lyophilised and suspended in PBS, SUP, sterile cell-free supernatant of SG277 grown overnight; SEC, preparative SEC fraction- Superdex 200 purified SG277 supernatant (*see* Section 4.2.2), naïve, animals received PBS buffer.

4.3 Discussion

Targeted restoration of the gut microbiota has proven efficacious for treatment of CDI but the mechanism of action has remained elusive and might include rebalancing the gut microbiota, competitive exclusion and the contribution of predatory bacteriophages (Lawley *et al.*, 2012; Orenstein *et al.*, 2016). It is shown here that these methods appear to have overlooked the contribution of aerobic spore-formers for control of CDI. There are several reasons for this. First, methods have employed sequence or metagenomic-based methods to identify candidates for bacteriotherapy, which have been shown unable to detect bacteria at concentrations below 10^5 bacteria/g (Lagier *et al.*, 2012). Second, a focus on anaerobic bacteria and the assumption that minority bacterial populations are unlikely to play a predominant role in the control of intestinal infections. The integral role played by anaerobes has been further supported by recent work which looked at the 7α-dehydroxylation activity certain groups of anaerobic bacteria display, for example *C. scindens* (Buffie *et al.*, 2015). The 7α-dehydroxylation activity demonstrated by subsets of the intestinal microbiota provides a strong mechanism in which the removal of the 7α-dehydroxyl group of primary bile acids results in the formation of *C. difficile* toxic secondary bile acids, thereby providing colonisation resistance against CDI in healthy people (Giel *et al.*, 2010). The administration of antibiotics perturbs this mechanism and results in the decrease of these secondary bile acids and a subsequent decrease in colonisation resistance against CDI.

By contrast, the approach described here has focussed on traditional microbiological methods for identification of bacteria with functional activity, coupled with a focus on aerobic spore-forming bacteria. Using *C. difficile* as a model gastrointestinal pathogen, the cohort of aerobic spore-formers from this work are *Bacillus* species and of course these would ordinarily be mostly undetectable using standard metagenomic or sequence-based approaches (Lagier *et al.*, 2012). These bacteria constitute an allochthonous population that implies our exposure to aerobic spore formers plays an important role in controlling CDI. It is possible that this same cohort of aerobic spore-formers might also play a role in controlling other intestinal infections in both humans and animals and this is an area worthy of future

exploration. Rates of CDI are highest in industrialised countries, most notably the USA followed by the UK (Bauer *et al.*, 2011; Lessa *et al.*, 2015) and it could be argued then that the increase in processed food and decreased exposure to environmental bacteria might be an important factor in why rates are so high in these countries. The hygiene hypothesis attempts to link changes in lifestyle in industrialised countries that have produced a decrease in infectious disease with a concurrent rise in allergic and autoimmune disease (Strachan, 1989; Kelly *et al.*, 2007; López-Serrano *et al.*, 2010). It could therefore be speculated that, broadly, the same phenomenon may be a contributing factor underlying the high rates of CDI observed in the USA and UK (Lessa *et al.*, 2012, 2015; Gupta *et al.*, 2014). Decreased exposure to environmental microbes through for example, diet and increased hygiene standards, might be expected to lower our exposure to *Bacilli* and may, in part, account for the increased rates of CDI. As noted by others the root cause is likely to be multifactorial (Bloomfield and Riley, 2016) but the contribution of exposure to environmental microbes and particularly aerobic microbes has, hitherto, been unnoticed. Although the lack of thorough or proper diagnosis may partly explain the low incidence rates of CDI in developing countries, it is intriguing that these are the same countries that have a record of abuse and overuse of antibiotics (Ayukekbong *et al.*, 2017). It is also interesting that CDI is now beginning to emerge in many developing countries in parallel with improved living standards, hygiene and nutrition (Collins *et al.*, 2013; Burke and Lamont, 2014). It could therefore be suspected that exposure to environmental bacteria plays a contributing role in controlling CDI and perhaps also in other gastrointestinal diseases.

The ability of *Bacillus* species, including *B. amyloliquefaciens* and *B. subtilis*, to produce antimicrobials is well known and the non-ribosomally produced, cyclic anionic, lipopeptides (iturins and surfactins and fengycins) are considered particularly powerful biosurfactants (Duitman *et al.*, 1999; Deleu *et al.*, 2008; Xu *et al.*, 2014; Mnif and Ghribi, 2015; Zhi *et al.*, 2017). A number of these are used commercially as anti-fungal agents for the control of plant disease (Klich *et al.*, 1994; Yoshida *et al.*, 2001; Kim *et al.*, 2004; Romero *et al.*, 2007). It is shown here

that the functional antibiotic produced by *B. amyloliquefaciens* and *B. subtilis* is a water-soluble complex that comprises different isoforms of iturin A, surfactin, mycosubtilin, chlorotetaine, fengycin A and B and potentially others. This antibiotic is therefore an antimicrobial lipopeptide (AMLP) and is particularly unusual because it comprises a mixture of different anionic biosurfactants. It has been shown that when combined the apparent molecular weight of the active fraction is higher than that of the individual monomers indicating that micelles are formed, a characteristic of many biosurfactants (Cui *et al.*, 2008). AMLP micelles have been shown to aggregate into nanostructures, are more stable and resistant to degradation, have enhanced solubility and carry a higher antimicrobial activity than the monomeric form (Makovitzki *et al.*, 2008; Horn *et al.*, 2012, 2013). These characteristics will be further explored in the subsequent chapter. It could be surmised then, that this antibiotic is most likely a mixture of different AMLPs that in some *B. amyloliquefaciens* strains is produced in sufficient concentrations to form micelles.

In Figure 4.10 and 4.11, a link between the presence of lipopeptides in the small intestine and the *ex vivo* exhibition of activity against *C. difficile* is demonstrated. This implies a mechanism for CDI resistance in healthy mice, the presence of lipopeptides aides in the suppression of *C. difficile* growth following spore germination. The activity demonstrated by the small intestinal extract from untreated mice differs from previous findings: Koenigsknecht *et al.* (2015) and Theriot, Bowman and Young (2016) both showed that following *C. difficile* spore germination, growth was supported in the small intestine of untreated mice. There could be several reasons for this divergence in the data; both of the mentioned publications diluted the intestinal extract 1:1 in PBS (w/v), centrifuged and then added spores and proceeded to use a single 6 hour time point to measure the change in CFU. In this study's method however, a more rigorous extraction procedure (90% MeOH) was used and CFU was measured over a shorter time frame (1, 2 and 3 hours). The use of an organic solvent in our extraction method may therefore be more effective at removing surface associated hydrophobic compounds, including some bile salts and lipopeptides. The passage of *C. difficile* through the small

intestine is 1-2 hours (Figure 4.7) and our data suggests that in this time frame there are compounds which would inhibit vegetative cell outgrowth, but it is possible that if left for a longer period of time (6 hours) that recovery and subsequent growth would occur.

It has been previously reported that surfactins can act synergistically with iturins (Razafindralambo *et al.*, 2002) and mycosubtilin (Jauregi *et al.*, 2013) and considering the elution of the complex occurs in the same high molecular weight fractions after Superdex 200 SEC separation, it is likely that mixed micelles are being formed. The presence of surfactins, fengycin and iturin in the small intestine of healthy mice was confirmed and their ability to form mixed micelles may provide a link between their inhibitory action and that of secondary bile acids. Bile acids function in the GI tract by forming mixed micelles which increase the uptake of cholesterol and fatty acid. Although to date there have been no studies describing mixed micelles forming between bile acids and bacterial lipopeptides, bile acids have been shown to form mixed micelles with other anionic surfactants (Poša and Ćirin, 2012; Faustino *et al.*, 2014; Jójárt *et al.*, 2014). This may suggest a mechanism by which bacterially produced lipopeptides combine with inhibitory secondary bile acids in mixed micellar complexes within the GI tract of healthy hosts to provide colonisation resistance against CDI via direct antimicrobial action against the vegetative cells. The interaction of iturin A with surfactin in mixed micelles has been previously shown to enhance the lytic activity against erythrocyte membranes (Thimon *et al.*, 1992). Furthermore, the increased presence of agents capable of forming micelles in the GI tract is likely to increase the chance of micelle formation; it being a concentration dependent phenomena, and it has been established that the micelle in solution is better than the corresponding monomers at membrane binding, while also showing preferential selectivity towards bacterial, rather than mammalian membranes (Lin and Grossfield, 2014, 2015; Bereau, 2015). By forming micelles, monomers increase their relative solubility thereby allowing for an increased concentration of compound per unit volume.

This chapter presents data showing that either a cocktail of *Bacillus* strains or a *B. amyloliquefaciens* strain alone, SG277, can be used as a probiotic *in vivo* to control CDI. Interestingly, using the active filtrate or the purified SEC fraction also confers protection against CDI, although not to the same degree. Using probiotics based on their antibacterial activity against CDI is fairly novel, and to our knowledge only shown before by Geeraerts *et al.* (2015). CDI was induced in all naïve groups successfully and was measured by toxin ELISA and EtOH resistant CFU counting in caecum samples. SG277 was administered 4 hours prior to challenge for mice, and 48 hours prior for hamsters to enable the bacteria to colonise the gut and start producing lipopeptides. When SG277 was administered at 5 x 10^9 cells/dose according to the regimen shown in Figures 4.12 and 4.15, full protection was seen in a mouse model with no toxins or CFU observed. The absence of *C. difficile* spores suggests that SG277 works by inhibiting *C. difficile* directly, and therefore toxin production. In hamsters, 83% survived when dosed with SG277 resuspended in PBS, and 100% when dosed with SG277 resuspended in supernatant. This may reflect the additional antimicrobial activity provided by the supernatant. Although some toxins and CFU are observed in non-moribund hamsters (3 out of 12), the levels are relatively low (symptoms were minor) and may be transitory, as due to hamster's sensitivity to *C. difficile* toxins a sustained, high level would certainly lead to death. Perhaps if the experiment had been elongated longer than the 12 days they would have succumbed to infection.

It has been proposed that only multistrain probiotics will be efficacious in the prevention of CDI (Hell *et al.*, 2013), but our data suggests a single strain of *B. amyloliquefaciens* is sufficient for near complete protection. The secretion of a lipopeptide complex with high antibacterial activity against *C. difficile* is seemingly the mechanism behind the protection. The effect of these compounds on the microbiota is yet to be investigated but could be an interesting avenue for future research. The effectiveness of SG277 as a probiotic suggests that it can survive clindamycin administration and colonise the gut, despite its sensitivity to the antibiotic. This could be due to the high proportion of spores within the dose, the number of doses and the diminishing levels of clindamycin within the host post

administration. This has been shown before with other bacterial species, including *Lactobacillus casei,* which has been shown to reduce the incident rate of AAD and CDI when taken in conjunction with antibiotic therapy (Hickson *et al.*, 2007; Wong *et al.*, 2014). In conclusion, it has been shown that the administration of SG277 as a probiotic is effective, however further work, perhaps investigating the potential of the strain to be used as a treatment for CDI, or for CDI relapse is required.

CHAPTER 5

Purification, Identification and Characterisation of *Bacillus* Antimicrobial Activity

5.1 Introduction

The intestinal microbiota is integral to the health of the gut, largely due to the protection provided by individual species against pathogens. Disruption of this complex, diverse ecosystem can leave the host susceptible to a wide range of gastrointestinal infections. The administration of antibiotics perturbs the intestinal flora and approximately 25% of recipients develop AAD (Bartlett, 1992) of which *C. difficile* accounts for 15 – 25% of these. It is the dysbiosis of the healthy gut microbiota by antibiotics that triggers CDI. The standard treatment for CDI is a cessation of antibiotic use, and if that is not effective metronidazole or vancomycin are employed. However, the use of antibiotics to treat an illness initially caused by antibiotics is contradictory and somewhat ineffective, with relapse rates as high as 30% (Cole and Stahl, 2015). There is therefore an urgent need to develop alternative, effective measures for controlling CDI; measures that do not further disrupt the microbiota. Probiotics meet these requirements, by repopulating the gut with microorganisms capable of protecting the host from pathogens, either by activation of the immune system, competition for space/nutrients or by the secretion of antimicrobial compounds. *Lactobacillus rhamnosus GG* and *Saccharomyces boulardii* have both been shown to be partially effective in treating AAD (Siitonen *et al.*, 1990; Armuzzi *et al.*, 2001; Kotowska *et al.*, 2005; Can *et al.*, 2006). However, both have had mixed results in the treatment of CDAD and the data thus far is not sufficient to merit any specific recommendations of using these bacteria as treatments.

Bacillus is a genus of Gram-positive, spore-forming bacteria which are often used in probiotic products. *Bacillus* probiotics have been found to be effective in the prevention of AAD, but the core underlying mechanisms have yet to be identified

(Hempel *et al.*, 2012). Many *Bacillus* species are able to produce abundant amounts of diverse biologically active compounds (Ongena and Jacques, 2008; Ilinskaya *et al.*, 2017). *Bacillus* is known to produce a wide range of antimicrobial substances, including cell wall lytic enzymes, exoenzymes, bacteriocins, RNA-degrading enzymes and lipopeptides (Korenblum *et al.*, 2005; Stein, 2005; Abriouel *et al.*, 2011). These compounds can either have a broad spectrum of activity, or specific activity against closely related species (Jack *et al.*, 1995). Due to the emerging antibiotic resistance of pathogens, discovery, identification, purification, characterisation and development of alternative antimicrobial compounds is necessary.

Bacillus, specifically *B. subtilis*, *B. cereus*, *B. licheniformis*, and *B. amyloliquefaciens* are major producers of non-ribosomally generated amphipathic lipopeptide antibiotics. *Bacillus* lipopeptides are synthesised by large, multi-enzyme, non-ribosomal peptide synthetases (NRPs), resulting in exceptional uniformity and heterogeneity of amino acid sequences, length/branching of the fatty acid chain and peptide cyclisation amongst the lipopeptides (Ongena and Jacques, 2008). The lipopeptides so far identified fall into one of three main groups; iturins, fengycins or surfactins (Yang *et al.*, 2015). Iturins are cyclic heptapeptides with tails comprised of C14-C17 β-amino fatty acids. Fengycins are cyclic decapeptides formed by lactonization, with β-hydroxy fatty acid chains of 14-17 carbons in length. Fengycin has antifungal activity against filamentous fungi (Meena and Kanwar, 2015). Surfactins are cyclic, heptapeptide lactone rings comprising C13-C16 β-hydroxy fatty acid chains. Lipopeptides are formed as families of closely related isoforms, with differences in the length and fatty acid side chain branching defining isoforms. Due to the amphipathic nature of lipopeptides, lipid bilayer membranes can be disrupted through the formation of pore and ion channels, thereby providing a reduced likelihood of resistance emerging when compared to antibiotics (Inès and Dhouha, 2015). Lipopeptides tend to have low toxicity and higher stability in terms of pH, temperature, enzymes and salinity, making them attractive options as antimicrobial agents (Banat *et al.*, 2000). The function and biological activity of lipopeptides is dependent on the amino-acid peptide sequence,

length/branching of the fatty acid chain and cyclisation of the peptide (Zhao *et al.*, 2018). Lipopeptides display a wide range of useful properties, including but not limited to anti-viral, anti-bacterial, anti-fungal, anti-inflammatory and anti-cancer activities. These properties make them commercially attractive in the agricultural, food and pharmaceutical industries. The majority of *Bacillus* species can produce at least one type of lipopeptide, with some producing two or three types (Chen *et al.*, 2017; Toral *et al.*, 2018).

In the preceding chapter, data was presented indicating that allochthonous *Bacillus* species can secrete soluble antimicrobials that i) inhibit the growth of germinating *C. difficile* spores within the small intestine and ii) have inhibitory activity against *C. difficile in vivo*. This chapter will report on the steps taken to identify these molecules, evaluate and postulate on their mechanism of action.

5.2 Results

5.2.1 Screening of *Bacillus* isolates and identification of activity

Our laboratory has a large number of *Bacillus* isolates from various sources. *Bacillus* isolated from humans faeces has previously been characterised by our laboratory (Hong *et al.*, 2009). Using an agar well-diffusion assay (Figure 5.1), isolates were screened for extracellular activity against *C. difficile* strain CD630 present in filtered (0.45 μm) supernatants. A total of 166 strains from the *Bacillus* genera were screened for activity against *C. difficile* CD630 using the agar well-diffusion assay. Of 166 strains, 80 belonged to *B. subtilis*, 20 to *B. megaterium*, 6 to *B. licheniformis* 40 to *B. pumilus* and 20 to *B. amyloliquefaciens*. Of the strains screened, only those of *B. subtilis* (27/80), *B. licheniformis* (3/6) and *B. amyloliquefaciens* (17/20) showed activity against *C. difficile*. *B. amyloliquefaciens* strains showed the highest level of activity, with *B. subtilis* and *B. licheniformis* demonstrating comparatively lower levels of activity. Examples of activity against *C. difficile* strain 630 (CD630) obtained in representative *Bacillus* strains is shown in Table 5.1.

Table 5.1. Anti-CD630 activity of human *Bacillus* isolates using a well-diffusion assay [a]

Strain[b]	Species	Diameter of zone of inhibition (mm)
PY79	*subtilis*	0
SG140	*subtilis*	10 ± 1.11
SG83	*subtilis*	10 ± 0.89
10A1	*amyloliquefaciens*	0
SG57	*amyloliquefaciens*	22 ± 2.00
SG137	*amyloliquefaciens*	22 ± 1.00
SG185	*amyloliquefaciens*	23 ± 0.58
SG277	*amyloliquefaciens*	27 ± 1.73
SG297	*amyloliquefaciens*	27 ± 2.65

[a] ± Standard deviation
[b] PY79 is a *B. subtilis* control strain and 10A1 is a *B. amyloliquefaciens* strain obtained from the *Bacillus* Genetic Stock Centre. Results are means of three individual assays.

A microdilution assay was developed to quantify levels of extracellular inhibitory activity against *C. difficile* CD630 (Table 5.2).

Table 5.2. Anti-CD630 activity using a microdilution assay

Strain	Species	Endpoint titre[a]		
		expt 1	*expt 2*	*expt 3*
PY79	*subtilis*	0	0	0
SG140	*subtilis*	1/20	1/20	1/10
SG83	*subtilis*	1/20	1/20	1/20
10A1	*amyloliquefaciens*	0	0	0
SG57	*amyloliquefaciens*	1/40	1/40	1/40
SG137	*amyloliquefaciens*	1/40	1/40	1/40
SG185	*amyloliquefaciens*	1/80	1/40	1/40
SG277	*amyloliquefaciens*	1/160	1/160	1/80
SG297	*amyloliquefaciens*	1/160	1/160	1/160

[a] Highest dilution factor showing inhibition.

As was the case with the agar well-diffusion assay, *B. amyloliquefaciens* showed the highest levels of activity. In the case of SG277 and SG297, activity was consistently 4 to 8-fold higher than *B. subtilis* strains SG140 and SG183. Using the agar well-diffusion assay activity was tested for against a selection of *C. difficile* strains (Table 5.3).

Table 5.3. Activity of SG277 and SG297 against a selection of *C. difficile* ribotypes by the welldiffusion assay[a] [b]

Test	*C. difficile* strain				
	CD630 *RT012*	CD196 *RT027*	R20291 *RT027*	VPI 10463 *RT087*	SH1 *RT078*
SG277	26 ± 0	26 ± 1.4	28.5 ± 2.1	27.5 ± 3.5	26.5 ±4.9
SG297	26 ± 1.4	24.5 ± 0.7	23 ± 2.8	25 ± 0	25.5 ± 0.7
PY79	0	0	0	0	0
10A1	0	0	0	0	0

[a] Inhibition of CD strains was made using the well diffusion assay and defined as diameter of zone of inhibition (mm). Results are means of three individual assays. *RT*, ribotype.
[b] ± Standard deviation

The data demonstrated that the extracellular activity of SG277 and SG297 was effective against a variety of *C. difficile* strains and ribotypes, including the hypervirulent 027 ribotype and ribotype 078, and the diameters produced by the extracellular activity were roughly equivalent to one another. Furthermore, an experiment was conducted to assess the inhibition of *C. difficile* cell growth by measuring the optical density after overnight growth of *C. difficile* strains in BHIS. *C. difficile* cultures were grown for 10 hours at 37^{o}C in BHIS and 18 ml was removed to a falcon tube followed by an addition of 2 ml of SG277 filtrate. The cultures were incubated for 18 hours at 37^{o}C after which the OD_{600} was read to assess the inhibitory activity against the strains (Table 5.4). SG277 showed strong inhibitory effects against all tested *C. difficile* strains. The level of inhibition suggests that SG277 activity is bacteriolytic, as the OD of the culture returned to baseline and the culture almost completely cleared suggesting lysis of the *C. difficile* vegetative cells.

Table 5.4. Activity of SG277 against various *C. difficile* strains using a co-culture assay[a]

C. difficile strain	Ribotype	OD_{600}		% inhibition
		Untreated[b]	+ SG277	
CD630	RT012	0.756	0.059	92
SH1	RT078	0.845	0.086	90
SH101	RT115	0.772	0.081	89.5
SH102	RT176	0.981	0.072	93
R20291	RT027	0.857	0.091	89
CD196	RT027	0.798	0.056	93
SH104	RT023	0.534	0.056	89.5
VPI 10463	RT087	0.687	0.054	92
CD10	non-tox	0.824	0.102	88
SH242	RT111	0.672	0.089	87
SH200	RT056	0.914	0.076	92
SH203	RT038	0.882	0.064	93
SH218	RT001	0.732	0.081	89
SH210	RT002	0.655	0.055	92
SH213	RT014	0.758	0.064	92
SH215	RT54	1.025	0.056	95
SH220	RT336	0.783	0.049	94
SH222	RT401	0.952	0.082	91
SH231	RT56	0.791	0.073	91
SH236	RT005	0.883	0.091	90
SH239	RT103	0.966	0.086	91
SH103	RT075	0.761	0.106	86
SH3	RT017	0.692	0.055	92

[a] SG277 filtrate was incubated overnight as a control and showed no growth

[b] Untreated control had 2 ml of BHIB added to 18 ml of culture prior to being incubated for 18 hours at 37ºC.

A notable feature of *B. amyloliquefaciens* is the formation of mucoid colonies which were more pronounced on some media. It was shown that SG277 and SG297 could produce proficient biofilms in LB, DSM as well as S7 medium (a minimal medium; (Saggese *et al.*, 2018)). Biofilms were always more pronounced when cells were grown at 30ºC rather than at 37ºC. When SG277 or SG297 were grown in S7 medium with no shaking a dark brown pigment was produced. Figure 5.1 shows scanning EM (SEM) images of SG277 and SG297 biofilms developed in S7 medium. Spores could be seen in the biofilms together with vegetative cells.

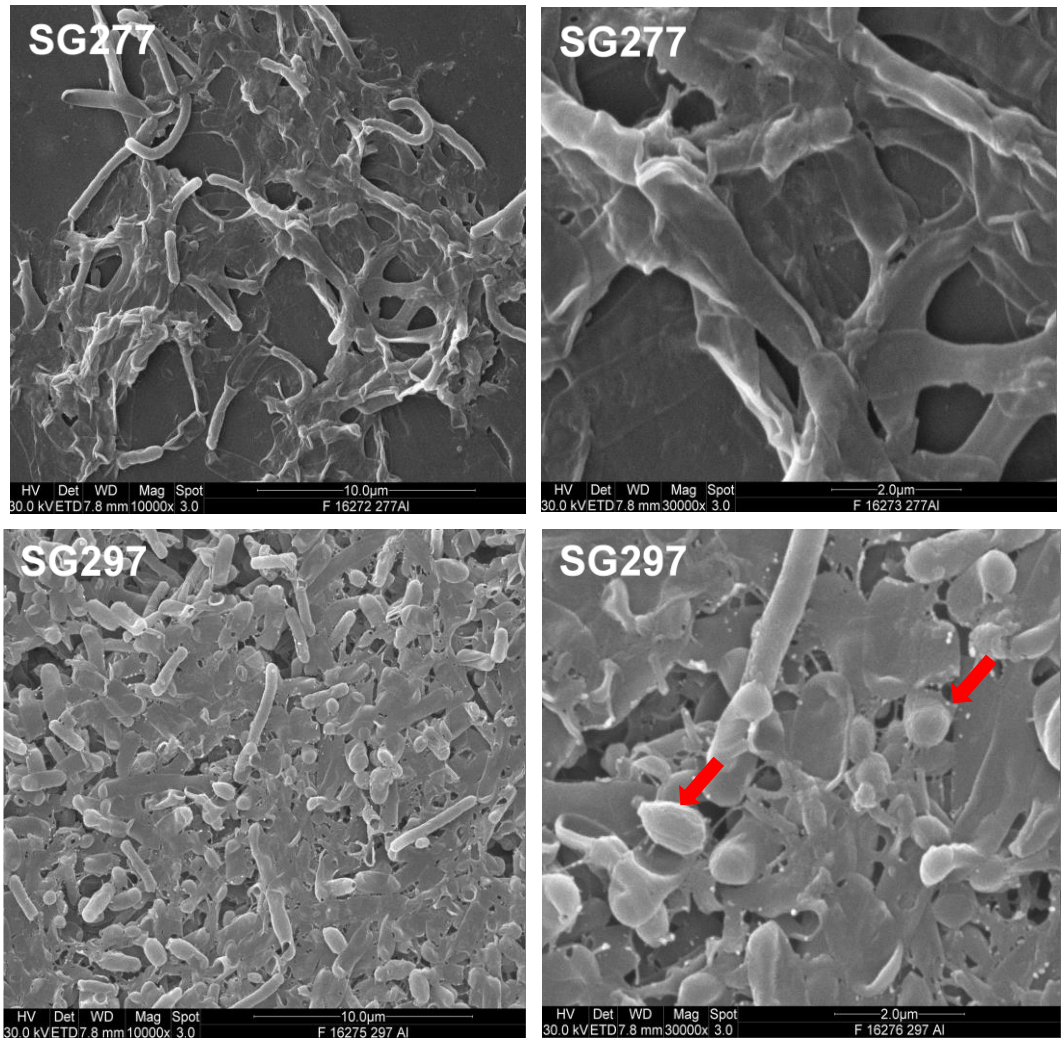


Figure 5.1. SG277 and SG297 biofilms developed in S7 medium imaged using SEM. Red arrows highlight spores in the biofilm. Image courtesy of Dr Ezio Ricca, Federico II University, Naples. Magnification at 10,000x.

5.2.2 Characterisation of anti-*C. difficile* activity

Table 5.4 suggests that the extracellular activity exhibited by *Bacillus* strains against *C. difficile* is bacteriolytic, as the decreasing OD suggested that the bacteria had lysed. Co-culture experiments were therefore conducted where SG277 and SG297 filtrates were added to exponentially growing cultures of CD630, VPI-10463, R20291 or SH1 (Figure 5.2).

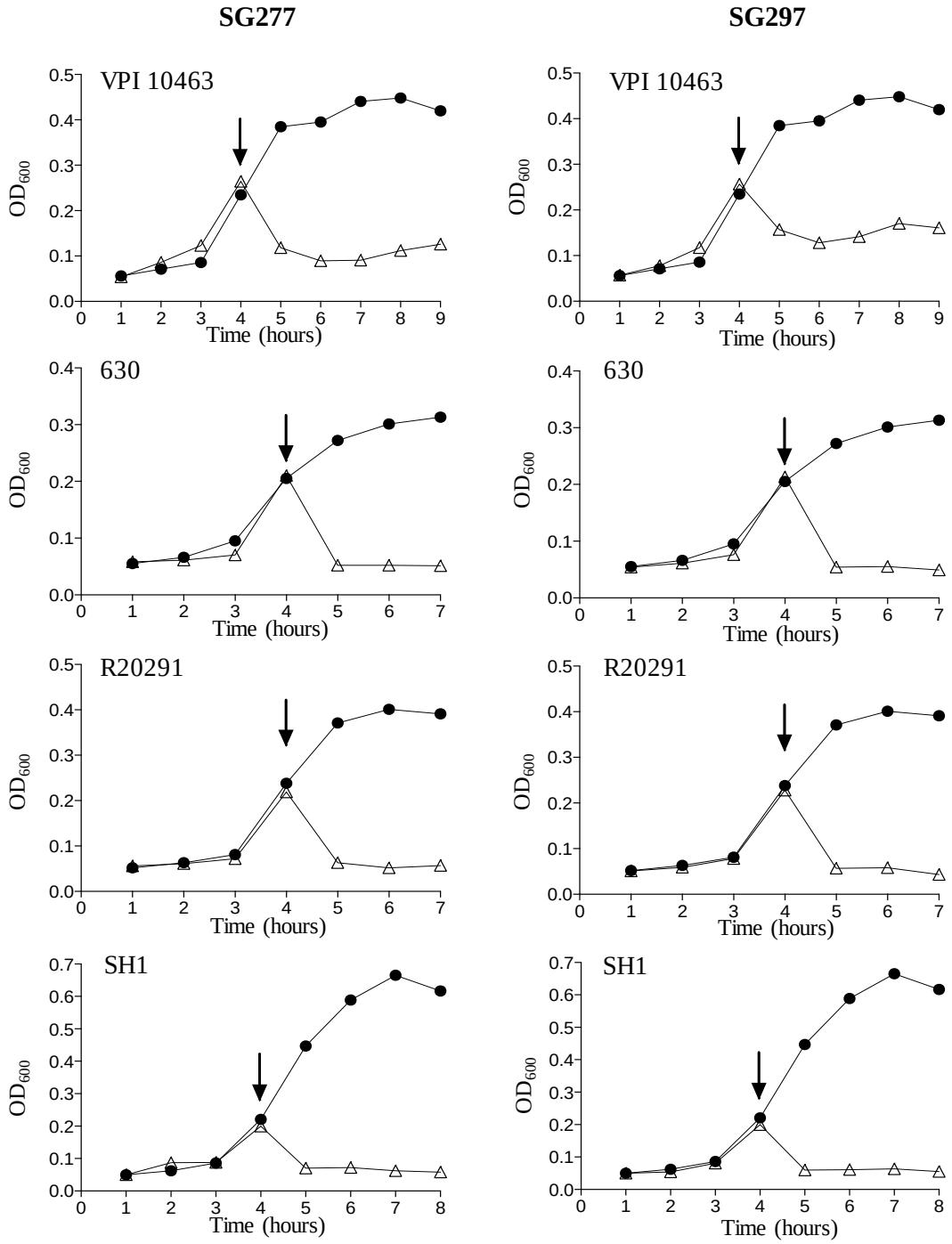


Figure 5.2. Co-culture assays using SG277 and SG297 filtrates against various *C. difficile* strains. Samples of sterile extra-cellular filtrates from either SG277 or SG297 were added (1:10, v/v) to exponential growing cultures of four different *C. difficile* strains (time of addition indicated by arrow). Cultures with or without addition of samples were grown at 37°C and OD_{600} readings taken every hour. ● indicates cultures without addition of filtrate and Δ indicates cultures with filtrate added. Strains used were VPI-10463 (RT087), CD630 (RT012), R20291 (RT027) and SH1 (RT078).

Upon addition of the filtrates the OD immediately dropped to baseline levels. Both SG277 and SG297 filtrates significantly reduced the optical density of all *C. difficile* strains confirming that they exhibited bacteriolytic activity resulting in almost complete lysis in < 1 hour.

To further characterise the activity exhibited by SG277, a simple stability assay was performed for the supernatant and lyophilised supernatant. Prior to storage (time point 0), fresh and lyophilised supernatants were tested for activity against CD630 in a microdilution assay. The endpoint titre for both was 1/160, and so all samples tested during the assay had their percentage of retained activity calculated according to this value. Table 5.5 shows the activity of fresh and lyophilised supernatants, stored under various temperature conditions over 8 weeks. Samples were removed weekly from their storage conditions and all tested at the end of the 8 weeks with the exception of the -20^{o}C samples (which were tested on a weekly basis). At the end of the experiment a sample were thawed, lyophilised samples resuspended in 1 ml dH_2O and samples either heated (65^{o}C, 15 min.) or left untreated for activity testing. Samples were heated prior to activity testing to measure whether prolonged storage had an effect on the heat resistance of the compound/s. The percentage of activity retained over time is displayed in Figure 5.3. The data showed that i) heating the samples (65^{o}C, 15 min.) prior to the microdilution assay had no effect on activity, ii) storage of fresh or lyophilised supernatants at -20^{o}C had no effect on activity, and iii) lyophilised samples exhibited better stability at 4^{o}C and RT than fresh samples.

The activity of SG277 and SG297 filtrates was further characterised (filter-sterilised, 0.45 µm) by a variety of treatments using a microdilution assay to measure inhibitory activity against CD630 (Table 5.6). The filtrates showed high levels of heat resistance, retaining activity at 90^{o}C for 30 min. and only losing some activity at 100^{o}C (1/40) and upon autoclaving. SG277 and SG297 filtrates showed resistance to all enzymes and solvents tested, as well as to simulated gastric fluid.

Table 5.5. Stability of SG277 lyophilised and fresh supernatant.

Supernatant	Storage	Week								
		0	1	2	3	4	5	6	7	8
Fresh	-20°C	1:160	1:160	1:160	1:160	1:160	1:160	1:160	1:160	1:160
	-20°C heated	1:160	1:160	1:160	1:160	1:160	1:160	1:160	1:160	1:160
	4°C	1:160	1:160	1:80	1:80	1:80	1:80	1:80	1:40	1:40
	4°C heated	1:160	1:160	1:80	1:80	1:80	1:80	1:80	1:40	1:40
	RT	1:160	1:40	1:40	1:20	1:10	0	0	0	0
	RT heated	1:160	1:40	1:40	1:20	1:10	0	0	0	0
Lyophilised	-20°C	1:160	1:160	1:160	1:160	1:160	1:160	1:160	1:160	1:160
	-20°C heated	1:160	1:160	1:160	1:160	1:160	1:160	1:160	1:160	1:160
	4°C	1:160	1:160	1:160	1:160	1:160	1:160	1:160	1:80	1:80
	4°C heated	1:160	1:160	1:160	1:160	1:160	1:160	1:160	1:80	1:80
	RT	1:160	1:80	1:80	1:80	1:40	1:40	1:40	1:40	1:20
	RT heated	1:160	1:80	1:80	1:80	1:40	1:40	1:40	1:40	1:20

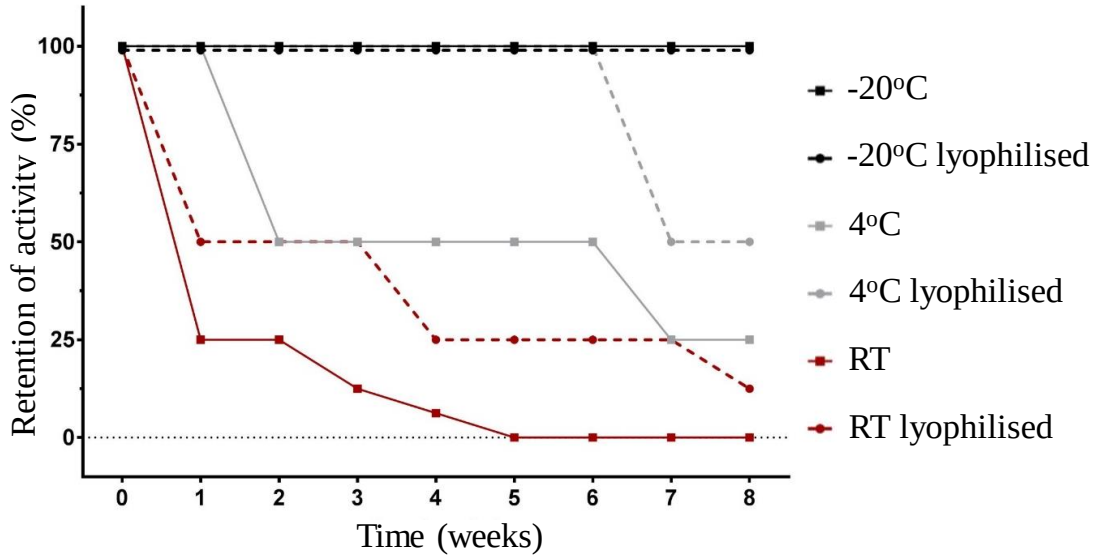


Figure 5.3. Retention of activity of SG277 lyophilised and fresh supernatants at different temperatures over time.

A slight loss of activity was observed after acetone treatment, with the endpoint titre halving to 1/40. The resistance to proteases, lipases and amylases suggests that the active molecule was not protein, lipid or carbohydrate based. Resistance to simulated gastric fluid indicated that the activity might remain stable if dosed orally *in vivo.* All concentrations of glutaraldehyde had no effect on the activity of the SG277 or SG297 filtrates, indicating that there were no amine groups for glutaraldehyde to crosslink with.

Table 5.6. Characterisation of extracellular activity[a]

Treatment		SG277 filtrate	SG297 filtrate
No treatment		1/80	1/80
Heat[b]	60°C	1/80	1/80
	70°C	1/80	1/80
	80°C	1/80	1/80
	90°C	1/40	1/80
	100°C	1/40	1/40
Autoclaving[c]		0	0
SGF[d]	pH2	1/80	1/80
	pH3	1/80	1/80
	pH4	1/80	1/80
Enzymes[e]	Lysozyme	1/80	1/80
	Lipase	1/80	1/80
	Amylase	1/80	1/80
	Pronase	1/80	1/80
	Trypsin	1/80	1/80
	Proteinase K	1/80	1/80
Solvents[f]	Toluene	1/80	1/80
	Chloroform	1/80	1/80
	Acetone	1/40	1/80
0.1% (w/v) SDS[g]		1/80	1/80
0.1% (v/v) glutaraldehyde[h]		1/80	1/80
0.25% (v/v) glutaraldehyde[h]		1/80	1/80
0.5% (v/v) glutaraldehyde[h]		1/80	1/80
1% (v/v) glutaraldehyde[h]		1/80	1/80

[a] The highest dilution factor that showed inhibitory activity is shown. Treatment only controls (no filtrate) was used for all tests. The no treatment control was diluted 1:1 in dH_2O (v/v) prior to activity testing, and all samples diluted by treatments were further diluted if necessary, with dH_2O so that supernatants were at a final concentration of 1:1 (v/v).

[b] Supernatants were incubated in an oven at the selected temperature for 30 min. and allowed to cool to RT before assay.

[c] 121°C, 20 psi, 20 min.

[d] For simulated gastric fluid three solutions at pH, 2, 3 and 4 were made using HCl to adjust pH. Supernatants were incubated with an equal volume of SGF and incubated for 1 hour at 37°C before assay.

[e] Supernatants were incubated with the following enzymes at 1 mg/ml final concentration for 1 hour at 37°C before assay, lysozyme, lipase, amylase. For the proteases, pronase, trypsin and proteinase K were used.

[f] Supernatants were vortexed for 1 min. 1:1 with 100% solvent (v/v) and incubated for 1 hour.

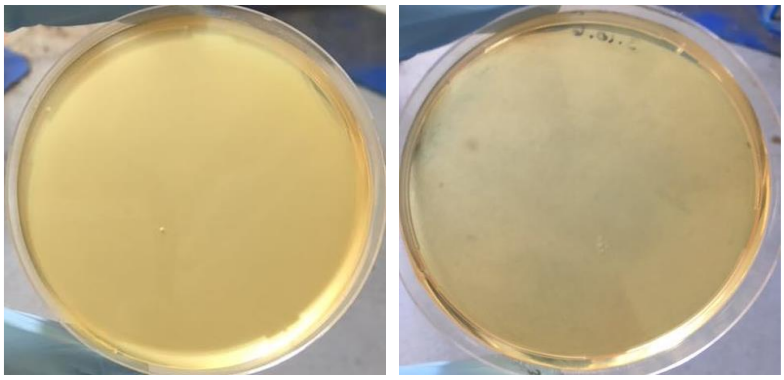
[g] Incubated at RT for 3 hours.

[h] Incubated at 37°C for 2 hours. Glutaraldehyde was neutralised by glycine at a molar ratio of 1:10.

Although the data showed that significant activity was associated with the extracellular material, whether activity was associated with the cell surface was not yet addressed. For this, SG277 was grown in BHIB for 16 hours at 37°C and the

cell and supernatant fractions were separated. The cell fraction (comprised of both spores and cells) was washed with PBS 3x and mixed with CD630 cells in soft agar (0.7%), poured onto petri dishes and then grown overnight anaerobically (Figure 5.4).

CD630 lawn **CD630 lysed lawn**



CD630	Plate Lysis
Untreated	-
+ SG277 supernatant	+
+ SG277 supernatant (80^{o}C)	+
+ SG277 washed cells/spores	+
+ SG277 washed cells/spores (80^{o}C)	+

Figure 5.4. Lytic activity is associated with the cell/spore surface. Activity was shown by complete lysis of the bacterial lawn. SG277 cell suspensions were mixed with soft agar and incubated overnight as a control and as aerobes showed no growth (not shown).

Plates carrying SG277 extracellular supernatant + CD630 showed complete lysis, as did the SG277 cell fraction + CD630 (Figure 5.4). Heat treatment (80^{o}C, 30 min.) of both the washed cell fraction and the supernatant had no effect upon the activity, with complete lysis still occurring. Heat treatment would kill SG277 vegetative cells indicating that activity must be associated, *in situ*, with the cell/spore surface, rather than with potential growth and secretion of the active inhibitory compound/s. Under anoxic conditions growth of SG277 cannot occur confirming that inhibitory activity must be present on the surface of the cells.

5.2.3 Purification of the active molecules

The final purification method used for identifying the active molecules has been described in Chapter 4 and shown in Figure 4.8. However, there were various other avenues which were explored in regard to the nature of the compounds responsible for the activity, and they are outlined further here. Figure 5.5 shows the various ways in which attempts were made to determine the properties of the inhibitory activity against *C. difficile*. Unless specified otherwise, all purification steps were performed using extra-cellular (filter-sterile, 0.45 µm) material obtained from 16-hour overnight cultures of SG277 grown in BHIB medium (37^{o}C). All functional testing was performed against CD630 in a microdilution assay.

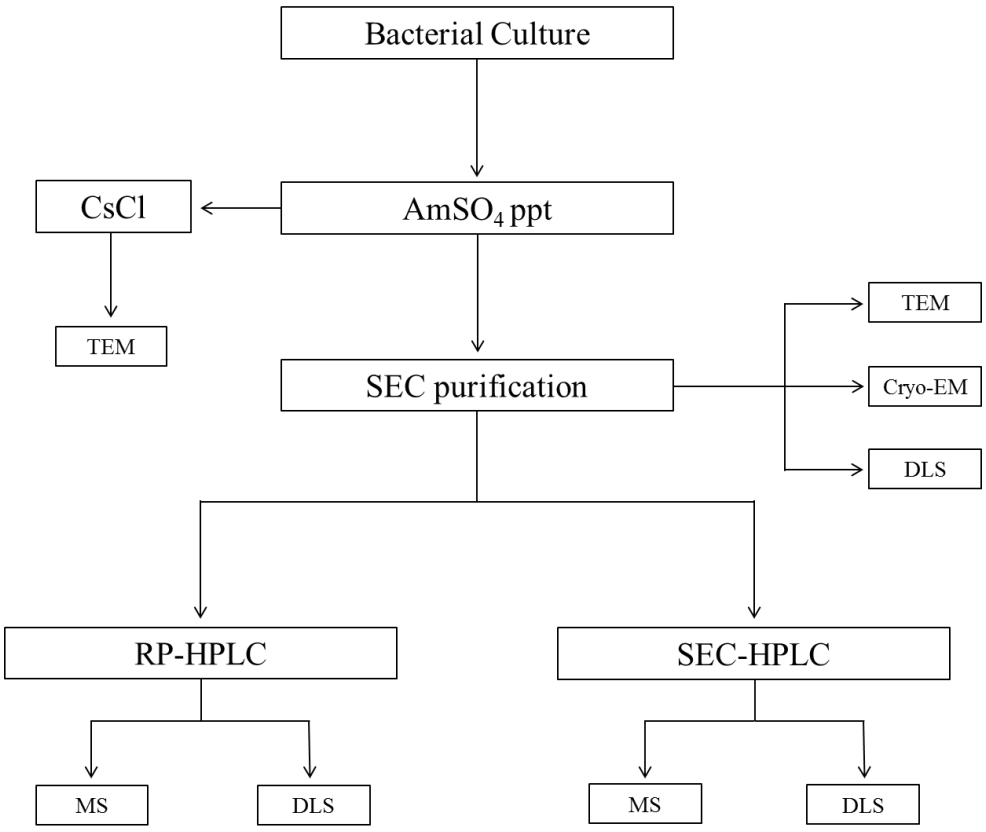


Figure 5.5. Purification of the active components responsible for anti-*C. difficile* activity. See methods for more details.

Initially, purification of SG277 filtrate was attempted using Vivaspin centrifugal molecular weight cut offs (MWCOs), to give an approximation of the molecular weight. Analysis of the SG277 filtrate using a sequence of MWCOs (100 kDa, 30 kDa and 10 kDa) and testing for activity against CD630 is shown in Table 5.7.

Table 5.7. Size fractionation of SG277 activity

Cut-off (kDa)[a]	Activity titre
Untreated	1/80
<10	0
10-30	0
30-100	1/40
>100	1/40

[a] sterile filtered (0.45 μm) culture supernatant passed through Vivaspin 6 centrifugal concentrators. Activity against CD630 measured using a microdilution assay.

Activity against CD630 was found in two fractions: 30-100 kDa and >100 kDa. This suggests that a relatively high molecular weight compound was responsible. The presence of the activity above and below 100 kDa suggested the formation of a complex, which might be physically labile and able to dissociate and pass into the flow through while retaining some activity.

It was found that activity could be precipitated using 20% $AmSO_4$ as well as with polyethylene glycol (PEG) and following centrifugation through CsCl gradients three distinct bands were present, of which one, band 2 (B2), carried functional activity against *C. difficile* (Figure 5.6).

SG277 filtered supernatant was precipitated using PEG and resuspended at a 100x concentration in SM buffer. Activity of the supernatant used was 1/80, total PEG ppt. was 1/320 and B2 had 1/320 activity, demonstrating retention of all activity from the PEG ppt. in B2.

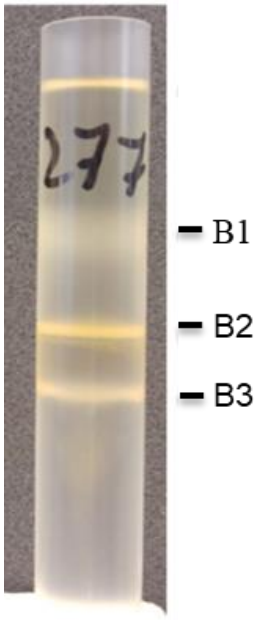


Figure 5.6. CsCl centrifugation of PEG precipitated SG277 filtrate. PEG precipitation was used to precipitate material from the SG277 sterile filtrate. Following ultra-centrifugation through CsCl gradients three bands were present with only band B2 having activity against CD630 using the microdilution assay.

Transmission EM analysis (TEM) of B2 revealed distinct aggregates present in the active fractions following CsCl gradient centrifugation (Figure 5.7). Interestingly, this analysis revealed granular clusters of approximately 20-30 nm in diameter. Also apparent from the TEM images was some indication of ordered assembly. These assemblies were abundant and if responsible for the activity seen against *C. difficile* then the observed size would be broadly consistent with the data from Table 5.7.

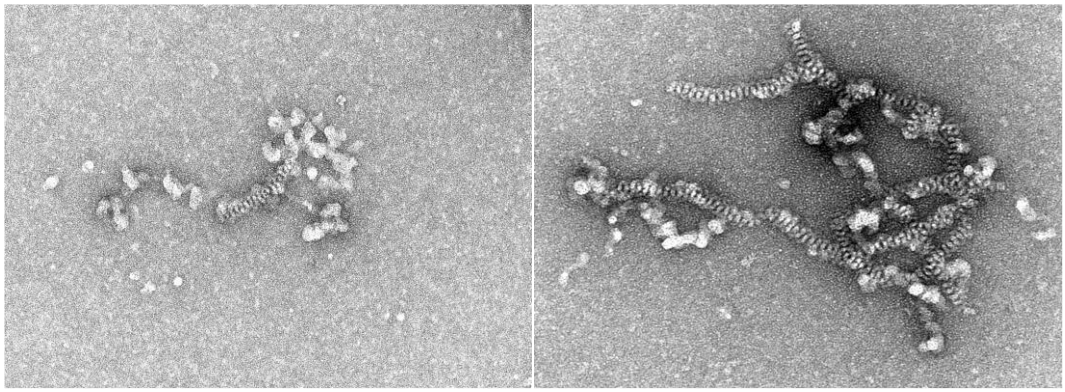


Figure 5.7. TEM analysis of CsCl band B2. Image courtesy of Dr Wilfried Meijer, CSIC.

$AmSO_4$ precipitation of the SG277 filtrate was made at final concentrations of 20% (w/v) and 70% (w/v), followed by incubation O/N at 4°C and resuspension of the precipitate in PBS at a concentration of 30x (for 30 ml of initial culture the precipitate would be dissolved in 1 ml of PBS). Precipitates were run on 12% SDS-PAGE and Coomassie stained (Figure 5.8A). A soft agar overlay assay was used to look for the region of activity (Figure 5.8B).

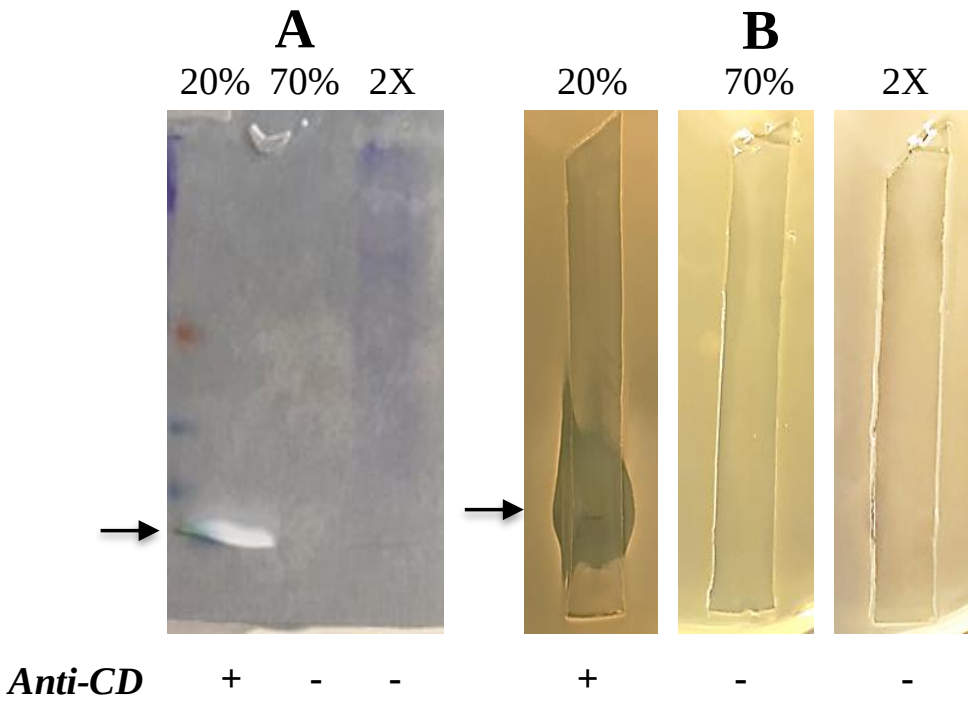


Figure 5.8. SDS-PAGE and soft-agar overlay analysis of $AmSO_4$ precipitates. SG277 extracellular material was $AmSO_4$ precipitated at concentrations of 20%, 70% or 20% followed by 70% (2X). Precipitates were run on 12% SDS-PAGE and Coomassie stained (**A**). A soft agar overlay assay was performed by excising all gel lanes and overlaying with soft BHIS agar and a logarithmic culture of CD630 (**B**). Arrow indicates presence of activity.

Figure 5.8A shows the presence of a white band close to the dye front, of approximately 1 kDa in molecular weight. This band is only present at a concentration of 20% $AmSO_4$ and is mirrored by the activity observed in Figure 5.8B, where the corresponding zone of inhibition is only seen with 20% $AmSO_4$. Following this observation, a 2-step $AmSO_4$ precipitation procedure was followed, first precipitating with 20% $AmSO_4$, followed by a 2nd precipitation at 70%; this abolished the activity seen initially at 20%. Precipitation at 20% $AmSO_4$ but not at

70% could be explained by several characteristics of the active molecules; it may occur due to the high hydrophobicity of the compounds resulting in oligomerisation and therefore precipitation at low concentrations, but at high concentrations the salt may coat the structure and repel interactions thereby preventing precipitation. Another potential explanation might be the differing pHs of the two solutions, with the pH at 20% $AmSO_4$ proving to be less favourable for solubilisation of the compound.

For identification of the active compounds using MALDI-TOF analysis, further purification would be necessary. As the first step in the purification process, $AmSO_4$ precipitation rather than PEG precipitation was selected due to a far greater yield of activity retained by $AmSO_4$ ppt. post PEG precipitation, a 100x concentrated solution (v/v) had an endpoint titre of 1/320, while $AmSO_4$ ppt. at a concentration of 30x had an endpoint titre of approximately 1/2560.

In order to further purify the active species, the $AmSO_4$ ppt. was separated using a Superdex 200 column (10,000 Da - 600,000 Da) in PBS + 0.1% SDS (w/v). This allowed for a preparative separation of high MW species. SDS was added to denature/linearise unwanted proteins, and an isocratic buffer containing 0.1% SDS (w/v) resulted in a purer separation (Figure 5.9A) when compared to buffer without SDS (Figure 5.9B). Figure 5.9A shows that SEC purification successfully removes protein content, and that activity does not correlate with the position of protein elution indicating that the active species are not proteinaceous.

Separation was performed without the presence of SDS so as to calculate the molecular weight using four internal standards; IgG (150 kDa), BSA (66.5 kDa), lysozyme (14.3 kDa) and insulin (5.8 kDa) (Figure 5.9C). SG277 active components had a retention time of 26 min. and solving the equation of the straight line for y gave a value of 4.81414 and $10^{4.81414}$ = 65,184 Da.

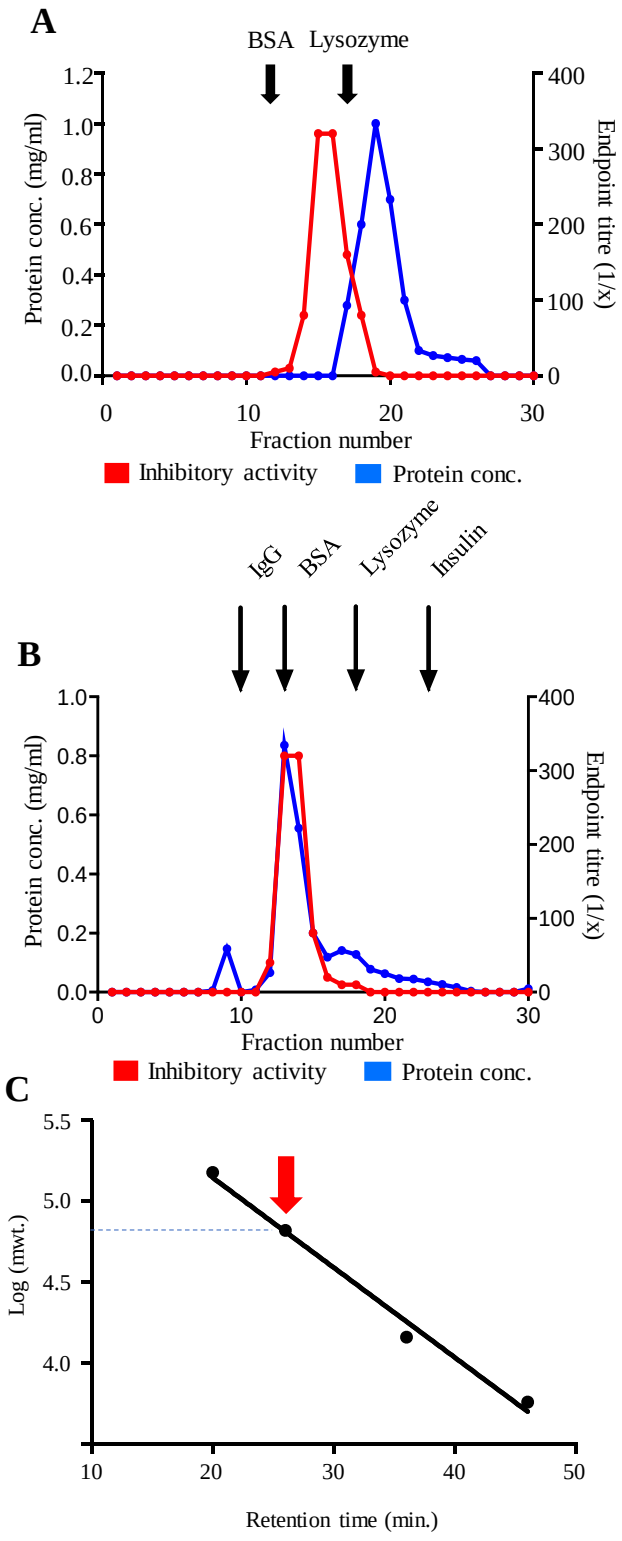


Figure 5.9. Analysis of SG277 $AmSO_4$ ppt. by preparative SEC. $AmSO_4$-precipitated material from SG277 cell-free supernatant was fractionated using SEC in the presence (**A**) or absence (**B**) of 0.1% SDS. Fractions were analysed for protein concentration (NanoDrop, A280nm) and inhibitory activity to *C. difficile* 630. The position of size standards is shown. The log (mwt.) of the four standards were plotted as a function of the retention time. A trend line was used to calculate the straight-line equation and was solved for *y* (SG277 retention volume: 26 min. as indicated by red arrow) to determine the molecular weight of the complex (65,184 Da) (**C**).

Following SEC separation active fractions were combined, washed and concentrated using a 10 kDa MWCO to a final volume of 1 ml. Activity testing of the preparative SEC fraction indicated that the endpoint titre for activity was approximately 1/1280. The preparative SEC fraction was further examined using TEM (Figure 5.10).

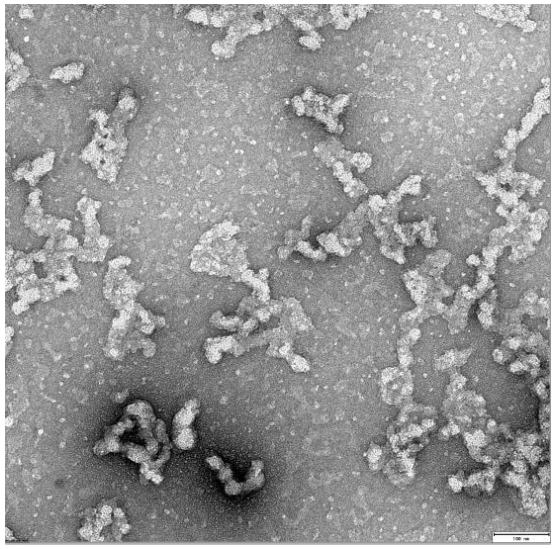


Figure 5.10. TEM analysis of SG277 preparative SEC fraction. Marker = 100nm. Image courtesy of Dr Wilfried Meijer, CSIC.

As was the case in Figure 5.7, there was an abundance of granular like clusters of approximately 20-30 nm in diameter.

The accumulated data indicated that the active species responsible were lipopeptides. *Bacillus* lipopeptides are low molecular weight cyclic, amphipathic antibiotics able to agglomerate and form large molecular weight micelles in aqueous solution. This would explain the discrepancy seen in the molecular weight; in Figure 5.8 where activity is observed to be approximately 1 kDa, yet the SEC separation (Figure 5.9) suggests a compound of higher molecular weight (65,184 Da). A large molecular weight complex is supported by Table 5.7, where activity is retained above a 30 kDa MWCO. It seems that in aqueous solution lipopeptides were forming micelles of large molecular weight while in denaturing conditions (SDS-PAGE) these micelles are broken down into their constituent, low molecular weight monomers. Lipopeptides have high biosurfactant activity, and in Figure 4.3A an association is seen between the level of anti-*C. difficile* and biosurfactant

activity using an oil dispersion assay. *Bacillus* are known to produce large quantities of lipopeptides (Yang *et al.*, 2015; Ilinskaya *et al.*, 2017) with antimicrobial activity (Meena and Kanwar, 2015) and so to determine whether micelles were responsible for the observed activity a modified method from Jauregi *et al.* (2013) was used. This method relies upon methanol for the disruption of the micellar aggregation of the surfactant molecules. A diagrammatic representation of the procedure is shown in Figure 5.11.

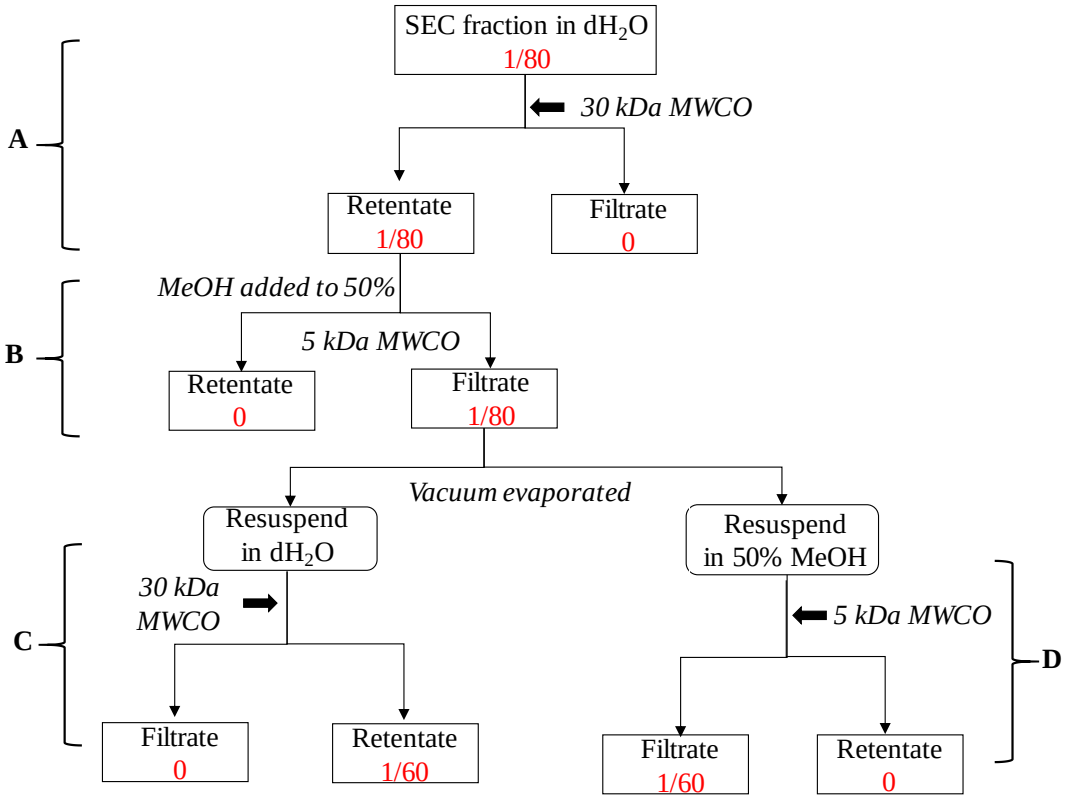


Figure 5.11. High molecular weight activity is due to micelle formation of lipopeptide surfactants. SG277 SEC fraction was centrifuged through a 30 kDa MWCO filter to ensure activity measured was above 30 kDa **(A)**. The retentate was collected and MeOH added to a final concentration of 50% (v/v) thereby disrupting the micelles **(B)** followed by centrifugation through a 5 kDa MWCO. The resulting filtrate was aliquoted into two equal volumes, vacuum evaporated and pellets resuspended either in dH_2O **(C)** or 50% MeOH (v/v) **(D)** and centrifuged through 30 and 5 kDa MWCO respectively. All fractions were normalised by volume and tested for activity against CD630 using a microdilution assay.

Figure 5.11 confirms the presence of lipopeptide micelles through the formation of high molecular weight structures in a polar solvent (dH_2O) and dissolution of the complex into low molecular weight monomers in an organic solvent (MeOH). The

dissolution of micelles by MeOH can be reversed by removing the solvent and replacement with a polar solvent.

Further purification of the preparative SEC was performed using RP-HPLC and SEC-HPLC on SG277 $AmSO_4$ precipitate. RP-HPLC separation was performed on a phenyl column using a MeOH (+0.1% (v/v) AcOOH) gradient; from 65% to 95%. The resulting chromatogram (Figure 4.9), peak collections, activity testing (Table 4.3) and MALDI-TOF identification (Table 4.4) was described in Section 4.2.2. MS analysis revealed different isoforms of the lipopeptides, iturin A, fengycin, surfactin and mycosubtilin. In addition, two additional lipopeptides, mojavensin A (Ma *et al.*, 2012) and kurstakin (Béchet *et al.*, 2012) were detected. Using the microdilution assay chlorotetaine, iturin, fengycin and surfactin were found to carry anti-CD630 activity albeit at reduced levels compared to the preparative SEC fraction. Furthermore, RP-HPLC fractionation was used to analyse the preparative SEC fractions from SG297 (*B. amyloliquefaciens*) and two *B. subtilis* strains, SG140 and SG83 (Figure 5.12).

RP-HPLC of the SG297 preparative SEC fraction demonstrated evidence for the presence of iturins, fengycins and surfactins, although the iturin and fengycin profiles differed slightly from that of SG277. *B. subtilis* strains SG83 and SG140 preparative SEC fraction contained fengycins and surfactins but no iturins. Abundance of fengycin/surfactin between SG83 and SG140 differed markedly but activity of the original filtrate is of an equivalent level (1/20; Table 5.2). The difference in quantity of fengycin/surfactin between SG297 and SG140 is also noticeable, although the difference in activity (1/160 vs 1/20) seems somewhat disproportionate according to the size of the peaks. Although iturins display very little individual activity (1/10; Table 4.3), they may play another role within the micellar complex hence exaggerating the activity exhibited by strains producing this lipopeptide in addition to surfactin and fengycin.

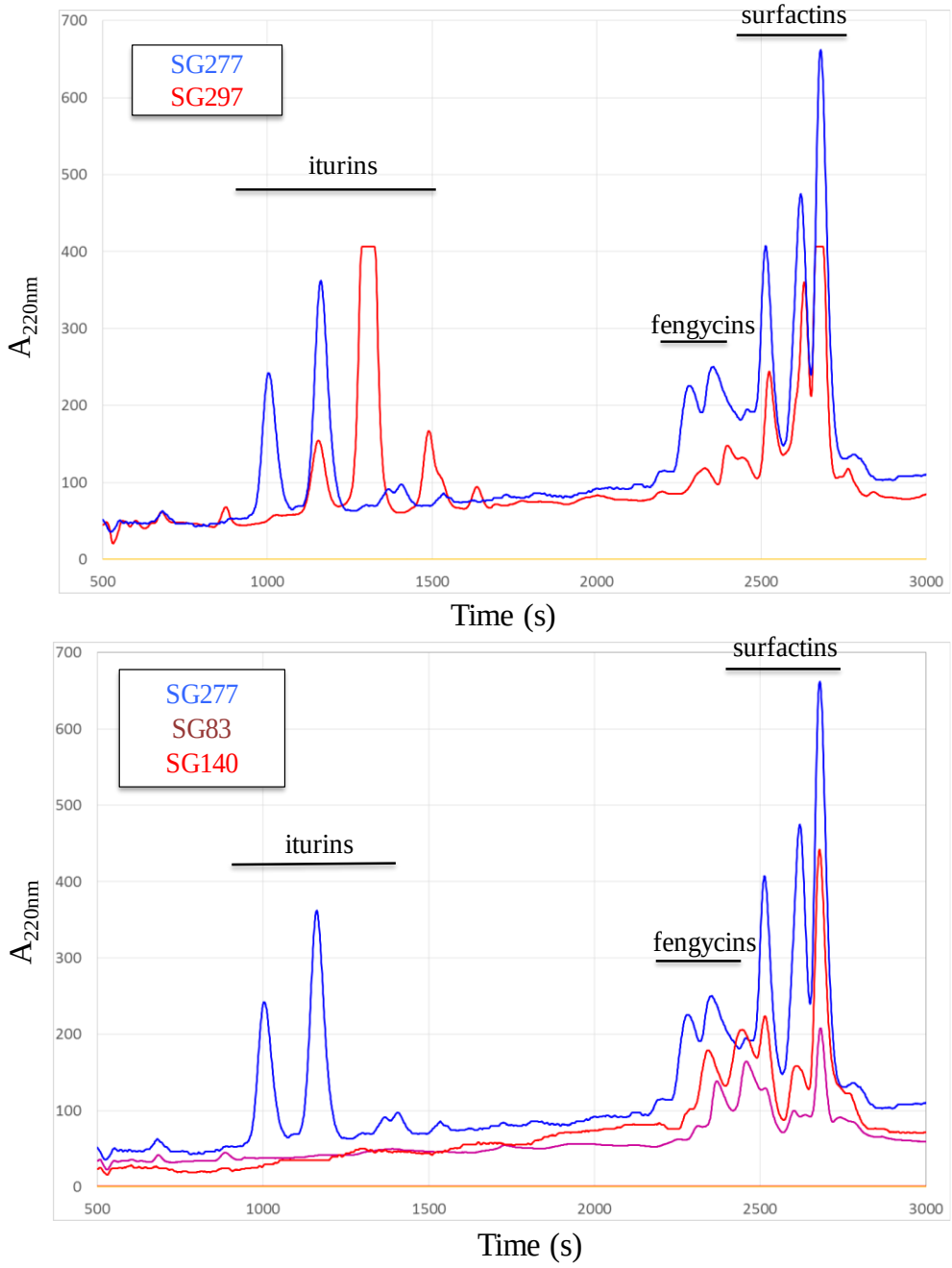


Figure 5.12. RP-HPLC separation of *B. amyloliquefaciens* and *B. subtilis* preparative SEC fraction. RP-HPLC separation of *B. amyloliquefaciens* strains SG277 and SG297 (**A**) and *B. subtilis* strains SG83 and SG140 (**B**).

To examine further the constituent species of the preparative SEC active fraction, separation was performed using SEC-HPLC on a TSK Gel G2500PW XL column (Figure 5.13). SEC-HPLC is more analytical than the preparative SEC performed using Superdex 200 resin described earlier (Figure 5.9) and separates molecules

between the range of 200-3,000 Da. The column was run under denaturing conditions, using a mobile phase of 65% ACN (+ 0.1% acetic acid).

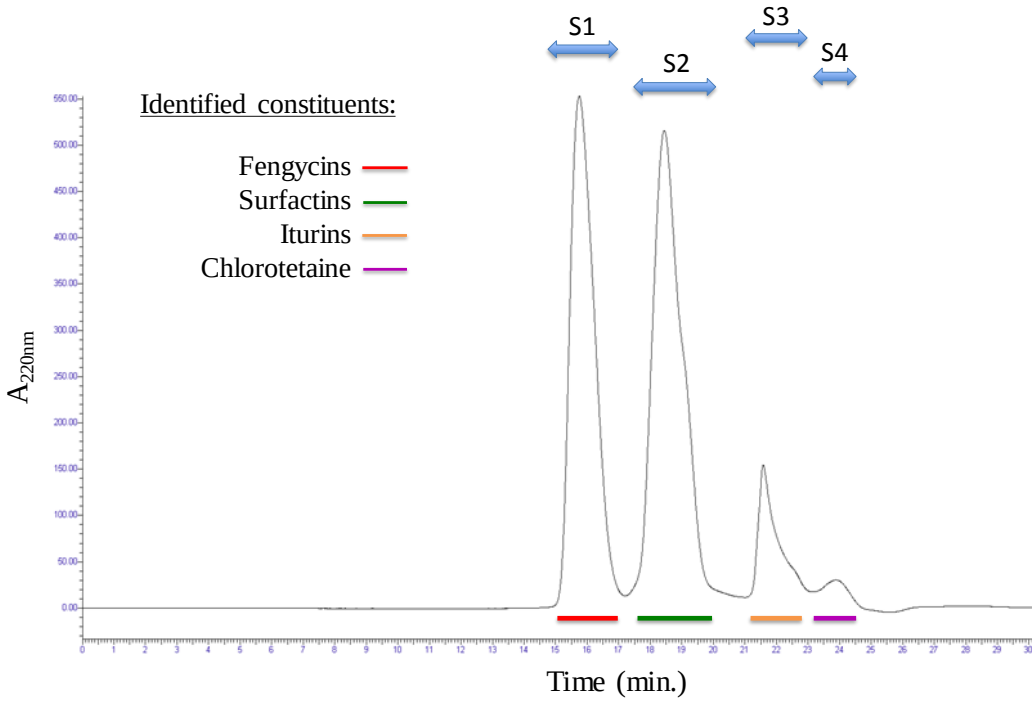


Figure 5.13. SEC-HPLC fractionation of SG277 preparative SEC. Analytical SEC-HPLC under denaturing conditions using HMW active SG277 SEC fraction. Non-polar mobile phase (65% acetonitrile + 0.1% acetic acid) breaks down the supramolecular lipopeptide aggregates to release LMW iturins, fengycins and surfactins. The LMW chlorotetaine elutes last. MALDI-TOF identification of material within peaks noted in the chromatogram. More in depth data of observed masses shown in Table 4.4.

SEC-HPLC analysis of high molecular weight species purified from SG277 growth medium using preparative SEC (Superdex 200) confirms their composition as a mixture of fengycins, surfactins, iturins and chlorotetaine (for MALDI-TOF data see Table 4.4). No additional high molecular weight components were identified (Figure 5.13).

It should be noted that absorbance at 220 nm, and the resultant peak sizes is not strictly quantitative if comparing different molecules. This is due to differential absorption of UV by biomolecules. Four peaks were observed during SEC-HPLC, collected and tested for activity against CD630 (Table 5.8).

Table 5.8. Anti-CD activity of SG277 SEC-HPLC fractions

Purification step	Anti-CD activity (endpoint titre)
Extracellular Filtrate	1/160
$AMSO_4$ (30x)	1/2560
Preparative SEC fraction[a]	1/1280
S1[a]	1/20
S2[a]	1/320
S3[a]	1/10
S4[a]	1/10

[a] Normalised by volume according to volume of $AmSO_4$ used.

The amount of activity of differing fractions separated by SEC-HPLC was equivalent to those separated by RP-HPLC, as would be expected. Surfactin displays by far the most activity (1/320), with fengycin, iturin and chlorotetaine providing significantly smaller amounts. Interestingly, the level of activity demonstrated by surfactin is of a much lower level than that of the preparative SEC fraction (1/1280) and considering that these various components elute in the same fractions during SEC, and appear to share the same, high molecular weight it is possible that they were forming mixed micelles.

A further experiment was performed to examine the mechanism of activity against CD630 in a co-culture assay (Figure 5.14). At an OD_{600} of 0.3-0.4, samples from various stages of SG277 purification, anti-*C. difficile* activity normalised (to an approximate end point titre of 1/100) were added to growing *C. difficile* cultures at a dilution factor of 1:10 and for every hour thereafter OD_{600} readings were recorded and CFU counts (plated on BHISS) enumerated. All samples showed equally rapid lytic activity against the log phase CD630 culture; within an hour the OD had dropped to the baseline and there were no detectable CFU.

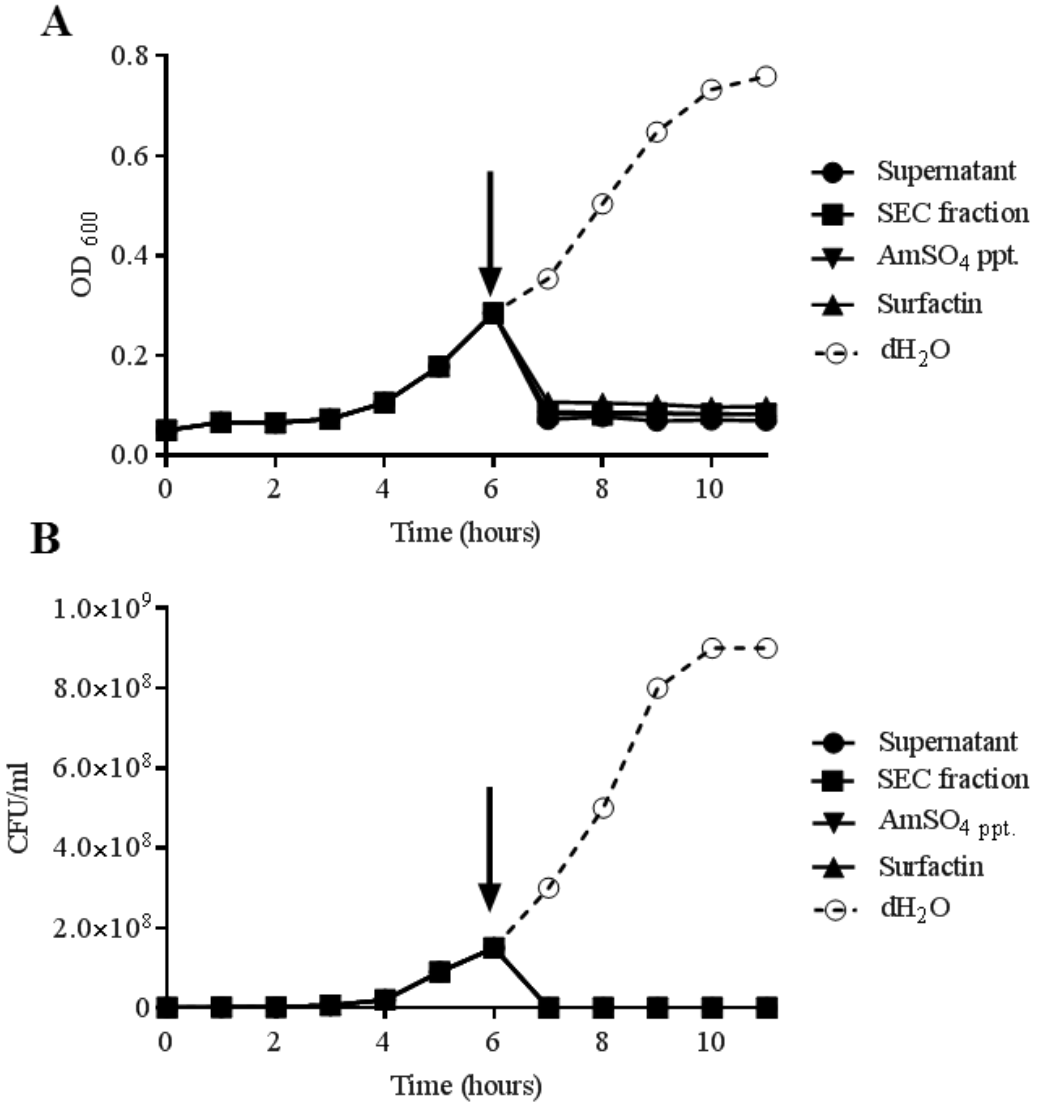


Figure 5.14. Co-culture assay using purified SG277 material against CD630. Samples of SG277 at various stages of purification; supernatant (0.45 µm filtered), dialysed 20% $AmSO_4$ ppt. in PBS, preparative SEC fraction and RP-HPLC purified surfactin were all diluted so activity against CD630 in a microdilution assay would be approximately 1/100. Samples were added to log phase CD630 cultures at a dilution of 1:10 (v/v) and OD_{600} readings were taken and CFU counts were enumerated hourly. The arrow indicates addition of sample.

5.2.4 Investigating the nature of the micellar structure

It has been reported that surfactins can act synergistically with iturins (Razafindralambo *et al.*, 2002) and mycosubtilin (Jauregi *et al.*, 2013). The interaction of iturin A with surfactin in mixed micellar structures has been shown to enhance the lytic activity against erythrocyte membranes (Thimon *et al.*, 1992). Figure 5.12 provided some evidence for iturin's mediatory role in the enhancement

of anti-*C. difficile* activity, with strains producing iturins in addition to fengycin/surfactin seemingly exhibiting disproportionately high levels of activity in comparison to strains lacking this single lipopeptide. Other studies have shown the complementary effect different amphipathic compounds can have on each other, and the resultant increase in antibacterial activity when they combine to form mixed micelles (Sheikh *et al.*, 2013). When surfactants mix within an aqueous solution, several physicochemical changes take place due to interactions between amphiphiles often resulting in enhanced interfacial and micellar properties. One example of a property which can be affected by the mixing of surfactants is the critical micellar concentration (CMC) (Sheikh *et al.*, 2013), the altering of which can affect the level of solubility and therefore the concentration of surfactants per unit volume and this can enhance their biosurfactant properties. Gram-positive bacteria lack an outer lipopolysaccharide layer but carry a thicker peptidoglycan and phospholipid membrane bilayer. Surfactin's detergent-like activity acts to create a permeable environment for the lipid bilayer, therefore disrupting and solubilising the bacterial membrane (Heerklotz and Seelig, 2001). For antibacterial activity, a significant concentration of surfactin is required (between 12 and 50 μg/ml) and so the CMC and solubility are integral to the active process. The resulting synergism from the formation of mixed micelles can lower the CMC, decrease the polarity of mixed micelles thereby enhancing interactions between the micelles and the bacterial membrane resulting in greater antibacterial activity. Sheikh et al. (2013) notes that their observed antibacterial activity only occurs above the CMC value, demonstrating the necessity of micelle formation for antibacterial activity.

It was therefore decided to investigate whether the combination of lipopeptides has a cumulative effect upon the activity against *C. difficile* (Figure 5.15). SG277 RP-HPLC purified material was collected according to identity (chlorotetaine, iturin, fengycin and surfactin), evaporated and tested against CD630 individually or in combination in a microdilution assay. Commercially produced (Sigma) lipopeptides were also tested alone and in combination against CD630.

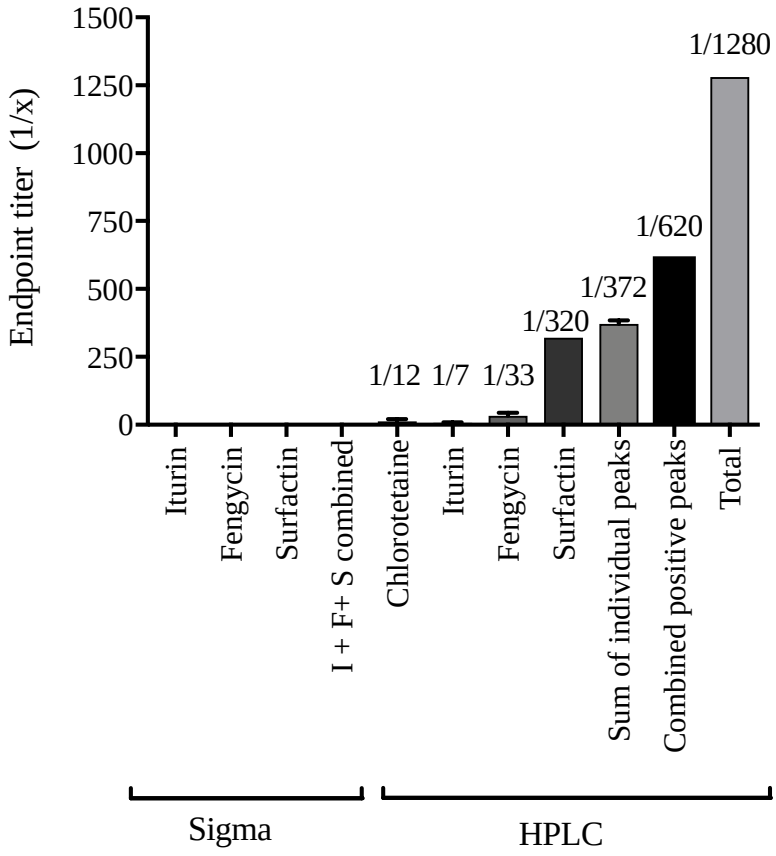


Figure 5.15. Cumulative activity of RP-HPLC purified SG277 material. SG277 SEC-HPLC separated material, individual or combined was tested for activity against CD630. Sum of individual peaks refers to mathematical addition of the activities of individual compounds, while combined positive peaks were pooled and then tested. All samples were normalised according to volume and represent the quantity found within the equivalent volume of SG277 preparative SEC fraction used for column injections. The average activity for three replicates is displayed above the relevant bars (standard deviation). Commercial compounds from Sigma were assessed for activity.

Interestingly, the amount of activity increased as the molecules were combined. Importantly, this was greater that the mathematical sum of the individual activities for each of the four compounds. This suggests that a synergistic effect between the compounds was taking place, and for an optimal level of activity the compounds needs to be in solution together. Using commercial compounds (Sigma), none demonstrated any activity, on their own or when combined.

To further discern the nature of these mixed micelles two techniques were employed. The first of which was Cryo-TEM analysis (Figure 5.16) which has been used to visualise micelles in solution (Vinson *et al.*, 1991; Won *et al.*, 2002). Surfactin has been previously imaged with Cryo-TEM displaying micelles of

approximately 5-9 nm at pH 7 (Isa *et al.*, 2007). The size and shape of surfactin micelles however were heterogeneous, with some appearing cylindrical with lengths up to 160 nm.

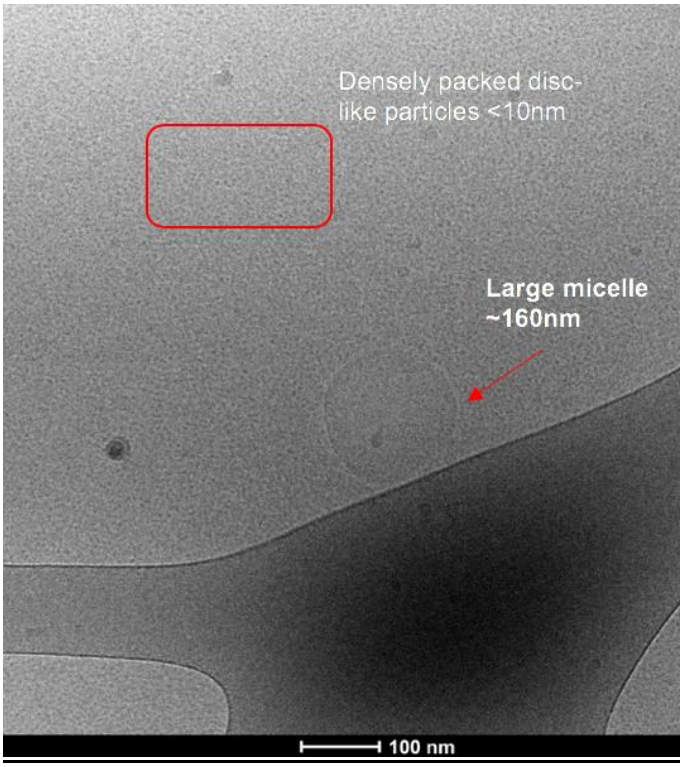


Figure 5.16. Cryo-TEM analysis of SG277 SEC material. Image courtesy of Professor Alain Brisson, University of Bordeaux.

Cryo-TEM analysis of the SG277 preparative SEC material showed disc-like objects tightly packed and <10 nm in size. Occasionally large size discs were apparent with an approximate diameter of 160 nm. These objects resembled micelles of <10 nm and ~160 nm in size. Individual RP-HPLC fractions were also imaged, corresponding to iturin, fengycin and surfactin but instead of showing micelles, displayed rod-like filaments (data not shown).

The second technique employed was dynamic light scattering (DLS) analysis. All analysis was performed using material in 150 mM sodium phosphate buffer (pH 7.2). The preparative SEC material was analysed and the presence of micelles with a hydrodynamic diameter of 6.8 nm was confirmed (Figure 5.17A). The micelles remained remarkably stable over 17 hours at RT during which no change in size was detected (Figure 5.17B). In Figure 5.17B, the intensity of the main 6.8 μm peak

increases slightly over time and the peaks indicated aggregates larger than 100 nm that disappeared completely after 17 hours, due to either re-assembly into smaller micelles or sedimentation over time. A freeze-dried SEC fraction was reconstituted in phosphate buffer and the size distribution measured by DLS was equivalent to the original sample, suggesting that the micelles could withstand lyophilisation and resuspension (Figure 5.18A).

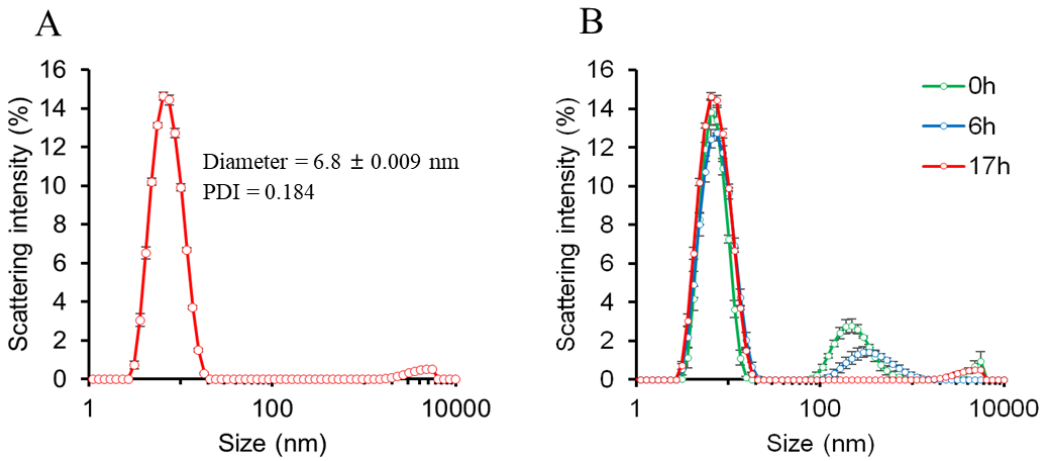


Figure 5.17. DLS analysis of SG277 preparative SEC material. Self-assembly of lipopeptides. Hydrodynamic size distribution of preparative SEC material prepared in 150 mM sodium phosphate buffer at pH=7.2 shows high molecular weight monodisperse complexes with an average diameter of 6.8 ± 0.009 nm (**A**). Size distributions of the SEC fraction at different times after dilution in 150 mM sodium phosphate buffer at pH = 7.2 (**B**). Data points are the average of three measurements with error bars representing SEM. DLS performed in collaboration with Dr Enrico Ferrari, University of Lincoln and Dr Mikhail Soloviev, Royal Holloway University.

In an attempt to identify the minimum molecular subset responsible for the self-assembly of the micellar complex, the high molecular weight preparative SEC fraction was further fractionated under fully denaturing conditions using either RP-HPLC (Figure 4.9) or SEC-HPLC (Figure 5.13). All the individual lipopeptides collected from the HPLC experiments were tested individually and mixed to reconstitute the original composition. When complete sets of collected fractions were recombined and analysed, the size distributions measured by DLS showed the same data as observed in the original SEC and freeze-dried reconstituted sample, suggesting that micelle formation is driven by spontaneous self-assembly of the low molecular weight lipopeptides (Figure 5.18A). However, HPLC reconstituted samples showed the presence of aggregates, suggesting that the exact conditions and molar ratios in which individual components agglomerate is most likely

important in the optimal construction of micelles with a narrow size distribution and equivalent yield to those formed in native conditions. Individually reconstituted HPLC fractions displayed high polydispersity due to aggregation and so rigorous analysis and fitting of the hydrodynamic diameter was not possible. However, DLS analysis provided the cumulants fit intercept (CFI) for all fractions and combinations (Figure 5.18B). CFI crudely quantifies the presence of scattering particles over the background noise, with a maximum value of 1 indicting extensive scattering from particles in suspension and values below 0.2 typically indicating no scattering. The cumulant fit analysis also provided the polydispersity index (PDI), which indicates how broad the size distribution is. PDI is greatly affected by the presence of multiple peaks, with values between 0.5 and 1 indicating extensive aggregation, and values below 0.5 indicating monodispersed distributions.

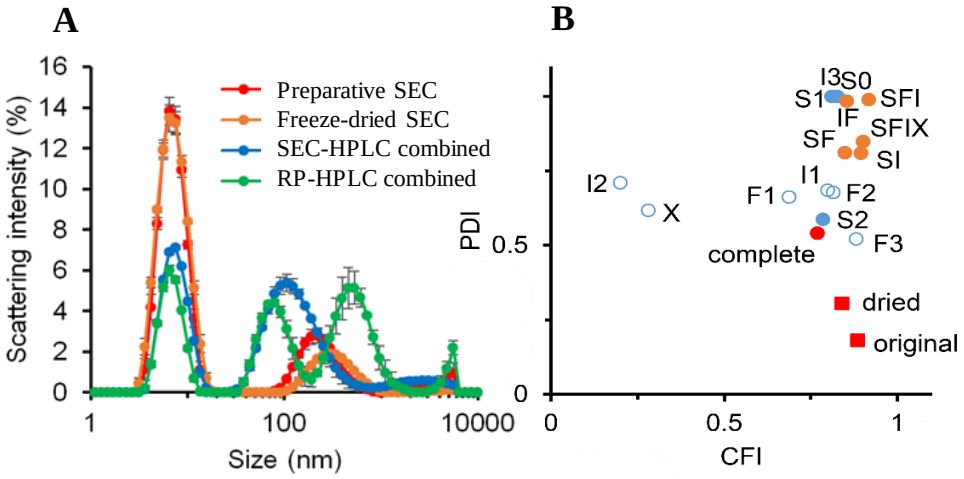


Figure 5.18. DLS analysis of HPLC purified SG277 material. Self-assembly of lipopeptides from original and freeze-dried preparative SEC fraction and from reconstituted combined HPLC preparations, containing all lipopeptides. The size distributions of samples reconstituted from HPLC fractions yields higher polydispersity and indicates limited aggregation (**A**). The polydispersity index (PDI) versus the cumulants fit intercept (CFI) of individual RP-HPLC fractions or combinations of them (**B**). Squares represent mixtures not separated by HPLC, circles correspond to RP-HPLC fractions and combinations thereof. All red symbols indicate the presence of all lipopeptides, blue symbols correspond to individual RP-HPLC fractions and orange symbols are defined mixtures comprised of different peaks S= surfactin, F= fengycin, I= Iturin, X= chlorotetaine; (SFI=S3+F3+I3; SFIX=S3+F3+I3+X; SF=S3+F3; IF=I3+F3; SI=S3+I3). Individual peak fractions used can be viewed in Figure 4.9. Filled shapes indicate preparations where a peak of 6.8 nm was visible by DLS, whereas open symbols represent preparations where no such nm peak was detected by DLS. Data points are the average of three measurements with error bars representing SEM. DLS performed in collaboration with Dr Enrico Ferrari, University of Lincoln and Dr Mikhail Soloviev, Royal Holloway University.

The scatter plot of PDI versus CFI revealed that iturins, fengycins and chlorotetaine on their own yield highly polydisperse aggregates. Individual surfactin fractions

eluted from RP-HPLC and combinations of surfactins with the other lipopeptide fractions do assemble into micelles to some extent (Figure 5.18B). However, no combinations yielded monodisperse and stable micelles as seen in the original preparative SEC fraction and with freeze-dried SEC, except for the combination of all the RP-HPLC fractions (complete), where size distribution was comparable to that of the preparative SEC material. RP-HPLC fraction F3 yielded size and PDI values similar to that of the combined RP-HPLC fractions (complete) and S2, which is not entirely unexpected as this fraction contains both fengycin and surfactins as confirmed by MALDI-TOF-MS and RP-HPLC analyses (Table 4.4 and Figure 4.9). It is therefore reasonable to conclude that surfactins are responsible for providing a micellar morphology to the mixture but the formation of the stable and monodisperse micelles observed in the original sample depends on the precise combination of multiple lipopeptides in a mixed micellar complex (Figure 5.19).

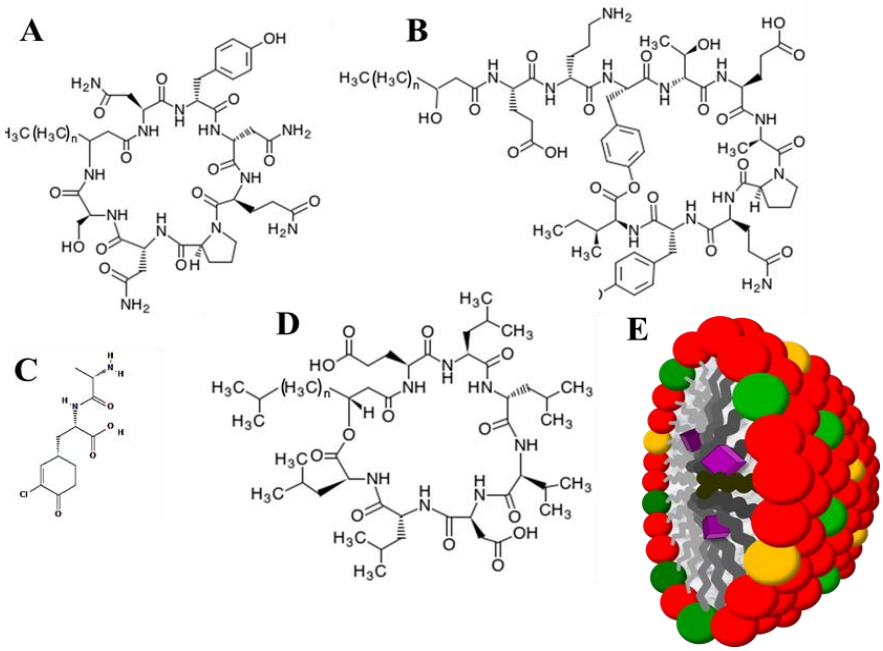


Figure 5.19. Micellar constituents. The individual, principal components of the micellar complex responsible for activity against *C. difficile* are shown, in addition to a hypothetical diagram of their association with each other. Iturin (**A**), fengycin (**B),** chlorotetaine (**C**), surfactin (**D**) and a hypothetical composite micelle formed by surfactins (red), fengycins (green) and iturins (yellow) (**E**). The micelle contains low molecular weight components (e.g. Chlorotetaine; purple), and may act as a delivery vehicle, increasing the bioavailability of all of the components. All images from KEGG; iturin- (KEGG: C12266. ChEBI:31737, PubChem: 14411) CAS: 52229-90-0, fengycin-(KEGG: C12042. ChEBI: 29583, PubChem: 14197. 3DMET: B05603, MW=1463.7122) CAS: 102577-03-7, surfactin-(KEGG: C12043. ChEBI:29681&71978, PubChem: 14198. 3DMET: B04413, MW=1036.3446) CAS: 24730-31-2.

5.3 Discussion

This chapter provides evidence for a complex of lipopeptides produced by strains of *B. amlyloliquefaciens*, *B. subtilis* and *B. licheniformis.* This complex exerts significant bacteriolytic activity upon the nosocomial pathogen, *C. difficile.* The principal lipopeptides responsible for this activity are iturin, fengycin and surfactin; surfactin seemingly being the predominant constituent evidenced by its proportionally high presence (Figure 5.13), high activity (Table 5.8) and responsibility in providing a stable, monodisperse micellar morphology in combination with the other lipopeptides (Figure 5.18B). These individual lipopeptides self- assemble to form mixed micelles thereby magnifying the activity exhibited against *C. difficile*. This effect has been noted previously, in the case of surfactin and mycosubtilin (Jauregi *et al.*, 2013) and surfactin and iturin A (Razafindralambo *et al.*, 2002; Thimon *et al.*, 1992). The observed synergy presented by the mixing of micelles is likely due to the complimentary effect that different amphipathic compounds have upon each other; physiochemical changes due to interactions between amphiphiles and the subsequent enhancement of interfacial and micellar properties resulting in an increase in antibacterial activity (Sheikh *et al.*, 2013). Physiochemical alterations of micelles can be inferred by the DLS analysis data presented in Figure 5.18B, where upon the mixing of differing lipopeptides, an increase in mono-dispersity and a decrease in aggregation suggest modifications to the solubility of the constituents. These physiochemical changes translate into increased antibacterial activity against *C. difficile* (Figure 5.15), where the mixing of the individual lipopeptides resulted in activity which was greater than the mathematical sum of the individual activities for each of the four compounds.

Interestingly, activity was associated with the outer surface of the bacterial cell and/or spore (Figure 5.4). This material is most probably associated with the polysaccharide matrix of the outer surface of the cell envelope, a characteristic of the mucoid *B. amyloliquefaciens*. *B. subtilis* strains which demonstrated activity against *C. difficile* also tended to share this mucoid characteristic in their colony morphology. Surfactin has been implicated in the motility and swarming of *B.*

subtilis by increasing surface wettability (Angelini *et al.*, 2009; Ghelardi *et al.*, 2012), and so it would follow that surfactins associated with the surface of the cell would provide benefit to the host bacterium by providing high and localised concentrations of the surface-active agent in the immediate vicinity of the cell. This surface association has intriguing implications when applied to protection against CDI *in vivo*. In Chapter 4 increased protection against CDI was observed when animals were dosed with SG277 cells and compared to the administration of the filtrate alone. This may be explained by the phenomenon of cell/spore surface associated activity; it is possible that the filtrate is retained within the GI tract for a lesser amount of time than spores or vegetative cells, which may form biofilms (Vogt *et al.*, 2018) or otherwise associate with the gut mucosa. This could result in comparatively higher persistence of antibacterial lipopeptides within the GI tract and therefore enhanced elimination of *C. difficile.* The provision of cells/spores with lipopeptides associated with the surface may therefore provide a longer lasting, and more consistent supply of antibacterial lipopeptides within the GI tract, resulting in greater elimination of *C. difficile* and enhanced protection against CDI. In addition, germination of spores, and outgrowth of cells may provide additional lipopeptides but in the case of the mouse study this is unlikely considering the short time span of the experiment and the rapidity at which *C. difficile* initiates infection within the host post-challenge.

Cryo-TEM analysis in Figure 5.16 presents micelles of the approximate diameter as determined by DLS analysis. Of further interest is the presentation of the micelles during TEM analysis of the CsCl band and of the SEC fraction (Figure 5.7 and 5.10). At the time of TEM analysis the identity of the active components had not been confirmed, and so TEM analysis was performed without full knowledge of the expected size of the compound/s. However, rather than the presence of circular micelles, extended granular clusters 20-30 nm in diameter were observed. These granular clusters most likely represent the formation of 'wormlike' micelles (Helvig *et al.*, 2015). Wormlike micelles are formed by the agglomeration of amphiphiles into flexible, elongated, self-assembled structures (Dreiss, 2007). Furthermore, the formation of wormlike micelles has been documented for surfactins (Arutchelvi *et*

al., 2014). Although surfactants and their structure have been well studied over the past century, the function and implications of the biophysical phenomenon of wormlike micelles is not well understood (Dreiss, 2007).

The activity shows remarkable stability against various chemical and enzymatic insults, in addition to resistance to high temperatures and acidic pH. This high stability is most likely due to the hydrophilic cyclical structure of the lipopeptides and the added stability in solution conferred by micellisation (Lin and Grossfield, 2015). The lipopeptides showed good stability against simulated gastric fluid, providing an explanation for the effectiveness demonstrated *in vivo* against CDI. 100% of the activity against *C. difficile* is retained for 1 week at 4^{o}C, and 50% for ~6 weeks. These properties provide an advantage over conventional antimicrobial peptides (AMPs), which are effective, broad-spectrum, thermally stable agents but are labile under high temperatures for extended periods of time (80^{o}C for 15 min.) and extremes of pH (Lin and Grossfield, 2015).

The activity displayed by these lipopeptides is bacteriolytic (Figure 5.2). This would imply the mechanism of action is similar to that of a detergent, where lysis of the cell occurs through a combination of surface layer protein solubilisation, pore forming and membrane lytic mechanisms (Bechinger and Lohner, 2006). Gram-positive bacteria are generally more susceptible to this action than are Gram-negative bacteria (Baker *et al.*, 1941). Surfactin has been shown to have detergent-like, membrane destabilising action upon lipid membranes, and this has been attributed to its preference for micelle formation over membrane insertion (Heerklotz and Seelig, 2001). The activity exerted upon *C. difficile* occurs rapidly, with almost complete lysis occurring in less than 1-hour post addition of SG277 or SG297 filtrate to growing *C. difficile* cultures. Extracellular material produced from *B. amyloliquefaciens* has previously been shown to have activity against *C. difficile* using a cross-streak method (Boottanun *et al.*, 2017) but the work presented here is the first to define the nature of the inhibitory activity.

CHAPTER 6

Discussion

6.1 Spore mediated colonisation of *C. difficile*

As is the case with many bacterial diseases, CDI involves host colonisation and subsequent toxin production. Colonisation is comprised of several distinct phases, starting with the ingestion and acquisition of *C. difficile* spores, germination, outgrowth and proliferation. Studies focussing on the colonisation of *C. difficile* have mostly examined the role of the vegetative cell, since it has been pre-supposed that the role of the spore is confined to transmission and dissemination of this obligate anaerobe. However, it has been shown that the spore may contribute to colonisation and infection and the spore coat protein, CotE, has been identified as an agent of mucus binding and colonisation.

The spore of *C. difficile* is a dormant entity that enables indefinite survival outside of the host. It is therefore vital for transmission and dissemination and accounts for the prevalence of this organism in humans and animals. It is assumed that *C. difficile* is acquired immediately prior to infection although there is evidence that humans can be asymptomatic carriers with low numbers of spores resident and latent in the GI tract (Deakin *et al.*, 2012). *C. difficile* strains defective in spore formation (*spo0A* mutants), fail to transmit disease, confirming the functional role of the spore in dissemination (Deakin *et al.*, 2012).

Prior to this work, there was preliminary evidence that the spore may play a role in colonisation and infection. The *C. difficile* spore exosporium carries three large collagen-like proteins that form hair-like filaments. One of these proteins, BclA1 has been shown to play a direct role in colonisation. In hamsters, *bclA1* mutant spores had a 2-log higher ID_{50} (50% infectious dose) than isogenic wild-type spores demonstrating that BclA1 plays a role in the initial colonisation of the host, prior to germination of the spore (Phetcharaburanin *et al.*, 2014). *C. difficile* has recently

been shown to form biofilms *in vivo* (Semenyuk *et al.*, 2014, 2015; Soavelomandroso *et al.*, 2017). Spores and vegetative cells were found in equal numbers in localised biofilms throughout the caecum and colon. The biofilm was shown to be enriched with polysaccharides, and to reside on the uppermost layer of mucus. In the case of R20291 the biofilm was both above the mucus layer and embedded within it. The presence of spores within the biofilm may therefore extend their role beyond that of only dissemination.

Inflammation is an important symptom of CDI and results from the action of both cytotoxins (Shen, 2012). However, there is evidence that factors (currently unknown) other than toxins contribute to pathogenesis (Buckley *et al.*, 2013). While *C. difficile* spores can survive within phagocytes for extended periods of time (Paredes-Sabja *et al.*, 2012), there is also evidence for invasion into epithelial cells (Goulding *et al.*, 2009). The ability to invade enterocytes is both intriguing and important since it could potentially contribute to the inflammatory response. This assumption is supported by hamster models performed using a *sigK*$^{-}$ mutant strain of *C. difficile*, in which administration of *sigK*$^{-}$ vegetative cells results in a marked reduction in virulence (Figure 3.21). The infected animals carried equivalent levels of toxins in the caeca as animals infected with spores, yet endpoints were reached much later for the *sigK*$^{-}$ mutant (168 hours) than for wild-type *C. difficile* (72 hours). This suggests that the spore, as well as the vegetative cell may a role in inflammation and therefore in the infection process.

Chapter 3 presents data in support of the spore's role in colonisation and infection. This process is mediated by the bifunctional protein, CotE, through its C-terminal chitinase. The CotE chitinase domain was shown to be required for the adhesion of spores to mucus *in vitro* (HT29-MTX cells) and *ex vivo* (pig mucus explants) and that antibodies specific to the C-terminus were able to reduce adhesion. Using an ELISA assay, it was shown that the recombinant C-terminal chitinase of CotE (rCotEC) bound to mucin, in addition to mucin's monomer constituents GlcNAc and GalNAc (Brockhausen *et al.*, 1985). This mechanism underlying colonisation has been previously found to facilitate infection by *Vibrio cholerae* (Kirn *et al.*,

2005). The chitin binding protein GbpA was found to be required for efficient intestinal colonisation by binding to GlcNAc present on the surface of intestinal epithelial cells. A *gbpA* mutant showed a 50% reduction in attachment to HT29 cells and a tenfold increase in the LD_{50} compared to wild type. To further test the importance of CotE in the colonisation of the host by *C. difficile,* GlcNAc or glucosamine hydrochloride was orally administered to mice (in drinking water) and was found to prevent colonisation by CD630. By administering GlcNAc in drinking water we aimed to competitively inhibit the binding of the chitinase domain to the GlcNAc present in mucin thereby preventing colonisation. The prevention of infection observed suggests that this is what occurred, and so underscores the importance of CotE mediated adhesion to mucin via interaction with GlcNAc in colonisation by *C. difficile*. A possible confounding variable in this experiment is the effect of GlcNAc on the microbiota. The ingestion of dietary trehalose and microbiota accessible carbohydrates (MACs) has been shown to have an enhancing, and inhibiting effect on CDI respectively (Collins *et al.*, 2018; Hryckowian *et al.*, 2018). The consumption by mice of MACs, either as a complex mixture or with inulin being the sole source, has demonstrated significant effects on the microbiota membership of the GI tract and its metabolic output. The outgrowth of MAC-utilising taxa results in the production of short chain fatty acids (SCFAs) and a subsequent decrease in fitness of *C. difficile.* It is therefore possible that the administration of GlcNAc had a similar effect and so further *in vivo* studies utilising concomitant metagenomic analysis of intestinal contents over time are required.

It was also demonstrated that wild type *C. difficile* spores were able to degrade mucin *in vitro* and that spores devoid of the CotE chitinase domain were unable to do so. Mucin degradation has also been displayed by *Vibrio cholerae*, where the secreted chitinase ChiA2 is used by the organism to hydrolyse intestinal mucin to release GlcNAc which is subsequently used for growth and survival within the GI tract (Mondal *et al.*, 2014). Interestingly, chitinases alone have been shown to have low mucolytic effects, and it is the additional presence of proteases and nucleases which amplify the degradation exhibited by chitinases (Sanders *et al.*, 2007). This perhaps suggests that the mucin degradation demonstrated by the CotE chitinase

may, in part, be due to assistance from either vestigial proteases/nucleases from the spore purification process or from enzymes resident on the spore surface layers. It could be imagined that the limited degradation of the mucin polysaccharide moieties by CotE renders mucin increasingly sensitive to protease degradation. Therefore, a direct role for CotE in mucus degradation remains to be established.

Previous data gathered by our laboratory demonstrated that CotE chitinase activity is at its highest during spore germination (Permpoonpattana *et al.*, 2011). Spore germination occurs within minutes and CotE is likely to be active in the spore coat integuments following release of the cell from the spore coat. It is therefore possible that the degradation of mucin by CotE provides nutrients for the newly germinated *C. difficile* cell. It has been previously shown that *C. difficile* is capable of metabolising mannose, fucose, galactose, GalNAc and GlcNAc for growth; all of which are components of mucin glycan side chains (Engevik *et al.*, 2015). To further test CotE's role in the degradation of mucin, wild type and CotE$^-$ spores could be assayed for degradation against various glycosylation profiles, purified from mouse models, pigs and human cell lines. The supernatants following treatment assays would then be analysed using ^{1}H NMR to identify the metabolites produced from the pattern of their chemical shift signatures (Crost *et al.*, 2013, 2018).

Studies in humans, have showed that compared to healthy subjects, CDI patients demonstrated decreased levels of MUC2, the major component of mucus (Engevik *et al.*, 2015). The hypothesis that the demonstrated ability of CotE to degrade mucin, and therefore mucus, may facilitate progression of toxins towards the epithelium could be tested by using *in vitro* organ culture (IVOC). This system is based on human intestinal biopsies and can be used to monitor for the appearance of "dark holes" devoid of MUC2 staining in infected tissue samples symbolising mucin proteolysis (Etienne-Mesmin *et al.*, 2019). This could be performed with wild type and CotE$^-$ spores and used to determine the effect of decreased mucus levels on toxin progression, with exogenous toxin added during the IVOC experiment, the mucus layer removed, and the biopsy epithelium quantified for

spores and toxins using ELISA or immunofluorescent staining (Etienne-Mesmin *et al.*, 2019).

Mice infected with CotE mutant spores, showed a marked reduction in colonisation relative to those infected with wild type spores, as demonstrated by the ID_{50} data. In a hamster model of infection, CotE clearly influenced the virulent phase of infection since the time from first presentation of symptoms to severe symptoms of CDI was significantly delayed in animals dosed with spores devoid of CotE. This phenotype was in marked contrast to that of a BclA1 mutant which showed no difference in the severity of CDI compared to wild type spores (Phetcharaburanin *et al.*, 2014), suggesting that BclA1 is involved in the primary stages of colonisation rather than the virulent phase. Interestingly, the level of caecal toxins and CFU of deceased hamsters were essentially equivalent whether animals were infected with wild type or CotE mutant spores. This suggests that CotE plays an important role in CDI by targeting the spore to the mucosa enabling subsequent steps in the infection process. It should be noted that the deletion of *bclA1* resulted in significant, morphological defects in the *C. difficile* spore coat (Phetcharaburanin *et al.*, 2014). This may suggest a role for BclA1 as a structural protein within the spore coat and exosporium, as is the case for the *B. anthracis* protein, BclB, which is vital for the integrity of the exosporium (Thompson and Stewart, 2008). It is therefore entirely possible that deletion of BclA1 results in a loss in the abundance of spore coat proteins, such as CotE, on the outer surface of the spore, thereby reducing the spore's ability to bind to mucus and colonise a host.

Chitinases and peroxiredoxins have both been linked to inflammatory responses (Lee *et al.*, 2011; Knoops *et al.*, 2016). It is possible peroxiredoxins, as antioxidants and reducers of superoxide dismutase could play a role in spore survival within phagocytes, as has been shown in the case of *Listeria monocytogenes* (Kim *et al.*, 2007). Chitinases have been linked to the uptake of bacteria by host cells (Chaudhuri *et al.*, 2010), thereby providing a possible reason as to why peroxiredoxin and chitinase are positioned within the same gene. Additionally, considering the inflammatory responses elicited by the rCotEN protein in our

studies, and the extremely high levels of spores that are produced concurrent with toxin secretion, spores could contribute to, and amplify, the inflammatory responses that are typical of acute CDI. In summary, our data combined with that of others suggest that CotE plays a direct role in *C. difficile* colonisation and virulence. That this attribute is associated with the dormant spore is both unique and unexpected.

To better determine if CotE contributes to the inflammation observed during CDI, a triple mutant devoid of both CotE and toxins A and B could be used. This could be created by introducing an insertional mutation of *cotE* into a *tcdA*$^-$ *tcdB*$^-$ clostron mutant using a plasmid-based system shown to work for construction of triple mutants (Kuehne *et al.*, 2014). A triple mutant would allow for comparisons between a *tcdA*$^-$ *tcdB*$^-$ and a *tcdA*$^-$ *tcdB*$^-$ *cotE*$^-$ mutant thereby isolating the *in vivo* inflammatory properties of CotE. This experiment could be performed in a mouse model at multiple time points, with symptoms being recorded. Colonic tissue could be isolated, examined histopathologically and spleens and mesenteric lymph nodes examined for induction of inflammatory cytokines using RT-PCR (Nguyen Van *et al.*, 2006).

The ability of CotE to bind oligosaccharides might provide a mechanism by which CotE contributes to biofilm assembly and colonisation. To test this hypothesis, a wild type and a *cotE*$^-$ mutant could be cultured anaerobically on polycarbonate discs, a technique shown to produce robust *C. difficile* biofilms (Semenyuk *et al.*, 2014). SEM could be used to assess the extent of the extracellular matrix and to check for the presence of vegetative cells and spores. ELISA assays using antibodies against spores, CotE and toxins A and B could be used to assess differences. To gain further insights into the receptors involved in the interaction with mucus, and to test the hypothesis that CotE is able to interact with biofilms, assays could be performed to test the binding of wild type and *cotE*$^-$ mutant spores to a range of mucins with different glycosignatures (Gunning *et al.*, 2016).

This work has been focussed on the chitinase domain of CotE, so studies could also explore the potential role of the N-terminal peroxiredoxin. As mentioned earlier,

peroxiredoxin has been implicated in intra-phagocyte survival through the detoxification of reactive oxygen species (ROS) which contribute to killing of intracellular microorganisms (e.g., H_2O_2 and potentially nitric oxide) (Harder *et al.*, 2006; Kim *et al.*, 2007). Interestingly, spores of *Bacillus subtilis* which share similarity with those of *C. difficile* are completely phagocytosed after several hours (Ceragioli *et al.*, 2009). Experiments to measure the degree to which CotE contributes to phagocyte survival could be performed. Uptake and internalisation of wild type and CotE$^-$ mutant spores could be monitored by fluorescence microscopy using Alexa Fluor 488 and biotin labelled spores (Paredes-Sabja and Sarker, 2012a; Paredes-Sabja *et al.*, 2012). Survival within phagocytes could be determined using direct counting of viable CFU within lysed cells. Studies could also be performed *in vivo* following histopathology of colonic samples from mice infected either with wild type of CotE$^-$ mutant spores. TEM could be used to examine infected gut material with a focus on phagocytic cells, and specific focus could be directed to detecting the presence of *C. difficile* in neutrophils and non-phagocytic cells in which *C. difficile* has been shown to invade (Goulding *et al.*, 2009).

Peroxiredoxins react with hydrogen peroxide using an active site peroxidatic cysteine which consequently becomes oxidised to a sulfenic acid. Peroxiredoxins can be classified into different groups via mechanistic differences (Rhee, 2016). A 2-Cys peroxiredoxin possess a 'resolving' cysteine which displaces the hydroxyl from the peroxidatic cysteine to form a disulphide bond which is usually reduced by a thioredoxin (Trx)/thioredoxin reductase (TrxR) system of proteins (Park *et al.*, 2014). 1-Cys peroxiredoxins lack the resolving cysteine and so the sulfenic acid is often reduced by small molecules such as glutathione or ascorbate (Rhee, 2016). CotE was originally assigned as a 1-Cys peroxiredoxin (Permpoonpattana *et al.*, 2011), however the presence of other cysteines in its peroxiredoxin domain and the more recent recognition that the resolving cysteine can have varying locations in different 2-Cys peroxiredoxins warrants further investigation (Perkins *et al.*, 2015). Experiments could be performed where the CotE peroxiredoxin domain is incubated in the presence of H_2O_2 and the capacity of Trx/TrxR/NADPH and small

molecule reductants to support peroxidase activity could be tested. UV/Vis spectrophotometry could also be used to monitor the reactions (Lu *et al.*, 2006). The presence of *cis*-regulation of activity by the partner domain should also be investigated, for example, by assaying the chitinase activity in the presence of H_2O_2.

Up until this point effort has almost exclusively been focused on the vegetative cell as the primary factor of colonisation and so the work presented here is new to the field. Measures to control CDI are mostly focused on the toxins and therefore, understanding that the spore interacts with the mucosa may open new avenues for research into disease prevention. Further work to understand how the spore interacts with the mucosa may provide strategies to prevent spore attachment and gut colonisation by *C. difficile*. The suggested studies in this chapter will help to further unravel how *C. difficile* spores colonise the host, and long-term this may provide novel approaches for rational drug design and more robust measures to control this human and animal pathogen.

6.2 On the role of allochthonous *Bacillus* in CDI

There is growing appreciation at the integral role played by gastrointestinal microbiota in preventing the colonisation and subsequent infection by pathogens (Guarner and Malagelada, 2003; Kamada *et al.*, 2013). The use of probiotics are frequently claimed to improve human health, and the implicated mechanisms behind this beneficial effect are mostly attributed to indirect effects, such as immune system modulation, intestinal epithelium enhancement and competition with pathogens for space and for nutrients (Macpherson and Harris, 2004; Gourbeyre *et al.*, 2011; Bermudez-Brito *et al.*, 2012). Evidence for direct effects exerted upon pathogenic bacteria by probiotics are less commonly found in the literature. It has been shown that bacteriocin-producing *E. coli* prevents colonisation of related pathogenic species in the inflamed gut of mice (Sassone-Corsi *et al.*, 2016). Evidence for non-bacteriocin probiotic interference of pathogens in humans is scarce, but a recent study by Piewngam *et al.* (2018) demonstrated that *Bacillus* produced cyclic lipopeptides that abolished colonisation by the pathogen *S. aureus* in humans by inhibiting quorum sensing.

Bacillus are a genus of soil bacteria, able to form highly resistant endospores which often integrate themselves into the human microbiota by exposure to the environment and consumption of vegetables (Tam *et al.*, 2006). These endospores can subsequently germinate into metabolically active, vegetative cells (Casula and Cutting, 2002) within the small intestine and temporarily colonise the GI tract (Duc *et al.*, 2004). The reported numbers of *Bacillus* spores in faeces are on average ~10^5 CFU/gram (Tam *et al.*, 2006). Several probiotic formulations include species of *Bacillus* (Hong *et al.*, 2005) but the mechanism of action remains poorly defined.

In this work *Bacillus* was isolated from the faeces of mice and it was demonstrated that several strains from three species, *B. amyloliquefaciens*, *B. subtilis* and *B. licheniformis* had extracellular activity against *C. difficile.* It was shown that this 'active' population decreased, both in the quantity per gram and as a proportion of the total isolated spore formers, in response to clindamycin treatment. Furthermore, *Bacillus* was shown to be an allochthonous population within the GI tract; the population must be replenished from environmental *Bacillus*. The activity against *C. difficile* coincided with the demonstrated biosurfactant activity of the strains, as assayed using an oil dispersion assay. Previously, it has been shown that the solvent extracted biosurfactant from *B. amyloliquefaciens* and *P. aeruginosa* had activity against a range of pathogens, including *S. aureus*, *E. coli* and *C. albicans* (Ndlovu *et al.*, 2017).

These findings provided a link to the hygiene hypothesis. The hygiene hypothesis attempts to link changes in lifestyle in industrialised countries that have produced a decrease in infectious disease with a concurrent rise in allergic and autoimmune disease (Strachan, 1989; Kelly *et al.*, 2007; López-Serrano *et al.*, 2010). It could therefore be speculated that the same phenomenon may be a contributing factor underlying the high rates of CDI observed in the USA and UK (Lessa *et al.*, 2012, 2015; Gupta *et al.*, 2014). Decreased exposure to environmental microbes through for example, diet and increased hygiene standards, might be expected to lower our exposure to *Bacilli* and may, in part, account for high rates of CDI. As noted others the 'root cause' is likely to be multifactorial (Bloomfield and Riley, 2016) but the

contribution of exposure to environmental microbes and particularly aerobic microbes has, until now, been unnoticed. To lend credence to this hypothesis, efforts were bifurcated into two simultaneous investigations; to discover the role of *Bacillus* within the GI tract in the suppression of *C. difficile* (Chapter 4) and the mechanism underlying the extracellular activity against *C. difficile* (Chapter 5).

Chapter 5 reports on the efforts made to identify the active molecule/s produced by *Bacillus* that inhibited *C. difficile*. For purposes of purification the high activity *B. amlyloliquefaciens* strain SG277 was used. Evidence was provided for a complex of lipopeptides produced by strains of *B. amlyloliquefaciens, B. subtilis* and *B. licheniformis* using RP-HPLC and MALDI-TOF analysis. The principal lipopeptides responsible for this activity were shown to be iturin, fengycin and surfactin and the ensuing complex displayed significant bacteriolytic activity upon *C. difficile*. Surfactin, the predominant constituent, demonstrated the highest activity and was responsible for providing a stable, monodisperse micellar morphology in combination with the other lipopeptides. These individual lipopeptides self-assemble to form mixed micelles thereby magnifying the activity exhibited against *C. difficile*. This combinatorial effect has been noted previously, in the case of surfactin and mycosubtilin (Jauregi *et al.*, 2013) and surfactin and iturin A (Razafindralambo *et al.*, 2002; Thimon *et al.*, 1992). The observed synergy presented by the mixing of micelles is likely due to the complimentary effect different amphipathic compounds have upon each other; physiochemical changes due to interactions between amphiphiles and the subsequent enhancement of interfacial and micellar properties resulting in an increase in antibacterial activity (Sheikh *et al.*, 2013). Physiochemical changes in the micelles can be inferred from the DLS analysis (Figure 5.17), and these changes translated into increased antibacterial activity against *C. difficile* (Figure 5.15), with combined micelles demonstrating higher activity than individual compounds.

In Chapter 4 previous findings were confirmed, in which *C. difficile* spores were shown to germinate in the small intestine regardless of antibiotic administration (Wilson *et al.*, 1985; Koenigsknecht *et al.*, 2015; Winston and Theriot, 2016).

Considering that antibiotics are a prerequisite to the development of CDI, the subsequent step was to then discover the fate of these newly germinated vegetative cells and so *ex vivo* experiment was designed to measure the 'killing' ability of small intestinal extract from either naïve, or clindamycin treated mice. We hypothesised that the increased presence of *Bacillus* in the small intestine of naïve mice would result in a higher quantity of *C. difficile* inhibitory lipopeptides and consequently an *ex vivo* killing effect. This experiment measured over time the effect of the extract upon taurocholate induced germinated CD630 spores and it was observed that only the intestinal samples from naïve mice reduced the count of *C. difficile.* RP-HPLC and MALDI-TOF analysis of the intestinal extract identified iturin, fengycin and surfactin in the naïve mice only, and it was shown that activity against *C. difficile* within the region corresponding to these lipopeptides was comparatively higher than the equivalent region in the small intestinal extract from clindamycin treated mice. Finally, the administration of SG277 was shown to confer full protection against CDI in a mouse and hamster model of disease therefore demonstrating the efficacy of this protective mechanism. The dosing of SG277 supernatant, or SEC purified material also conferred protection (to a lower degree), indicating that an increased concentration of these lipopeptides within the GI tract results in suppression of CDI.

Current literature provides strong evidence for the key role bile salts play as a regulatory mechanism fundamental to the life cycle of *C. difficile*. Current opinion places the site of germination for *C. difficile* spores as within the small intestine, and the site for *C. difficile* inhibition in a healthy host is the caecum/large intestine where anaerobic bacteria enzymatically synthesise inhibitory secondary bile acids to suppress the development of CDI (Theriot *et al.*, 2014; Koenigsknecht *et al.*, 2015; Shen, 2015; Winston and Theriot, 2016). However, *C. scindens*, a bacterium heavily implicated in the possible prevention of CDI via enzymatic synthesis of *C. difficile* inhibitory deoxycholate is unable to confer full protection against CDI (Buffie *et al.*, 2015). This has recently been supported by the observation that *C. scindens* is often present in the GI tract of CDI patients (Amrane *et al.*, 2018). These observations therefore allow room for other factors, in addition to bile salt

regulatory mechanisms, to underly the resistance displayed by healthy individuals against CDI. An intriguing possibility could be based on the ability of bile acids to form mixed micelles with other anionic surfactants (Poša and Ćirin, 2012; Faustino *et al.*, 2014; Jójárt *et al.*, 2014). This may suggest a mechanism by which bacterially produced lipopeptides combine with inhibitory secondary bile acids in mixed micellar complexes within the GI tract of healthy hosts to provide colonisation resistance against CDI via direct antimicrobial action against the vegetative cells. Recent work by Dubois *et al.* (2019) found that sub-lethal concentrations of deoxycholate, perhaps encountered by *C. difficile* following termination of antibiotic treatment induces the formation of biofilms. The authors therefore hypothesised that production of deoxycholate by the intestinal bacterial community induces biofilm formation while suppressing spore and toxin production. This was demonstrated experimentally using *in vitro* biofilm assays, where growing *C. difficile* and *C. scindens* together resulted in increased biofilm formation by the former in response to higher levels of deoxycholate. This perhaps provides an explanation for the partial protection observed against CDI in adoptive transfer experiments involving *C. scindens*; if the deoxycholate levels are insufficient then protection will not be complete due to the formation of biofilms. It follows then, that the presence of additional antibacterial agents, lipopeptides produced by *Bacillus*, able to form mixed micelles with deoxycholate would increase the relative concentration of inhibitors in the GI tract thereby eliminating *C. difficile* outright and increasing resistance to CDI. The capability of deoxycholate to synergise with antimicrobials produced by residents of the GI tract and thereby enhance the activity of these compounds has recently been demonstrated by Kang *et al.* (2019). This hypothetical 'synergy' may hold intriguing implications for both the administration of *Bacillus* to those at risk of CDI and the underlying mechanism by which a healthy microbiota staves off *C. difficile* outgrowth.

In conclusion, this work has expanded on our understanding of *C. difficile* pathogenesis with two important findings. First, that the spore of *C. difficile* is not a silent participant in the infection process but instead plays an active role in infection. Second, that environmental factors play an important role in our

susceptibility to infection. With regard to the role of the spore it is clear that the spore should be considered in treatment strategies, whether this be drug or vaccine and perhaps partly explains why vaccine strategies focused on targeting the toxins have, to date, failed. Regarding the link to the environment we can propose that the rise over the last 30 years of infection rates may be linked to a change in lifestyle. This is important because it suggests that the Western lifestyle may reduce our exposure to environmental factors enabling not only *C. difficile* to emerge but also the possibility that other diseases may, in the future arise. It is interesting that two recent studies also link *C. difficile* infection to lifestyle, the first being a link with a fibre-less diet (Hryckowian *et al.*, 2018) and a second to increased consumption of dietary glucose (Kumar *et al.*, 2019) (Figure 6.1).

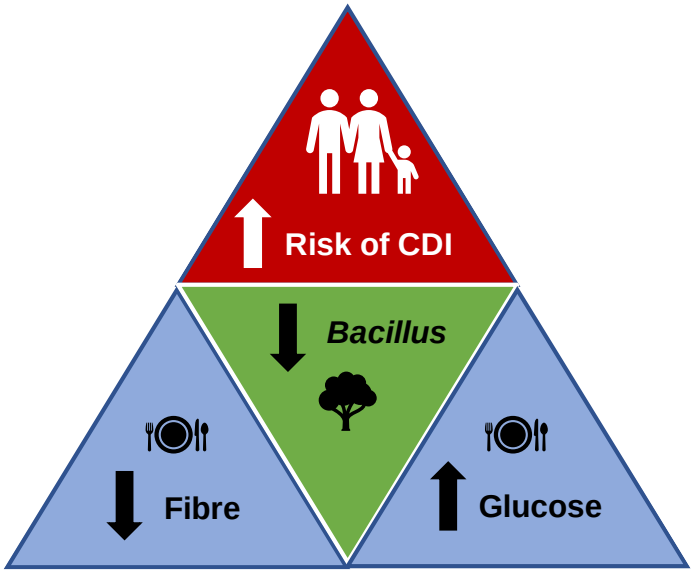


Figure 6.1. Schematic representation of the three risk factors driving CDI.

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Appendices

Appendix A

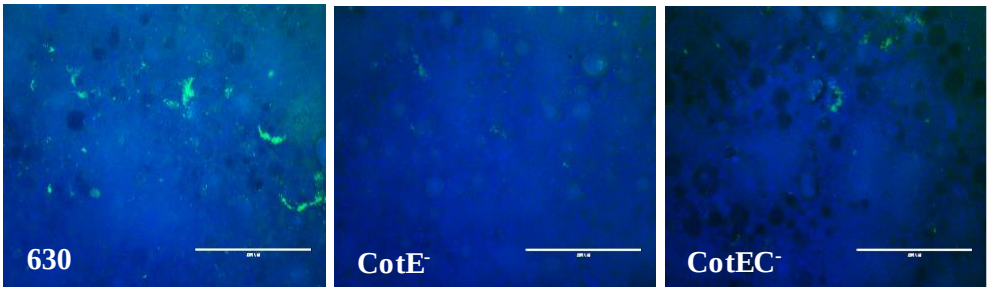

Figure 1. Adhesion of spores to mucus monolayers. Image analysis (20x magnification) of day 14 cell monolayers probed with anti-*C. difficile* spore antibodies (green). Scale bar = 200 mm. Spores of *C. difficile* were added to seeded cells (MOI = 100) and incubated for 2 hours. After washing with DPBS to remove non-adherent bacteria, cells were fixed with 4% paraformaldehyde (Alfa-Aesar) in PBS for 15 min. at RT using 250 µl/well. Cells were then washed twice with DPBS and permeabilised with 0.5% triton-X-100 in PBS for 10 min. at RT, 200 µl/well. After two washes with DPBS they were incubated with 1:100 dilution of mouse antiserum against *C. difficile* spores in 1% (w/v) BSA in PBS for 1 houe at 37ºC, 5% CO_2 using 200 µl/well. Cells were washed 3-times with DPBS and then incubated with a 1:1000 dilution of anti-mouse IgG-FITC antibody (Sigma) in 1% BSA in PBS for 1 hour at 37ºC, 5% CO_2 using 200 µl/well. Cells were washed 3-times with DPBS and incubated with a 1:1000 dilution of DAPI (1 µg/ml) (Sigma-Aldrich, UK) using 200 µl/well for 5 min. in the dark followed by three washes with DPBS. Coverslips were mounted using Vectashield mounting medium (Vectorlabs, UK) and sealed using clear nail varnish. Slides were visualised using an EVOS FL digital microscope (Life technologies). Assay was performed by fellow student, Saba Anwar.

Appendix B

List of publications

Hong, Huynh A*, **Ferreira, W. T***., Hosseini, S., Anwar, S., Hitri, K., Wilkinson, A. J., Vahjen, W., Zentek, J., Soloviev, M. and Cutting, S. M. (2017) The spore coat protein CotE facilitates host colonization by *Clostridium difficile*. *Journal of Infectious Diseases*. 216 (11), 1452–1459.

* These authors contributed equally to this work

Kotowicz, N., Bhardwaj, R.K., **Ferreira, W. T**., Hong, Huynh A., Olender, A., Ramirez, J., Cutting, S. M. (2019) Safety and probiotic evaluation of two *Bacillus* strains producing antioxidant compounds. *Beneficial microbes*. 10 (7), 759-771.

The Journal of Infectious Diseases

MAJOR ARTICLE

The Spore Coat Protein CotE Facilitates Host Colonization by *Clostridium difficile*

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Clostridium difficile infection (CDI) is an important hospital-acquired infection resulting from the germination of spores in the intestine as a consequence of antibiotic-mediated dysbiosis of the gut microbiota. Key to this is CotE, a protein displayed on the spore surface and carrying 2 functional elements, an N-terminal peroxiredoxin and a C-terminal chitinase domain. Using isogenic mutants, we show in vitro and ex vivo that CotE enables binding of spores to mucus by direct interaction with mucin and contributes to its degradation. In animal models of CDI, we show that when CotE is absent, both colonization and virulence were markedly reduced. We demonstrate here that the attachment of spores to the intestine is essential in the development of CDI. Spores are usually regarded as biochemically dormant, but our findings demonstrate that rather than being simply agents of transmission and dissemination, spores directly contribute to the establishment and promotion of disease.

Keywords. *Clostridium difficile*; spores; colonization; chitinase; virulence; mucus.

Clostridium difficile is a Gram-positive, spore-forming human and animal pathogen and one of the leading causes of nosocomial antibiotic-associated diarrhoea in developed countries [1]. The disease results from antibiotic-induced dysbiosis of the gut microflora that allows spores of *C. difficile* to germinate, proliferate, and produce at least 2 inflammatory cytotoxins (toxins A and B), resulting in tissue damage [2, 3]. It has been demonstrated that vegetative cell adherence is important and a number of adhesins have been found, including SlpA [4], flagellin [5], and Cwp84 [6]. However, whether it is necessary for the spore to attach to the intestine prior to germination is unknown.

During the acute stage of infection, there is transient production of enormous numbers of spores (approximately 6 logs higher than the infective dose) in the intestine [7]. However, the number of spores produced can vary significantly for different strains. A number of clues suggest that the spore may contribute to *C. difficile* infection (CDI). First, hamsters challenged with spores of a non-toxigenic strain of *C. difficile* (CD1342) experienced cell damage in the cecum as well as an inflammatory response [8]. Second, Kansau et al [9] found that in a mouse model, the sporulation process of hypervirulent strain R20291 was initiated earlier compared to non-hypervirulent strains. Finally, a *C. difficile spo0A* mutant that cannot produce spores was shown to be unable to persist within, and effectively transmit disease between mice [10].

Other than being an agent of transmission, an intriguing question that has not been addressed is whether the spore per se plays a role in the development of disease. The outermost layer of the spore carries a number of enzymes, but the CotE protein is particularly interesting [11]. It is an 81-kDa bifunctional protein that carries 2 distinct domains, an N-terminal peroxiredoxin domain and a C-terminal chitinase domain [11, 12]. The peroxiredoxin domain resembles a 1-Cys peroxiredoxin, suggesting it may be involved in reducing hydrogen peroxide arising as a by-product of SodA-mediated cross-linking of spore coat proteins [11]. The chitinase domain, belonging to the glycohydrolase family 18, might prima facie play a nutritional role in the turnover of macromolecules such as chitin. Intriguingly, CotE may have a more sophisticated function and contribute directly to pathogenesis.

In this article, we show in vitro that CotE enables spore adhesion to mucus via direct binding to the mucin glycoproteins GlcNAc and GalNAc. Our in vivo data demonstrate that spore attachment is important for developing CDI and that CotE is required for efficient intestinal colonization. Surprisingly, CotE increased the severity of CDI in a hamster model; animals dosed with spores lacking CotE exhibited significantly delayed symptoms of CDI. Taken together we suggest that CotE, and thus the spore, plays an integral role in attachment to the gut mucosa to initiate the infection and the ensuing virulence of CDI, a finding that is both important and hitherto unnoticed.

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[a]H. A. H. and W. T. F. contributed equally to this work.

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METHODS

Strains, Reagents, and General Methods

Two wild-type strains of *C. difficile* were used in this work: 630Δerm and 630. The 630Δerm strain is a spontaneously cured derivative of 630 (*tcdA*$^+$ *tcdB*$^+$) that is erythromycin sensitive [13]; 630 (erythromycin resistant) was isolated from a patient with pseudomembranous colitis during an outbreak of CDI [14]. The 630 strain is used in animal experiments as the 630Δerm strain is sensitive to clindamycin. Two previously described [12] *cotE* ClosTron mutants, isogenic to 630Δerm, were used: ET46 (*cotE::CT220s*) and ET24 (*cotE::CT1203s*). A *sigK* ClosTron mutant JP051 (*sigK::CT266s*) carrying an insertion after codon 87 of the coding open reading frame was constructed in this laboratory and will be described elsewhere. The CotE mutants were complemented with wild-type copies of the respective genes using pRPF185 as described previously [12]. All spores used were purified through a 20%–50% Histodenz gradient. Expression of CotE on the spore surface was verified by Western blotting (Supplementary Figure 1*C*). Antibodies (mouse) recognizing the N-terminus of CotE (M1-K234) have been described previously [12] while antibodies to the C-terminus of CotE were raised in mice by 3 intraperitoneal injections of rCotEC (P381-F712) (Supplementary Figure 1). Methods for growth and sporulation of *C. difficile* and previously described methods are shown in the Supplementary Methods.

Adhesion of Spores to HT29-MTX Cells

The method was performed as described previously [15]. In brief, cells were seeded at 4×10^4 cells/well in 24-well plates for 14 days at 37°C with 7.5% carbon dioxide. On the day of experimentation, cells were washed once with phosphate-buffered saline (PBS). Spores were added to the wells and incubated for 2 hours at 37°C. Non-adherent spores were removed by washing with PBS 5-times and cells lysed with Triton X-100 (0.1% w/v) in PBS for 10 minutes before enumeration of adherent spores by plating on brain heart infusion broth supplemented with yeast extract, cysteine and sodium taurocholate (BHISS) plates. Percentage adhesion was calculated using the formula [% adhesion = (colony-forming units [CFU] count / initial number of spores added) × 100]. Data were presented as the percentage proportion of binding demonstrated by strain 630 spores. For antibody-blocking adhesion experiments; the same procedure as described above was used, but spores were preincubated with anti-CotEC antibody (1/10) for 30 minutes at 37°C before adding to the wells.

Ligand Binding

High binding enzyme-linked immunosorbent assay (ELISA) plates were coated with ligands conjugated to human serum albumin (Dextra, Reading, United Kingdom) at a concentration of 10 μg/mL overnight at 4°C. For mucin, porcine stomach type III (M1778, Sigma), a solution was made fresh in PBS (pH 7.4) and used to coat plates at 1 μg/mL (overnight at 4°C). After blocking the plate with 2% (v/v) bovine serum albumin (BSA) in PBS (1 hour at 30°C), recombinant proteins (rCotEN or rCotEC) were diluted in 1% (v/v) BSA at a concentration of 0.5 μM and incubated for 2.5 hours at 30°C. rCotEN (mouse) and rCotEC (mouse) were used as primary antibodies and anti-mouse immunoglobulin G–horseradish peroxidase (DAKO P0447) was used as the secondary antibody.

Mucin Degradation

Three milliliters of sterile molten 0.5% (w/v) agarose containing 0.5% (w/v) of mucin (porcine type III, Sigma M1778) was overlaid onto sterile microscope slides (76 × 26 mm) rapidly. The sterile slide was placed into a petri dish and the agarose left to solidify, after which 3-mm-diameter holes were punched out of the medium. Ten microliters of spores (1×10^{10}/mL) was added to the wells. The petri dished was closed and sealed with parafilm and incubated for 48 hours at 37°C. Before staining with amido black solution for 30 minutes (Sigma A8181), the agarose was compressed to a thin layer using a 500-g weight (30 minutes with Whatman No. 3 between agarose and weight). The slide was then de-stained several times with 7% (v/v) acetic acid.

Animal Experiments

All animal procedures were performed under the UK Home Office project license PPL 70/8276. For animal experiments, we followed methods from those described previously [15, 16].

Hamsters

Female Golden Syrian Hamsters were 16–18 weeks old (Harlan UK Ltd). For the hamster challenge, animals were dosed by intragastric gavage (i.g.) with clindamycin (clindamycin-2-phosphate, Sigma) 30 mg/kg body weight. After 16 hours, they were dosed i.g. with spores or vegetative cells of wild-type or mutant strains. Animals were monitored for symptoms of disease progression and culled upon reaching the clinical endpoint. The symptoms of CDI were scored as severe/clinical endpoint (wet tail >2 cm, high lethargy), mild (wet tail <2 cm), or healthy. Feces were sampled daily and ceca taken from culled animals.

Mice

The 50% infectious dose (ID_{50}) was determined in mice (6–8 weeks old, female, Harlan UK Ltd) as described previously [17]. Animals were dosed i.g. with clindamycin (30 mg/kg) and challenged with spores (10^1, 10^2, and 10^3 CFU) of strain 630 or *cotE* mutants. Ceca were removed 24 hours post-infection and colonization was defined by the presence of toxins and spore CFU counts 2 logs higher than the initial dose.

Statistical Analysis

Statistical analysis was calculated and significance determined ($P < .05$) using the Welch *t* test for unequal variance. All statistical analyses were performed using GraphPad Prism software.

RESULTS

Generation of CotE Mutants and Their Complements

Two mutants of *cotE* and their complements have been described previously [12]. *cotE::CT220s* carries an insertion (*cotE::CT220s*) at the N-terminus preceding both the peroxiredoxin and chitinase domains, while the insertion in *cotE::CT1203s* is centrally positioned and precedes the C-terminal chitinase domain (Supplementary Figure 1*A*). For simplicity, these mutants will be described here as CotE$^-$ (*cotE::CT220s*) and CotEC$^-$ (*cotE::CT1203s*). To demonstrate the integrity of both mutants, we showed that each carried 2 copies of the *erm* gene, whereas the isogenic 630Δerm parent strain carried 1 copy (Supplementary Figure 1*B*). This confirmed the presence of 1 ClosTron insertion on the chromosome, which we further verified using DNA sequencing. Therefore, the phenotypes we observe for the 2 *cotE* mutants can be directly assigned to the absence of CotE or of course due to a pleiotropic effect (eg, disruption or missassembly of the spore coat resulting from the absence of an intact CotE). Using polyclonal antisera raised in mice to the N-terminal (residues 1–234) and C-terminal (residues 381–712) domains of CotE, we confirmed that neither mutant produced CotE (Supplementary Figure 1*C*). In previous work, we have reported a pRPF185-CotE complementation plasmid that carried full-length *cotE* [12]. The *cotE* mutant cells carrying this plasmid produced spores that expressed CotE (Supplementary Figure 1*D*). Functional complementation of the CotE mutant phenotype was demonstrated in this work and is described in more detail below.

CotE Enables Binding of Spores to Mucus

Adhesion of mutant and wild-type spores to mucus was evaluated using the HT29-MTX human cell line that secretes MUC2 mucins typically found in the small and large intestine and therefore more physiologically relevant for studies of pathogen–mucus interactions [18]. Wild-type 630Δerm spores exhibited higher levels of adhesion (15%–20%) to HT29-MTX cells compared to both *cotE* mutants, which showed significantly ($P < .001$) lower levels of adhesion (Figure 1A). In *cotE* mutant strains carrying the pRPF185-CotE plasmid, levels of adhesion were equivalent to wild type demonstrating functional, in trans, complementation of the CotE phenotype. Image analysis of antibody-labeled spores adhering to HT29-MTX cell lines (day 14) was also used to confirm that wild-type spores were more abundant on mucus-producing HT29-MTX monolayers (Supplementary Figure 2). In a second, ex vivo, approach we measured adhesion of wild-type and mutant spores to ileum and cecum biopsies obtained from a piglet (Supplementary Figure 3). For both *cotE* mutants, spore adhesion to ileum and cecum explants was significantly reduced compared with adhesion of wild-type spores at both incubation times. A further experiment, confirming that *cotE* is involved in binding of spores to mucus, was performed using wild-type spores preincubated with antibodies recognizing the C-terminal domain of *cotE* before being added to HT29-MTX cells. The data showed that adhesion of wild-type spores could be significantly reduced by preadsorption with CotE antibodies (Figure 1B).

CotE Mediates Spore Binding to Mucin and Enhances Colonization

To dissect CotE's affinity for mucus, we used an ELISA-based method to examine binding of recombinant CotE protein fragments to mucin, the main component of mucus, as well as GlcNAc and GalNAc. GlcNAc is the repeating unit in chitin and is also present in mucin glycoproteins [19] and glycosylated proteins present on intestinal epithelial cells [20]. GalNAc is

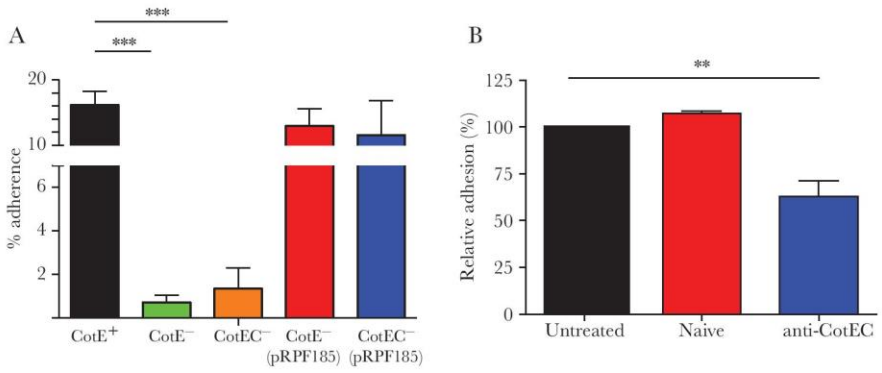

Figure 1. CotE is essential for spore binding to mucus-producing HT29-MTX cells. *A*, Spores of wild-type (630Δerm, *cotE*+), *cotE*–, and *cotEC*– isogenic strains or *cotE* mutants carrying the pRPF185-CotE plasmid were added to mucus-producing HT29-MTX monolayers (multiplicity of infection of 100:1) aged 14 days. Non-adherent spores were removed by washing and the adherent spores were determined by plating on brain heart infusion broth supplemented with yeast extract, cysteine and sodium taurocholate agar. The experiment was independently repeated 3-times. Error bars represent standard deviations. $^{**}P \leq .01$, $^{***}P \leq .001$ by the Welch unequal variance *t* test. *B*, Spores of 630Δerm (CotE+) were preincubated with anti-CotEC antibody or naive serum before adding to HT29-MTX cells. Non-adherent spores were removed by washing, and adherent spores were determined by plating on BHISS agar. Data for untreated spores and spore incubated with naive of anti-CotEC serum are shown. The experiment was independently repeated 3 times. Error bars represent standard deviations.

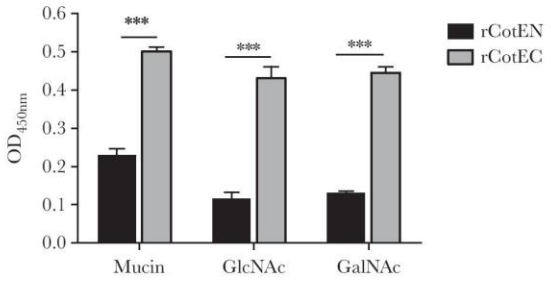

Figure 2. CotE binds to mucin via GlcNAc and GalNAc. The binding of proteins corresponding to the N-terminal (rCotEN) and C-terminal (rCotEC) domains of CotE to mucin or ligands was measured by enzyme-linked immunosorbent assay (ELISA). Mucin or ligands were first coated onto ELISA plates and either rCotEN or rCotEC then applied. Bound protein, after washing, was measured by ELISA. The experiment was independently repeated 3 times. Error bars represent standard deviations. ***$P \leq .001$ by the Welch unequal variance *t* test. Abbreviation: OD_{450}, optical density 450 nm.

the C4 epimer of GlcNAc and is found (together with GlcNAc) in mucin. We observed significantly more binding of rCotEC, comprising the C-terminal chitinase domain, to mucin, GlcNAc, and GalNAc compared to rCotEN that carried the N-terminal peroxiredoxin domain (Figure 2).

In many pathogenic bacteria, binding to mucus and mucin is a mechanism for attachment to the intestinal epithelium and host colonization [19, 21]. We hypothesize that CotE enables *C. difficile* spores to accomplish the same. To address this, we determined the ID_{50} of mutants in a murine model (Table 1) as previously described by Phetcharaburanin et al [17]. In this model, animals do not show symptoms of disease, and colonization is measured by the presence of toxin and CFU (2–3 logs higher CFU than initial dose) in the cecum. Wild-type 630 spores had an ID_{50} of 10^1 whereas values for the *cotE*$^-$ (10^3) and *cotEC*$^-$ ($10^{2.3}$) mutants were 1–2 logs higher, demonstrating that CotE had a role in the ability of spores to infect and colonize the host.

CotE Facilitates Degradation of Mucin

Chitin-binding proteins in bacteria can also function as mucolytic enzymes and therefore we investigated CotE for this trait [22, 23]. Using a modified mucin degradation assay [24], we showed mucin degradation mediated by CotE. After 48 hours of incubation, a zone or halo of mucin degradation was observed in amido black–stained mucin agarose wells containing wild-type spores (Figure 3). By contrast, very limited degradation was apparent in wells containing an identical number of spores for either of the *cotE* mutants. This phenotype, however, was restored in *cotE* mutant spores carrying pRPF185-CotE. Vegetative cells of *C. difficile* have been shown previously to be unable to degrade mucin [25], and we have confirmed this also with our wild-type and mutant strains (data not shown). Degradation of mucin by CotE was confirmed using fluorescently labeled mucin and reverse-phase high-performance liquid chromatography analysis (Supplementary Figure 4).

Table 1. The 50% Infectious Dose of *Clostridium difficile* Strain 630 and Mutant Spores in a Murine Model[a]

Strain	50% Infectious Dose
CotE$^+$	10^1
CotE$^-$	10^3
CotEC$^-$	$10^{2.3}$

[a]Mice are considered positive for *Clostridium difficile* infection when cecum tests positive for toxin and colony-forming units 2 logs higher than initial dose.

CotE Enhances Virulence of CDI in Hamsters

Degradation of mucin by intestinal pathogenic bacteria has been shown to contribute to virulence [19]. Accordingly, we investigated the effect of CotE on virulence using the hamster model of infection [26]. In this model, the colonization of the host can be observed from specific symptoms, or the presence of toxins and/or spore CFU in the cecum or feces. Virulence is reflected by survival rate and/or by the time from colonization to clinical endpoint (severe symptoms). In both mutants, 17% of hamsters were not colonized and survived until the end of the experiment (10 days) whereas with wild type, 100% of hamsters were colonized and eventually succumbed to infection (Figure 4A). While the time from colonization to clinical endpoint for 630 was approximately 6 hours, with CotE$^-$ and CotEC$^-$ the time was 28 and 27 hours, respectively (Figure 4B). Interestingly, among colonized hamsters (5/6), there was 1 hamster from each of the mutant groups which was positive for toxin in feces but developed no symptoms and only reached clinical endpoint after approximately 90 hours (Figure 4B). At 82 hours post-inoculation, 100% of hamsters infected with strain 630 spores had reached clinical endpoint, whereas with *cotE*$^-$ and *cotEC*$^-$ only 34% and 50%, respectively, had become moribund (Figure 5A–C). Once hamsters reached clinical endpoint, ceca were checked to confirm the presence of toxins and *C. difficile* spores at roughly equivalent levels between groups (Supplementary Figure 5). To rule out the possibility that the delay in symptoms and morbidity of hamsters could be due to a difference in toxin production, we checked the kinetics of toxin production both in vitro and in vivo and confirmed that they were of essentially equivalent levels between all 3 strains (Supplementary Figure 6). The germination rate of the mutant and wild-type spores has also been checked and confirmed to be the same (Supplementary Figure 7). The reduced virulence of *cotE* mutant spores observed here in hamsters has been independently repeated (Supplementary Figure 8).

To investigate whether sporulation affects virulence, we used vegetative cells of wild type and a sporulation defective *sigK*$^-$ mutant to infect hamsters following the same dosing regime as that used above with spores. Mutation of *sigK* (encoding a sporulation-specific sigma factor controlling

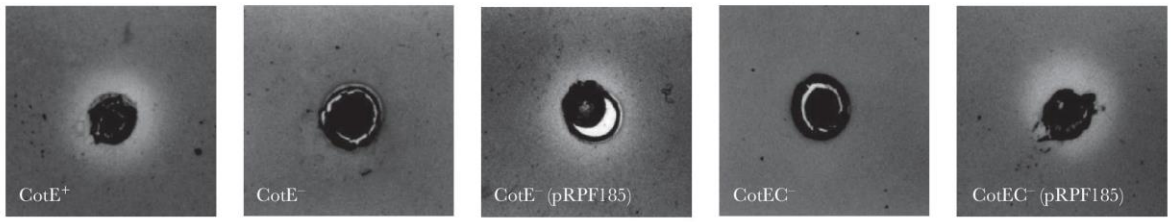

Figure 3. CotE promotes degradation of mucin. Mucin degradation assays for wild-type (630Δerm), *cotE* mutants, and complemented strains (carrying pRPF185-CotE). Spores were applied to wells cut in agarose containing mucin and were incubated aerobically for 48 h at 37°C before staining with amido black.

spore coat biosynthesis [27]) will affect the terminal stages of sporulation-specific gene expression in the mother cell chamber and, unlike *spoOA*, is less likely to exert pleiotropic effects on expression of additional virulence genes. While it took 72 hours for all strain 630–infected hamsters to reach clinical endpoint, 168 hours was required for all *sigK*$^-$ infected animals to succumb (Figure 6). Since hamsters dosed with *sigK*$^-$ cells exhibited delayed mortality, we ruled out the possibility of differential toxin production in vivo by measuring toxin kinetics in mice (Supplementary Figure 9). Note that in cecum samples, a low spore CFU count was found as the *sigK*$^-$ mutant being oligosporogenous is presumably able to produce a very low level (approximately 4 logs less than 630 per gram of feces) of functional spores (Figure 6) [28]. These results suggest that (*i*) spores contribute to the virulence of CDI, and (*ii*) CotE, and potentially additional spore-associated proteins, are involved in colonization.

DISCUSSION

Our study provides important insights into the role of the spore in CDI: first, that the spore is responsible for initial colonization by targeting and binding mucus enabling the establishment of infection and, second, that the spore is an active agent in the acute stage of infection. Although an interaction of the spore with mucus might not be unexpected, we can now attribute this to at least 1 spore-associated protein, CotE, a bifunctional peroxiredoxin/chitinase that is embedded in the outermost layers of the spore. What is surprising is that this spore enzyme is functionally active and able to bind directly to mucin monomers (the glycoproteins GlcNAc and GalNAc) and directly or indirectly facilitate mucin degradation. Some chitinases are thought to degrade mucus [22], and chitin binding proteins are considered colonization factors that have been found in several other important pathogens including *Listeria monocytogenes* [29], *Legionella pneumophila* [30], and *Escherichia coli* [31]. In each case, the proteins are secreted and have a direct role in virulence, colonization, and infection. *Vibrio cholerae* is a well-studied example where 2 proteins, GbpA and ChiA2 (1 of 2 chitinases in *V. cholerae*), act synergistically to bind and degrade both chitin and mucin. GbpA is a chitin-binding protein that is able to recognize GlcNAc present in chitin as well as on the surface of epithelial cells, enabling colonization of host cells by *V. cholerae* [21].

The adhesion of intestinal pathogenic bacteria to the gastrointestinal tract is a crucial aspect of host colonization as it prevents them being mechanically cleared [32]. In the case of *C. difficile*, the spore is the transmissible entity and here we show that for the successful establishment of infection, the spore must also adhere to the host. Interestingly, Theriot et al demonstrated that, within the small intestine, spores are able to germinate

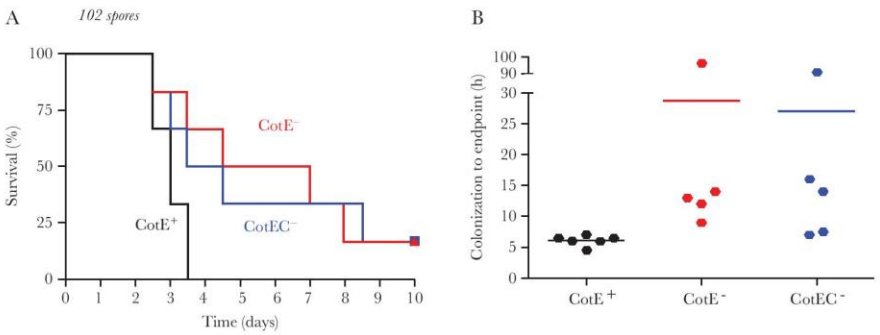

Figure 4. Absence of CotE slows progression of *Clostridium difficile* infection. Groups of hamsters (n = 6) were administered clindamycin and, 16 h later, dosed by intragastric gavage with spores (10^2) of *C. difficile* 630 (wild-type, *cotE+*), *cotE–*, and *cotEC–*. *A*, Kaplan–Meier survival. *B*, Time from initial symptoms (wet tail and/or presence of fecal toxins) to clinical endpoint (virulent phase) are shown. Note that 1 hamster from each mutant group was not colonized and did not show symptoms.

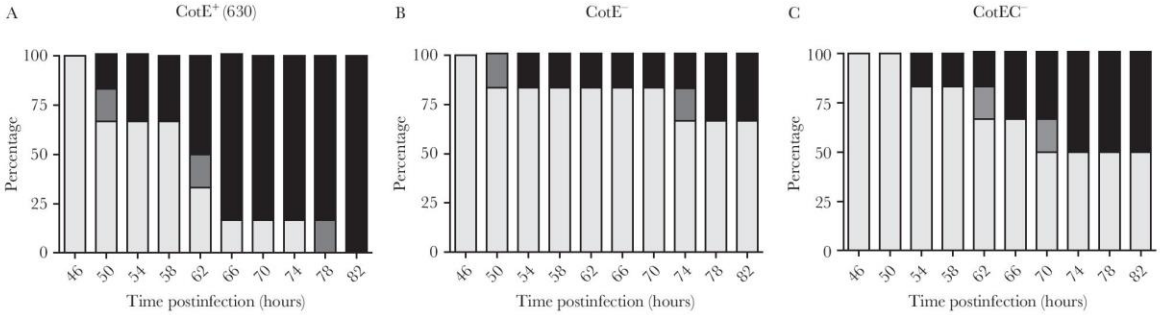

Figure 5. Reduced symptoms with the *cotE* mutants. Symptoms in the hamster study of Figure 4 displayed according to severity. *A*, Animals dosed with wild-type 630 spores. *B*, CotE⁻ spores. *C*, CotEC⁻ spores.

regardless of antibiotic perturbation [33]. It is therefore possible that spores are able to initiate colonization in the small bowel in the absence of microbiota disruption and that an intact microbiota is required to suppress vegetative *C. difficile* outgrowth to prevent clinical disease. Hong et al recently found that mucosal antibodies against the C-terminal of toxin A also recognize the spore and vegetative cells of *C. difficile* and prevents CDI in hamsters by blocking colonization [15]. We demonstrate that the spore binds to the gastrointestinal tract prior to germination to initiate CDI via interactions mediated by CotE. In our study, animals (both mice and hamsters) infected with spores lacking CotE show a marked reduction in colonization. It is important to consider that several adhesins may be responsible for initial spore attachment. In support of this, Phetcharaburanin et al identified hair-like appendages comprised of the BclA1 protein that make "first contact" with the host mucosa [17]. However, this study saw no differences between the severity of CDI caused by mutant BclA1 and wild-type 630 in hamsters.

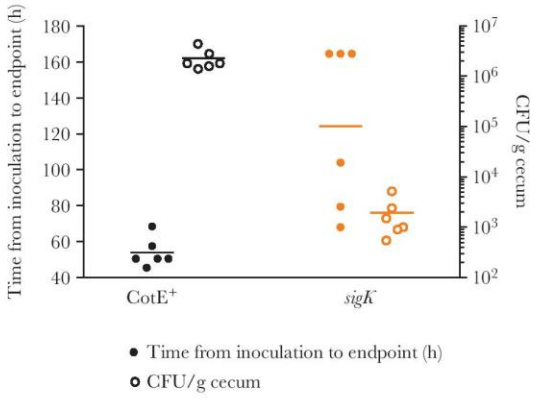

Figure 6. *sigK* mutants display lower virulence in hamsters. Groups of hamsters were administered clindamycin and then, 16 h later, dosed by intragastric gavage with vegetative cells (10^8) of *Clostridium difficile* 630 (wild-type, *cotE+*) or JP051 (*sigK–*). Data represented as time from inoculation of *C. difficile* vegetative cells until time of clinical endpoint combined with heat-resistant spore counts from cecum. Abbreviation: CFU, colony-forming units.

In our study, spores appear able to enhance the severity of disease once initial colonization has been established. This is clear from hamster studies showing that the time required to develop symptoms in colonized animals was markedly delayed in animals dosed with spores devoid of CotE. To explain this, we can envisage several scenarios. One possible explanation could be that by degrading mucus, CotE facilitates transit of toxins to the underlying epithelial cells and so mucin degradation is an integral step in pathogenesis. Another possibility is that the severity of disease is due to the combined action of toxins *and* spores interacting with the large number of macrophages and neutrophils produced during the acute stages of infection leading to the production of inflammatory cytokines [3]. In support of this we have shown, in vitro, that spores are inflammatory and that this response is significantly reduced in the absence of CotE (Supplementary Figure 10). The spore acting as a virulence factor has been shown in *Bacillus anthracis* where a spore surface superoxide dismutase enhances virulence by as much as 40-fold in a mouse model of anthrax [34].

Over the past decade, a multitude of studies have been conducted to understand the severity of epidemic and hypervirulent 027/B1/NAP1 strains, but precise causative mechanisms have yet to be found and links between high sporulation rates and hypervirulence remain inconclusive [35, 36]. A direct role for the spore in acute infection has implications for the concept of hypervirulence, a phenomenon that so far lacks clarity. Most compelling are recent in vivo studies showing (*i*) increased persistence of hypervirulent 027 strains (R20291) in the gastrointestinal tract [17] and (*ii*) increased levels of intestinal sporulation that correlated with increased severity of disease [9]. Using isogenic strains, we show that administration of cells of a *sigK* mutant that exhibits very low levels of spores results in an extended period of infection, post-colonization. One straightforward explanation is that increased spore numbers together with greater intestinal persistence exacerbate symptoms of CDI by CotE-mediated mucin degradation and/or inflammation.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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