CHARACTERISATION OF LIPOPROTEINS OF *CLOSTRIDIUM DIFFICILE* AND THEIR ROLE IN VIRULENCE

Submitted by

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Andrea Kovacs-Simon

“Men love to wonder, and that is the seed of science.”

Ralph Waldo Emerson

Abstract

Antibiotic-associated diarrhoea (AAD) and colitis, with the causative agent being the Gram-positive anaerobe, *Clostridium difficile*, are some of the most important hospital-acquired infections and significant burdens to healthcare services worldwide. Treatment of the infection is often ineffective and currently no vaccine is available against *C. difficile* infection (CDI). Research to identify novel virulence factors potentially leads to the development of new therapeutic and prophylactic drugs. As lipoproteins have been shown to play key roles in the virulence of several pathogens, the aim of this project was to investigate whether lipoproteins are involved in the virulence of *C. difficile*.

Lipoproteins are anchored to the extracellular side of the cytoplasmic membrane in Gram-positive bacteria. Two enzymes are involved in the biosynthesis of lipoproteins: lipoprotein diacylglycerol transferase (Lgt) attaches lipoproteins to the membrane, and lipoprotein signal peptidase (Lsp) cleaves the signal peptide from the amino-terminus of lipoproteins. In order to study lipoprotein processing in *C. difficile*, *lgt* and *lsp* mutants of the *C. difficie* 630Δ*erm* strain were generated using the ClosTron system. Antibody reactivity of 14 *C. difficile* lipoproteins was also investigated. It was shown in this study that *lgt* mutation caused changes in the lipoproteome of *C. difficile*. Therefore, inactivation of the *lgt* gene allowed investigation of the global contribution of lipoproteins to bacterial processes. The physiology and virulence of the *lgt* mutant was studied *in vitro* and *in vivo*. Surprisingly, many of the assayed phenotypes were not significantly affected by disruption of the *lgt* gene. Nevertheless, the ability of the *lgt* mutant to adhere to Caco-2 cells was markedly reduced. In addition, the phenotype of the *lgt* mutant observed in mice suggests that the faecal shedding of *C. difficile* is affected by Lgt inactivation. In further studies, the CD0873 lipoprotein as a potential adhesin of *C. difficile* was identified by *in silico* approach. Contribution of the CD0873 lipoprotein to the adherence of *C. difficle* was investigated by several different assays

and the results strongly suggest that the CD0873 lipoprotein is directly involved in adhesion.

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Declaration

The following co-workers were involved in this project:

All mass spectrometric analyses of the protein samples were undertaken by Hannah Florance at the University of Exeter Mass Spectrometry Facility.

The chemical biology experiments were performed by Thomas Charlton at the Imperial College London.

Protein microarrays for immunogenic studies were carried out at Novartis Vaccines and Diagnostics, Siena, Italy by Manuele Biazzi and myself under the supervision of Cesira Galeotti. Nine recombinant lipoproteins were prepared at the Cloning Unit, Novartis. The recombinant proteins were printed onto microarray slides by Manuele.

The scanning electron microscopy was performed by Peter Splatt at the University of Exeter Bioimaging Facility.

The CDI mouse experiment was carried out by Zoe Seager and myself under the supervision of Neil Fairweather at the Imperial College London.

The confocal immunofluorescence microscopy was undertaken by Magdalena Kasendra and Rosanna Leuzzi at Novartis Vaccines and Diagnostics, Siena, Italy.

The *lspA* and *lspB* mutants of *C. difficile* were generated by Edward Farries under the supervision of Stephen Michell and myself.

The results of all the above mentioned experiments were analysed by myself.

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Abbreviations

AAD	antibiotic-associated diarrhoea
ABC	ATP-binding cassette
ADP	adenosine-diphosphate
AFLP	amplified fragment length olymorphism
ANOVA	analysis of variance
ATP	adenosine-triphosphate
BHI	brain heart infusion
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	bovine serum albumin
CA	cholic acid
CCNA	culture cytotoxicity neutralization assay
CDAD	*C. difficile*-associated diarrhoea
CDI	*C. difficile* infection
CDM	chemically defined medium
CdtLoc	*C. difficile* toxin locus
CFU	colony forming unit
CTP	cytosine-triphosphate
DAPI	6-diamidino-2-phenylindole
DCA	deoxycholic acid
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide-triphosphate
DSF	differential scanning fluorometry
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme immunoassay
EMEM	Eagle`s minimal essential medium
FBS	fetal bovine serum
g	gravity
GDH	glutamate dehydrogenase
GTP	guanidine-triphosphate
h	hour

IEF	isoelectric focusing
Ig	immunoglobulin
IPTG	isopropyl beta-D-thiogalactopyranoside
kDa	kilodalton
LB	Luria-Bertani
LCT	large clostridial toxin
LD	lethal dose
Lgt	lipoprotein diacylglycerol transferase
LIC	ligation-independent cloning
LMW- and HMW-SLP	low- and high-molecular weight surface layer protein
Lnt	lipoprotein N-acyl transferase
Lsp	lipoprotein signal peptidase
MFI	mean fluorescence intensity
MIC	minimum inhibitory concentration
ml	millilitre
MLST	multilocus sequence typing
mM	millimolar
MOI	multiplicity of infection
MRSA	methicillin-resistant *Staphylococcus aureus*
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTZ	metronidazole
NEAA	nonessential amino acids
nm	nanometer
NPV	negative predictive value
OD_{590}	optical density at 590 nm
ORF	open reading frame
Pal	peptidoglycan-associated lipoprotein
PaLoc	pathogenicity locus
PAMP	pathogen-associated molecular pattern
PBP	penicillin-binding-protein
PCR	polimerase chain reaction
PFGE	pulsed field gel electrophoresis
PMC	pseudomembranous colitis
PBS	phosphate-buffered saline

RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcriptase PCR
SBP	solute-binding protein
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	scanning electronmicroscopy
SOE PCR	splicing by overlap extension PCR
TEV	Tobacco Etch Virus
TLR	Toll-like receptor
U	unit
UV	ultra-violet
X-Gal	5-bromo-4 chloro-3-indol-β-D-galactopyranoside
YT	yeast/tryptone
2DGE	two-dimensional gel electrophoresis
µl	microliter
µm	micrometer
µM	micromolar

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Chapter One

Introduction

1.1. The *Clostridium* genus

The *Clostridium* genus belongs to the Firmicutes phylum and is closely related to the *Bacillus* genus (Read *et al.*. 2003, Shimizu *et al.*. 2002). These two genera are thought to represent the first bacterial populations on Earth (Fox *et al.*. 1980). The *Clostridium* genus consists of approximately 100 species of anaerobic Gram-positive bacteria that are able to form endospores (Sauer *et al.*. 1995). The name *Clostridium* is derived from the Greek word *kloster* (κλωστήρ) meaning spindle which refers to the rod shape of the bacteria in this genus. The majority of the genus is non-pathogenic and has importance in both medical and industrial applications. For example, some species have received attention due to their potential for sustainable biofuel production, such as *Clostridium acetobutylicum*, *Clostridium beijerinkii* and *Clostridium saccharolyticum* which are able to produce butanol (Durre 2008), and the ethanol-producing *Clostridium thermocellum*, *Clostridium cellulyticum* and *Clostridium phytofermentans* (Demain *et al.*. 2005). Other species or their products offer novel approaches for treating cancer (*Clostridium sporogenes*, *Clostridium novyii*) (Minton 2003), muscular dysfunction (botulinum toxin of *Clostridium botulinum*) (Arimitsu *et al.*. 2003) and Dupuytren's disease (collagenase from *Clostridium histolyticum*) (Hurst *et al.*. 2009). However, the genus includes certain species that are important animal and human pathogens. *Clostridium perfringens* causes a wide range of gastrointestinal diseases, such as food poisoning, gas gangrene and enterotoxemia, in humans and animals, (Collie *et al.*. 1998). *Clostridium tetani* is the causative agent of tetanus, a disease characterised by painful muscular spasms (Poudel *et al.*. 2009). While *C. botulinum* toxin is widely used in the cosmetic industry and has numerous therapeutic applications, it is one of the most toxic substances known to man; the toxin of *C. botulinum* is designated as a bioterrorism agent and causes botulism, a muscle-paralysing disease (Schantz & Johnson 1992). Finally, diseases associated with

Clostridium difficile infection (CDI) also cause major and increasing health problems, especially in recent years.

1.2. *Clostridium difficile*

C. difficile was first detected in 1935 in the faecal flora of healthy newborns. Initially, it was named *Bacillus difficilis* (Hall 1935) and thought to be nonpathogenic for almost 40 years. Although the severe pseudomembranous colitis (PMC) was first described in 1893, *C. difficile* was not identified as the causative agent of the antibiotic-associated PMC until 1978 (Bartlett 1994, Bartlett *et al.*. 1978).

C. difficile exists in two forms, vegetative cells and spores. Vegetative cells are motile, rod-shape cells and are able to produce toxins, which are primarily responsible for the disease. Capsule and fimbria-like structures are produced by some strains (Borriello *et al.*. 1990). *C. difficile* is widely distributed in the environment (e.g., rivers, soil and hospital) and present in the gastrointestinal tract of animals and humans (al Saif & Brazier 1996).

Several biochemical reactions are carried out by *C. difficile*, such as liquefaction of gelatine, production of H_2S, fermentation of various organic acids and carbohydrates, and conversion of tyrosine to P-cresol (Nakamura *et al.*. 1982, Phillips & Rogers 1981). Cultured *C. difficile* produces a distinctive "horse-dung" odour (phenolic odour due to the production of P-cresol) (Brazier 1998). Selective medium for *C. difficile* was developed by Brazier (Brazier 1993), which contains p-hydroxyphenylacetic acid to enhance the production of P-cresol, egg yolk for differentiation of *C.difficile* from lecithinase-positive Clostridia, cyloserine, cefoxitin, and lysed horse blood for recognition of the yellow-green fluorescence of *C. difficile* colonies under UV-light.

1.2.1. Typing

In order to manage and control the infections and epidemiology associated with *C. difficile*, typing of the *C. difficile* strains was introduced. A range of typing methods are available, which were developed in different countries. Early typing techniques were based on phenotypic analyses, which distinguished between antibiotic resistance, surface components (Heard *et al..* 1986, Wust *et al..* 1982), bacteriophage and bacteriocin patterns (Sell *et al.* 1983), slide agglutination schemes (Delmee *et al.* 1985) and antigenicity of surface proteins of the strains (Kato *et al.* 1993). As technology developed, genotypic methods have emerged and, due to their improved reproducibility, have largely replaced the phenotypic assays (Brazier 2001). The most frequently used methods are PCR ribotyping, pulsed field gel electrophoresis (PFGE), multilocus variable number tandem repeat analysis (MLVA), restriction endonuclease analysis (REA) and toxinotyping. Other, less frequently used techniques are multilocus sequence typing (MLST) and amplified fragment length polymorphism (AFLP) (Rupnik *et al.* 2009). Application of a technique mainly depends on how quickly the result is needed. MLST and MLVA are time consuming and preferred for long term epidemiology studies, while REA and PCR ribotyping allows rapid categorisation of the strains (Kuijper *et al.* 2006b).

1.3. *Clostridium difficile* infection (CDI)

1.3.1. Symptoms

Although, many individuals encounter *C. difficile* during their lifetime, most of the people do not become ill; the bacteria either transiently pass through the gastrointestinal tract or lead to asymptomatic carriage. It is believed that *C. difficle* becomes part of the intestinal gut flora of approximately 60 % of individuals shortly after birth but they do not typically develop symptoms of CDI. *C. difficile* in general clears from the

gastrointestinal tract by the age of 3 years and only 3 to 4 % of the people remain asymptomatic carriers at adult age (Kelly *et al.* 1994, Poutanen & Simor 2004). However, the minority of individuals who come into contact with *C. difficile* manifest symptoms of CDI, which is highly variable depending on the patient and the strain factors. Infection with a toxigenic *C. difficile* causes a spectrum of diseases ranging from mild diarrhoea through more severe illnesses including abdominal pain and fever, to life-threatening pseudomembranous colitis (PMC). PMC is characterized by inflammatory lesions and the formation of pseudomembranes in the colon, and potentially the development of toxic megacolon, peritonitis and perforation of the intestine (Poxton *et al.* 2001). The mortality rate in the case of fulminant CDI is approximately 30%.

Although it rarely occurs, extracolonic manifestations of *C. difficile* infection have been described. These include bacteraemia, osteomyelitis, wound infections, peritonitis and urogenital infections (Jacobs *et al.* 2001).

Recurrent diarrhoea has been reported in 40 % of the patients with CDAD (*C. difficile*-associated diarrhoea), which usually occured a month after the end of the therapy (Poutanen & Simor 2004). 50 % of these cases were due to re-infection, the other 50 % were believed to be caused by the persistence of *C. difficile* spores in the intestine.

C. difficile colonisation is common in all age groups, especially in newborns. A study by Holst *et al.*. (1981) revealed that 64 % of neonates are asymptomatic carriers and in many cases the colonised strains are toxigenic (Holst *et al.* 1981). Neonates are suggested to aquire *C. difficile* after birth in the hospital environment. A few reasons have been proposed for the symptomless colonisation of newborns. It is supposed that the toxin receptors are masked in the neonatal intestine thereby the toxins are not able to attach to the epithelium and cause disease (Bolton *et al.* 1984, Donta & Myers 1982). It is also likely that the toxin receptors are not yet present in the neonatal gut (Cooperstock

et al. 1983). Another hypothesis is that newborns might be protected from CDI by antibodies to *C. difficile* they acquired from the mother during their foetal life (Cartman *et al.* 2010). The carriage rate is lower in healthy young adults, adults and elderly individuals (15.4%, 3 to 4% and 7%, respectively) (Nakamura *et al.* 1981, Poutanen & Simor 2004). However, these proportions are increasing with the time spent in hospital (McFarland *et al.* 1989).

1.3.2. Risk factors

The risk of CDI is associated with several factors. In contrast to the intestinal microflora, *C. difficile* is resistant to a wide range of antibiotics, which enables the bacterium to colonise and cause disease symptoms following exposure to broad spectrum antibiotics, especially clindamycin, cephalosporins, penicillins and fluoroquinolones (Rupnik *et al.* 2009). Other risk factors include gastrointestinal surgeries, treatment with specific drugs (such as H_2-receptor antagonists, e.g., methotrexate, and proton-pump inhibitors that increase germination rate) (Dalton *et al.* 2009, Nachnani *et al.* 2008), and exposure to immunosuppressive agents and chemotherapeutic agents, particularly the ones with antimicrobial properties (Dial *et al.* 2005, Poutanen & Simor 2004). Relapse of the disease is considered to be a risk factor for a subsequent episode. The likelihood of illness increases in the elderly (usually over the age of 65) during hospitalisation because it brings together multiple CDI risk factors (e. g., exposure to antibiotics, spore-contaminated environment and highly susceptible elderly patients) (Barbut *et al.* 1996). In general, *C. difficile* does not cause disease in healthy individuals. However, any illness and medical intervention (both chemical and mechanical) that disturbs the normal gut flora may increase the risk of CDI (Riley 1998).

1.3.3. Epidemiology

The epidemiology of CDI has changed dramatically during recent years; the number of incidences as well as the severity of the disease have increased. *C. difficile* is now a huge burden to healthcare services across the United States, Canada, Europe and some parts of Asia, where several outbreaks have been described in the last decade (Kuijper *et al.* 2007, Kuijper *et al.* 2006a, McDonald *et al.* 2006, Pepin *et al.* 2004). In the UK, the increase in CDAD was striking. A report of the Health Protection Agency showed a massive rise in the rate of CDI, from less than 1,000 in the early 1990s to 55,620 cases in 2006 (http://www.statistics.gov.uk, website for the Office of National Statistics). In 2007, *C. difficile* contributed to five times more deaths (8,324) than methicillin-resistant *Staphylococcus aureus* (MRSA) (1,593) and replaced MRSA as the most common cause of healthcare-associated infections (http://clostridium-difficile.com/cdiff/statistics.htm, website for Hyperdiff). The numbers of deaths caused by *C. difficile* increased until 2007 in England and Wales (from approximately 1,000 deaths in 1999 to almost 9,000 in 2007), then it started to decline. Other countries, for example the Netherlands, Belgium, Germany, France and Spain, have reported similar trends (Cartman *et al.* 2010, Kuijper *et al.* 2007, Suetens 2008). The statistical decrease in incidences in the recent years is thought to be the result of the improved testing, thereby the reporting of less false-positive results. However, there has clearly been a significant rise in CDI in the last two decades. Some reasons are suggested for this increase, such as the emergence of strains with increased antibiotic resistance, the increasing age of the population, thereby increasing in the number of individuals who are at risk, the emergence of hypervirulent strains, overcrowding, and lower standards of hygiene in hospitals (Cartman *et al.* 2010). CDI in populations that are thought to be at lower risk for infection than elderly, such as children and pregnant women, was also emerging. In addition, rise in CDI of individuals who are belived to be barely sensitive

for developing disease, such as youngsters without previous contact with hospital environment and antibiotics, was also reported (Kim *et al.* 2008, Rouphael *et al.* 2008). Furthermore, *C. difficile* is now being identified as an important pathogen in a number of wild and domestic animals (Clooten *et al.* 2008, Keel *et al.* 2007, Songer & Anderson 2006). Interestingly, strain types (e.g., some 078 ribotypes) causing disease in animals were also isolated from humans (Debast *et al.* 2009, Goorhuis *et al.* 2008).

In recent years, outbreaks and severe cases in the United States, Canada and Europe have most frequently been associated with type B1/NAP1/027 strains (Kuijper *et al.* 2006a), which are now considered hypervirulent strains. Depending on the method, the strains belong to the type BI by REA, NAP1 by PFGE or 027 by PCR-ribotyping. Other strains, such as some 001, 053 or 106 ribotype strains, were also emerging in a number of countries (Borgmann *et al.* 2008). Interestingly, symptoms of patients (usually youngsters) infected with ribotype 078 strains are very similar to the ones associated with 027 infections. The pattern of the toxin production may explain the high severity of the disease caused by the 078 and 027 ribotypes. These strains belong to the toxinotype III group and possess genes coding for toxin A, toxin B and the binary toxin (see under section 1.4.3.1). Moreover, a deletion is found in both ribotypes in the *tcdC* gene, which encodes a negative regulator of toxin A and B, which presumably results in increased toxin production. This is confirmed by a study where it was shown that the 027 strains produce much more toxin than the other strains described so far (Vohra & Poxton 2011).

1.3.4. Diagnosis

Suspicion of the infection by *C. difficile* first occurs upon clinical observations. Antibiotic-associated diarrhoea (AAD), colitis and pseudomembranous colitis are caused by *C. difficile* in 20 - 30 %, 50 - 75 % and more than 90 % of the cases,

respectively (Kelly *et al.* 1994). Apart from the clinical symptoms, patient factors are also considered in the diagnosis, including the age, the clinical history (e.g., medication) and the residence (e.g., nursing home) of the patient. Once the patient is considered to be infected with *C. difficile*, various diagnostic tests are available to prove the presence of *C. difficile* and the toxin in stool samples.

C. difficile toxins can be detected by a cell culture cytotoxicity neutralization assay (CCNA), which detects the cytotoxic activity of the toxins on cultured cells. CCNA is performed either directly on the faecal eluate (detects the presence of toxins but not the strain) or on the isolated selective culture (detects the strain that produce toxin) (Rupnik *et al.* 2009). Although, CCNA is sensitive (detects 1 pg of toxin), because it is slow, enzyme immunoassays (EIAs) have nowadays largely replaced this method, as they are rapid and easy to perform. However, sometimes EIAs have low sensitivity (Eastwood *et al.* 2009). Alternative approaches for detecting *C. difficile* toxins are the PCR assays which seem to be the most reliable methods (Tenover *et al.* 2010). All techniques have their advantages and disadvantages. As strains producing only one type of toxin (either toxin A or B) have been reported to be virulent, methods detecting both toxins are preferable. However, toxin testing on its own misses over 40 % of the positive CDI cases. Therefore, a two-step process was introduced in some laboratories to enhance the sensitivity of the testing. In this process, samples are subjected to an initial screening test that have a high negative predictive value (NPV), for example detection of glutamate dehydrogenase (GDH) constitutively produced by *C. difficile*. Samples that are GDH-positive are then tested for *C. difficile* toxins (Ticehurst *et al.* 2006). However, toxin positive patients may be carriers and their symptoms could be unrelated to the presence of the organism. Therefore, for the most accurate diagnosis, determination of the faecal lactoferrin level is a recommended adjunct to the GDH and toxin testing. Lactoferrin is a breakdown product of neutrophils and there is a direct correlation

between faecal lactoferrin levels and intestinal inflammation (Caccaro *et al.* 2010). Other tests, such as analysis of faecal leukocytes, blood tests and endoscopy provide further confirmation of the disease in uncertain situations. Since false-positive and false-negative results have major implications for the patient care (unnecessary treatment, or delay in treatment and cross-infection) and the hospital budget, the use of rapid and accurate laboratory strategies for diagnosing diseased patients is essential.

1.3.5. Treatment and prevention

Diarrhoea resolves without specific antimicrobial therapy in 15 – 23 % of the patients with mild CDAD after discontinuation of the precipitating agent (antibiotics or other drugs) (Olson *et al.* 1994). Therapeutic treatment is primarily used for those with moderate and severe CDAD. In these cases, patients usually start to recover 3 - 4 days after the commencement of the treatment. Administration of the antimicrobial agents should be combined with improved hygiene. Antiperistalics have to be avoided as these agents enhance the effect of the toxins and consequently cause more damage to the gut mucosa. Currently two antibiotics, metronidazole and vancomycin, are available for treating CDAD. Metronidazole used to be the initial drug of choice because of its lower cost. However, recently, there have been some reports of *C. difficile* strains that are less susceptible to metronidazole (Baines *et al.* 2008, Brazier *et al.* 2001, Brazier *et al.* 2008, Pelaez *et al.* 2005) Thus, vancomycin has become the first-line therapy. However, there are concerns over using it. *Enterococcus* species of the gut flora are widely resistant to vancomycin (Hurley & Nguyen 2002) and most of the antibiotic resistance genes are encoded on mobile elements increasing the chance of a potential gene transfer between enterococci and *C. difficile* (Launay *et al.* 2006, Roberts *et al.* 2001, Stinear *et al.* 2001). If the transfer of the vancomycin-resistant genes occured, it would have terrible consequences for the treatment of CDAD.

CDAD can recur in 5 - 30 % of the patients shortly after the antibiotic treatment of the previous episode has been stopped. First recurrence is usually treated with the same agent used for treating the original episode (Olson *et al.* 1994). Effective treatment for multiple relapses is presently under development. Administration of vancomycin in the combination with probiotics decreased relapse rates when *Saccharomyces boulardii* or oligofructose were used as a prebiotics (Surawicz *et al.* 2000, Lewis *et al.* 2005). Tolevamer, a toxin-binding agent, not only appeared to have some beneficial effects on of recurrence, but also reduced the severity of the disease in hamsters (Barker *et al.* 2006). Although administration of antibiotics is the main therapy, several other treatment and prevention approaches are also being investigated, including the use of flora-sparing antibiotics, vaccines, monoclonal antibodies, immunomodifying agents, phage-based approaches, and reconstituation of the protective flora with fecal infusions (Critchley *et al.* 2009, Hedge *et al.* 2008, Rohde *et al.* 2009). In addition, a number of clinical prediction tools have been developed to avoid CDI, and treatment of hospitalized patients is carried out depending on whether the patient is at high risk of primary, recurrent or severe CDI (e.g., careful application of antibiotics and other drugs, considering physical interventions) (Kelly & Kyne 2011). Some of the severely ill patients, for example the ones with toxic megacolon, may require surgery where removal of the colon is the only option for recovering from the disease. However, surgery is performed only in the most severe cases and, regrettably, 30 - 50 % of the surgical interventions become unsuccessful (Morris *et al.* 1990).

Immunotherapy seems to be a promising approach to treat CDAD as higher levels of serum immunoglobulins against the toxins are thought to protect against the disease in patients who are asymptomatically colonised (Kyne *et al.* 2000). Several studies investigating the role of passive immunization have been reported, including Phase I (Babcock *et al.* 2006) and Phase II studies (Leav *et al.* 2010, Lowy *et al.* 2010) in which

antibodies to toxin A and B were tested in hamsters and CDI patients, respectively. Active vaccination studies have so far tested toxin analogues as precipitants of antibody responses against the *C. difficile* toxins and a toxoid vaccine is now under Phase II clinical trials (clinicaltrials.gov NCT00772343 and NCT01230957). Research in recent years has identified non-toxin vaccine candidates, such as Cwp84 (Pechine *et al.* 2005), FliD, FliC and SlpA (Pechine *et al.* 2007).

1.4. Pathogenesis of *C. difficile*

1.4.1. Antibiotics

The more than 500 species in the healthy intestinal microflora provides the most significant defence against *C. difficile* colonisation (Borriello & Barclay 1986). However, when the microbial flora is disrupted, the gut potentially becomes overgrown by *C. difficile*. Exposure to antibiotics is considered to play the major role in the alteration of the gut flora and recognised as the main predisposing factor of CDAD because, in contrast to the intestinal microflora, *C. difficile* is resistant to a wide range of antibiotics (e.g., erythromycin, clindamycin, lincomycin, tetracycline, chloramphenicol). In addition, studies suggest that certain antibiotics, such as fluoroquinolones and clindamycin, induce germination of the *C. difficile* spores, which further contribute to the establishment of the disease (Gor *et al.* 2002, Miyajima *et al.* 2007). Figure 1.1. illustrates the effect of the antibiotic treatment on the normal gut flora and on the possibility of CDI. Clindamycin was the first reported antibiotic that is associated with PMC (Yawata *et al.* 2005). Since its identification, almost all antimicrobials have been described as potent precipitants of CDAD, some of them carrying higher risk for getting CDAD than others. Administration of clindamycin, cephalosporins, penicillins (Poutanen & Simor 2004) and fluoroquinolones (Muto *et al.* 2005) appear to be the most common antibiotics associated with disease.

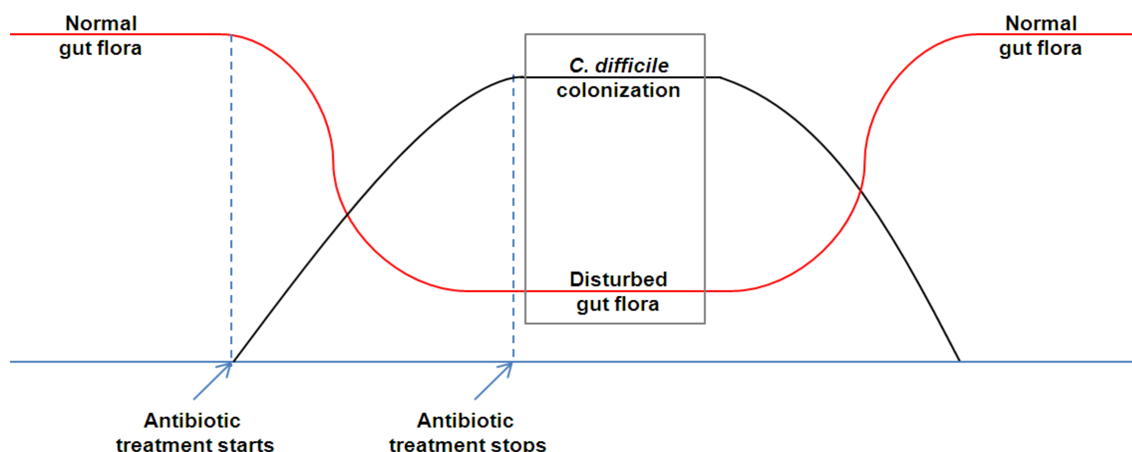


Figure 1.1. Effect of antibiotic treatment on the normal gut flora and on the likelihood of the CDI. Administration of therapeutic agents with antimicrobial properties, particularly antibiotics, disrupts the normal intestinal flora and increases the possibility of CDI. At the same time, in susceptible patients, *C. difficile*, which is resistant to a wide range of antibiotics, starts to overgrow the natural microflora in the colon. When antibiotics are no longer given, colonisation of the gut by *C. difficile* decreases and the gut flora becomes restored after a period of time. Length of the squared period is variable which depends, in part, on the administered antibiotics. Modified from (Rupnik *et al.* 2009).

1.4.2. Life cycle of *C. difficile*

C. difficile spores are formed in order to allow the bacteria to persist outside of the intestinal anaerobic environment and in the acidic environment of the stomach after ingestion. Spores of *C. difficile* are able to remain viable for long periods of time under harsh conditions as they are extremely resistant to desiccation, extreme temperatures, acidic environments and many chemicals (Rupnik *et al.* 2009). Alternation between the vegetative cell and the spore forms plays a critical role in the pathogenesis of CDAD. Spores are responsible for transmission of the bacteria and survival. Once *C. difficile* spores are ingested, they germinate into vegetative cells in the colon upon exposure to bile acids. Germination in some cases is very rapid, 78 % of the spores are able to germinate in one hour in the lower part of the intestinal tract (Miyaji *et al.* 2003). If the normal intestinal flora is altered, vegetative cells multiply, colonise the gut and cause disease in susceptible individuals through the action of the toxins. Figure 1.2

summarizes the three-step process of CDI (Johnson & Gerding 1998): (i) disruption of the normal intestinal microflora is usually the result of antibiotic treatment; (ii) acquisition of a toxigenic *C. difficile*, usually in a hospital environment, from symptomatic and/or asymptomatic infected patients; (iii) symptomatic or asymptomatic colonisation by *C. difficile*, depending on the susceptibility of the host (Kyne *et al.* 2000). Patients who develop disease symptoms do so perhaps no longer than 7 days after acquisition of the strain (Shim *et al.* 1998).

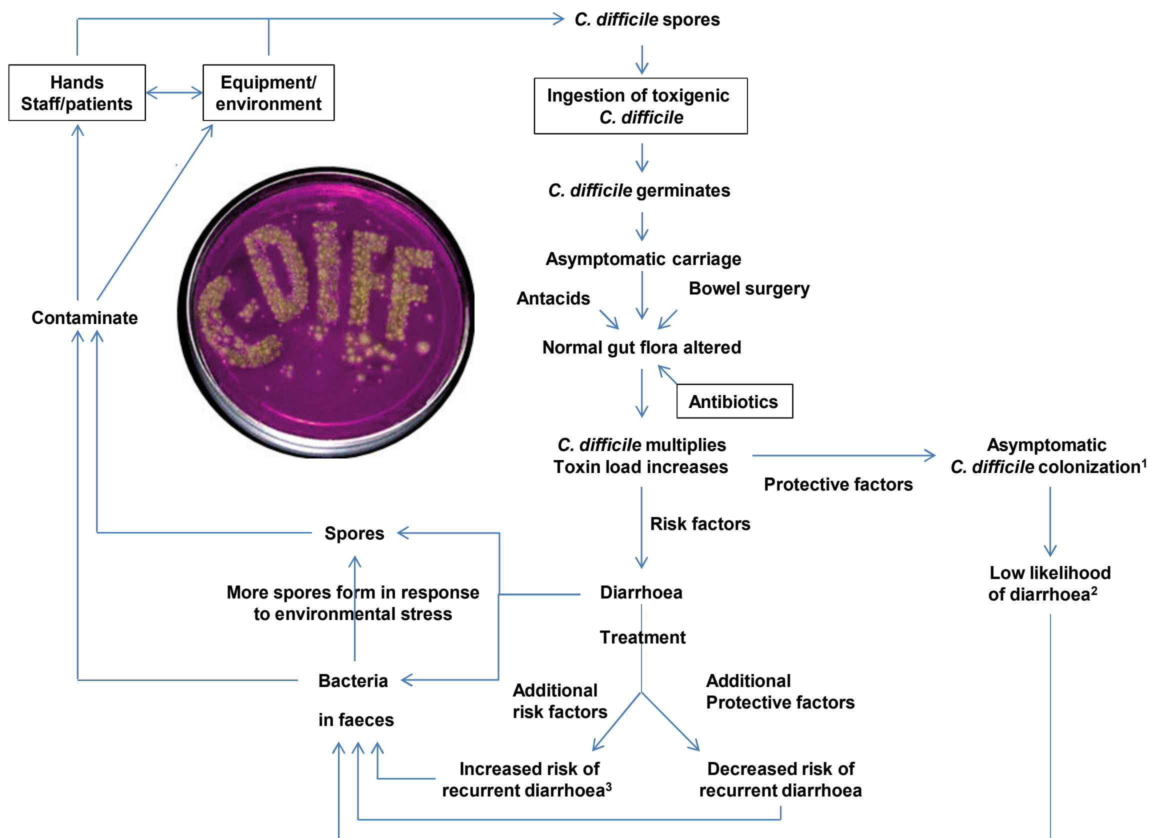


Figure 1.2. Schematic overview of *C. difficile* infection (CDI). Protective factors of CDI: high serum antibody response, mild underlying illness, age < 65 years, no exposure to additional antibiotics; Risk factors of CDI: low serum antibody response, severe underlying illness, age > 65 years, exposure to additional antibiotics. [1] carrier state; [2] may act as reservoir for spread of *C. difficile*; [3] 50% due to reinfection, 50% due to relapse. ▭ = indicates where the cycle can be broken. Modified from Leeds teaching hospital and http://www.cdiff-support.co.uk/about.htm website.

1.4.3. Virulence factors

The pathogenesis of CDAD is a multi-factorial process which invoves sporulation of the vegetative cells, germination of the spores, colonisation of the gut by *C. difficle* and production of the toxins.

1.4.3.1. Toxins

Diarrhoea and, in the worst case, pseudomembranous colitis are caused by the toxins produced by the vegetative cells (Lyerly *et al.* 1988). During infection, *C. difficile* toxins damage the host tissues which cause the symptoms of the disease, and activate signalling cascades of both the innate and acquired immune responses. *C. difficile* produces two toxins, toxin A and toxin B, which are encoded on the pathogenicity locus (PaLoc). This region consists of five genes: *tcdA* and *tcdB* encodes toxin A and B, respectively, *tcdR* encodes a positive, while *tcdC* a negative transcriptional regulator and *tcdE* encodes a protein presumably involved in releasing the toxins from the bacterial cell. In non-toxigenic strains, the PaLoC region is not present in the genome.

Toxin A and B are cytotoxic and belong to the family of large clostridial toxins (LCTs). Both toxins are single-chain proteins and comprise three domains. Domains responsible for binding of the toxins to the receptor and presumably to cell surface carbohydrates are located at the carboxy-terminus of the proteins (Ho *et al.* 2005). The catalytic domain is located at the amino-terminus, and the middle region carries a putative translocation domain (Jank *et al.* 2007). Following receptor binding and endocytosis, the catalytic domain of the toxins are cleaved and translocated from the endosome into the cytoplasm in response to the endosomal acidic environment (Barth *et al.* 2001). In the cytoplasm, toxins glucosylate, thereby inactivate, small GTPases (Rho and Ras). As a consequence, the cell cytoskeleton becomes disorganised which leads to epithelial cell destruction and opening of the tight junctions (von Eichel-Streiber *et al.* 1996). Action of the toxins leads to the release of various proinflammatory cytokines from the

intestinal epithelial cells, probably via activation of MAP kinases. Cytokines then penetrate the mucus layer which was previously altered by the toxins, recruit and activate inflammatory cells, and affect the enteric nerves. These activities lead to fluid secretion (and consequently watery diarrhoea), colitis and the formation of pseudomembranes (Voth & Ballard 2005).

Toxin production by the bacterial cells depends on the strain and is influenced by different environmental signals. In general, expression of the toxins is enhanced under stress *in vitro*. These stress conditions include nutrient-depleted conditions, such as modest depletion of biotin (Yamakawa *et al.* 1996), subinhibitory levels of some antibiotics (e.g., penicillin and vancomycin) (Nusrat *et al.* 2001) and exposure to different temperatures (Rupnik *et al.* 2009).

There is ambiguity over the role of the large toxins in CDI. Initially, toxin A was thought to be primarily responsible for CDAD (Lyerly *et al.* 1985). Contradictory, later findings, such as that significant numbers of clinically relevant strains produce TcdB but not TcdA (Cartman *et al.* 2010), and that TcdB was found to have more damaging effect on the human colonic tissue than TcdA (Riegler *et al.* 1995), suggested that TcdA might have less important role in the disease than previously thought. These results are consistent with more recent findings which demonstrated that $TcdA^{-}$ $TcdB^{+}$ mutants but not $TcdA^{+}$ $TcdB^{-}$ mutants remained virulent in a hamster infection model (Lyras *et al.* 2009). Most recently, results of another research group concluded that both toxin A and toxin B alone are sufficient for the development of the disease (Kuehne *et al.* 2010). The final conclusion of whether the toxin A or the toxin B is the more important factor in CDI is still awaited.

Approximaetly 6 % of the clinically isolated *C. difficile* strains produce a third type of toxin called CDT, which belongs to the group of the clostridial binary toxins and was

discovered in 1988 in the CD196 strain (Geric *et al.* 2006, Popoff *et al.* 1988). CDT is encoded in the binary toxin locus (CdtLoc) comprising three genes: *cdtA*, *cdtB* and *cdtR*. CDT is composed of two proteins, CdtA and CdtB which are encoded by *cdtA* and *cdtB*, respectively. CdtR has regulatory function (Carter *et al.* 2007). CdtA is the enzymatic component with an actin-specific ADP ribosyl transferase activity and causes disorganisation of the cytoskeleton, while CdtB is responsible for binding of the toxin to the host cell and translocation of CdtA into the cytosol (Perelle *et al.* 1997). The role of CDT is not well-known as strains producing CDT but not TcdA or TcdB do not seem to be virulent as investigated in hamsters (Geric *et al.* 2006). It has been proposed that CDT enhances virulence, as it is produced by many emerging strains, such as by the hypervirulent 027 type (Cartman *et al.* 2010), possibly by inducing morphological changes in the host intestinal epithelial cells which facilitates increased adherence of the bacterial cells (Schwan *et al.* 2009).

1.4.3.2. Surface proteins

Whilst toxins are the main virulence factors, some surface proteins also contribute to the virulence of *C. difficile* by being involved in colonisation. To colonise the gut, *C. difficile* first penetrates the mucus layer (possibly with the flagella and extracellular proteases) then adheres to the intestinal epithelial cells (by adhesins). In the laboratory, *C. difficile* is able to attach to different cell lines (e.g., Caco-2, Vero, HeLa and KB cells) and extracellular matrix components (e.g., fibronectin, laminin, collagen, vitronectin and fibrinogen) (Cerquetti *et al.* 2002, Karjalainen *et al.* 1994), and to caecal mucus *in vitro* and *ex vivo* (Eveillard *et al.* 1993, Karjalainen *et al.* 1994). *C. difficile* cells preferably attach to the basolateral surface of Caco-2 cells. The basolateral surface of enterocytes is not accessible in the healthy gut but after disruption of the intestinal epithelium by toxins, bacterial cells are able to reach their preferred site (Cerquetti *et al.*

2002). Colonisation of a pathogen is thought to be multifactorial and some of the factors involved in the colonisation of *C. difficile* have already been identified, for example, Cwp66, SlpA, Cwp84, Fbp68, GroEL, FliC and FliD.

Cwp66

A clue about the existence of Cwp66 was found in 1993 following the discovery of that binding of *C. difficile* to Caco-2 cells and to the mucus-secreting HT29-MTX cells is enhanced when the bacteria is grown in the presence of blood and heat shocked at 60°C (Eveillard *et al.* 1993). A year later, Karjalainen *et al.*. (1994) demonstrated the same effect of heat treatment on the binding of *C. difficile* to Vero, HeLa, and KB cells and that the enchanced bacterial adherence was mediated by proteins which become surface exposed after heat shock (Karjalainen *et al.* 1994). A few years later, it was shown that the adherence of *C. difficile* was increased by various other stresses (Waligora *et al.* 1999). Finally, in 2001, a cell wall protein with a molecular mass of 66 kDa (Cwp66) that mediates adherence of *C. difficile* was identified by immunoscreening of heat treated *C. difficile*. The role of Cwp66 in adherence was confirmed in an adherence inhibition assay in which fragments of the protein and anti-Cwp66 antibodies partially inhibited adherence of *C. difficle* to Vero cells (Waligora *et al.* 2001). Cwp66 was the first adhesin identified in *C. difficile*.

SlpA

The S-layer (surface-layer) consists of low (32 to 38 kDa)- and high (42 to 48 kDa)-molecular weight surface layer proteins (LMW- and HMW-SLP) in *C. difficile* which assemble the H/L protein complexes at the surface of the bacterial cell (Cerquetti *et al.* 2000, Cerquetti *et al.* 1992, de la Riva *et al.* 2011). These components derive from a single protein (SlpA) through post-translational cleavage (Calabi *et al.* 2001). It has

been shown that both the native and the recombinant SLPs can bind to HEp-2 cells and to human and mouse gastrointestinal tissue sections, and that binding of the HMW-SLPs was more effective than that of the LMW-SLPs. Binding of HMW-SLPs to collagen I, thrombospondin and vitronectin has also been described (Calabi *et al.* 2002).

Cwp84

Cwp84 is a cysteine protease and contributes to the colonisation of *C. difficile* by degrading extracellular matrix components. The protein is synthesised inactive and activated by autocatalytic cleavage. Cwp84 has a proteolytic effect on vitronectin, laminin and fibronectin, which can be inhibited by a specific inhibitor (E64) and anti-Cwp84 antibodies (Janoir *et al.* 2007).

Flagella

As in several other bacteria, the flagellum plays an important role in the motility of *C. difficile*. Some *C. difficile* strains possess numerous flagella, while others none or only a few (Tasteyre *et al.* 2000). Flagella also play a role in biofilm formation and colonisation of many species (Yildiz & Visick 2009). A flagellum is composed of a basal body, a hook and a filament made of flagellin proteins, such as FliC (flagellin) and FliD (flagellar cap protein). In *C. difficile*, FliC is posttranslationally modified by a glycosyltransferase enzyme (CD0240) which seems to be important in the function of the flagellum as mutation of the glycosyltransferase gene resulted in a non-motile phenotype (Twine *et al.* 2009). Although, the binding of *C. difficile* to Vero cells was not affected in inhibition assays using anti-FliC sera (Tasteyre *et al.* 2000), recombinant FliC, FliD and crude flagella have been shown to bind to mouse mucus (Tasteyre *et al.* 2001). In addition, flagellated strains colonised more effectively than strains without flagella (Tasteyre *et al.* 2001). Interestingly, inactivation of the flagellar components

(FliC and FliD) increased the *in vitro* adherence of *C. difficile* to intestinal Caco-2 cells. On the other hand, FliC and FliD mutants of *C. difficile* colonised the caecum at the same rate as the wild type in a hamster infection model (Dingle *et al.* 2011). As a conclusion, the role of the flagella in adherence is apparent but more studies are required to determine how colonisation is affected by FliC and FliD.

GroEL

Similarly to the Cwp66 protein, the 60 kDa heat-shock protein, GroEL (Hsp60), was found to be delocalised after heat treatment of *C. difficile*; it becomes membrane associated and is released from the cell. It was also shown that the expression of GroEL is increased after heat shock, acidic shock, iron starvation, high osmolarity, subinhibitory concentrations of ampicillin (Hennequin *et al.* 2001a) and by contact with eukaryotic cells. On the basis of these results in conjunction with the previous finding that adherence of *C. difficile* is enhanced under various stress conditions (Eveillard *et al.* 1993, Karjalainen *et al.* 1994, Waligora *et al.* 1999), it was suggested that GroEL is involved in adhesion. The role of GroEL in adhesion was confirmed in inhibition assays after demonstration of that both the purified protein and anti-GroEL antibodies decreased the adherence of *C. difficile* to Vero cell line (Hennequin *et al.* 2001b).

Fbp68

Based on homology to fibronectin binding proteins of other bacterial species, a putative fibronectin-binding protein, Fbp68, was identified in *C. difficile* and the attachment of Fbp68 to both soluble and immobilised fibronectin was shown in subsequent experiments (Hennequin *et al.* 2003). Interaction of Fbp68 with fibronectin depends on the presence of manganese, which stabilises the protein and it also increases its resistance to proteases. In addition to binding to fibronectin, Fbp68 might have a role in

adherence to Caco-2 cells based on the finding of that adherence of *C. difficile* was partially reduced when Caco-2 cells were preincubated with the C-terminal domain of the Fbp68 protein (Lin *et al.* 2011). In contrast, the *fbp68* mutant of *C. difficile* showed increased adherence to Caco-2 cells and HT29-MTX cells (Barketi-Klai *et al.* 2011). The role of Fbp68 in faecal shedding and colonisation of mouse caecum was also studied and it was shown that the phenotype of the *fbp68* mutant depends on which infection model is used.

Apart from contributing to adherence, surface proteins elicit immune responses and trigger host defence mechanisms in the host during infection. Immunoblots of the *C. difficile* cell wall extracts with patient sera using IgG, IgA and IgM as secondary antibodies showed that surface-layer proteins were immunoreactive indicating that SLPs are targets of the adaptive immune system (Wright *et al.* 2008). Patient antibody responses were also detected to flagellar proteins and to Cwp84 (Pechine *et al.* 2005). SLPs affect the production of preinflammatory mediators (Ausiello *et al.* 2006). However, no difference was found between the levels of the preinflammatory mediators stimulated by epidemic and non-epidemic strains, suggesting that the severity of the disease is not associated with the levels of these preinflammatory molecules (Bianco *et al.* 2011).

1.4.3.3. Other virulence factors

Spores are important for the survival and transmission of *C. difficile*. As CDAD and PMC are caused by the toxins produced by vegetative cells, spores must germinate in the gut following ingestion. Therefore, molecular determinants which are necessary for sporulation and germination are considered to contribute to the virulence of *C. difficile*.

Spo0A is required for the sporulation of vegetative cells (Heap *et al.* 2007), while SleC is essential for the germination of *C. difficile* spores (Burns *et al.* 2010).

CDAD is highly dependent on environmental factors and a numbers of patient factors. In addition, the outcome of the infection is determined by the type of the infecting strain. As with many pathogens, not all the strains are toxigenic and even the toxigenic ones are not equally virulent (Borriello *et al.* 1987). For example, *C. difficile* strains can be grouped by the diversity of the PaLoc and CdtLoc region coding for TcdA, TcdB and CDT, known as toxinotypes (Rupnik *et al.* 2009). Genomes of strains belonging to various toxinotypes encode all, some or none of the toxins and the expressed toxins could have different properties. Variability of the toxins determines, in part, the characteristics of the disease, but other factors also affect the course of the disease. There is variability between the surface proteins of the strains, for example, in the SLPs, which are suggested to determine the ability of the strain to cause disease. This hypothesis is supported by the fact that certain S-layer type strains are more often associated with disease (McCoubrey & Poxton 2001, Poxton *et al.* 2001), which might be related to the differences in their immunomodulatory and/or adherence properties (Cartman *et al.* 2010, Rupnik *et al.* 2009). Furthermore, importance of the spores in bacterial survival and, therefore, indirectly in virulence, is crucial; however, there seem to be no correlation between the sporulation and germination characteristics of the epidemic and non-epidemic strains (Burns *et al.* 2011, Heeg *et al.* 2012).

To date, there have been no studies on *C. difficile* lipoproteins and their roles in virulence.

1.5. Bacterial lipoproteins

Lipid modification of the bacterial proteins facilitates anchorage of the hydrophilic proteins to the hydrophobic cell membranes through hydrophobic interactions between the membrane phospholipids and the acyl chains attached to the N-terminal of the proteins. While anchored to the hydrophobic membrane through the amino terminus, lipoproteins are able to effectively fulfil their functions in the surrounding aqueous environment. Lipidation of a protein at the amino terminus was discovered in 1973 in the outer membrane of *Escherichia coli* and the identified protein was called Braun`s lipoprotein, named after the discoverer (Hantke & Braun 1973).

1.5.1. Biosynthesis

Both in Gram-negative and Gram-positive bacteria, lipoproteins are initially translated as preprolipoproteins (Figure 1.3) with an N-terminal signal peptide of around 20 amino acids possessing the typical characteristic features of the signal peptides of secreted proteins (n-, h- and c-region). The majority of preprolipoproteins are exported unfolded by the Secretory (Sec) pathway (Driessen & Nouwen 2008) and obtain tertiary structure entirely after the Sec pore. However, translocation of lipoproteins by the Tat system, which is responsible for the transport of the folded proteins, has also been described in the high-GC Gram-positive bacteria (*Actinomycetes*) (Widdick *et al.* 2006). However, how the lipoprotein biosynthetic pathway couples to translocation is not known.

The conserved sequence at the c-region of the signal peptides ([LVI][ASTVI][GAS]C), referred to as lipobox, is modified through covalent attachment of a diacylglycerol moiety to the thiol group on the side chain of the indispensable cysteine residue (Babu *et al.* 2006). This modification is catalyzed by the lipoprotein diacylglyceryl transferase (Lgt) enzyme. The resulting prolipoprotein (Figure 1.3) consists of a diacylglycerol moiety linked by a thioester bond to the protein. Lipid modification is suggested to be

initiated at the cytoplasmic side of the membrane (Selvan & Sankaran 2008), but due to the insufficient information about Lgt, this is only speculation. Comparison of the Lgt sequences from phylogenetically distantly related microorganisms (*Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Haemophilus influenzae*) revealed several highly conserved regions that might be important for the function of the enzyme (His-103 and Tyr-235) (Qi *et al.* 1995, Sankaran *et al.* 1997). Lgt was predicted to be an integral membrane protein in the inner membrane of *E. coli* (Daley *et al.* 2005). However, further studies indicated hydrophobic interactions between the membrane and enzyme surface and now it is rather supposed that the enzyme is loosely associated with the cytosolic side of the membrane, and the role of the transmembrane sequences is possibly to provide stronger contact with the membrane (Selvan & Sankaran 2008).

Lipoprotein signal peptidase (Lsp or SPaseII) is responsible for cleaving off the signal peptide from the amino terminus of the prolipoprotein by leaving the conserved cysteine in the lipobox sequence as the new N-terminal residue (Tokunaga *et al.* 1982). It is assumed that this process generally occurs after lipidation. Lsp is a transmembrane enzyme and belongs to the aspartic proteases (Dev & Ray 1984, Munoa *et al.* 1991). Lsp has five conserved sequence regions (Tjalsma *et al.* 1999) and six functionally important residues (Asp-14, Asn-99, Asp-102, Asn-126, Ala-128, and Asp-129) (Dev & Ray 1984). Globomycin, the specific inhibitor of Lsp, is important in studying the activity and function of Lsp enzymes (Dev *et al.* 1985). The mechanism for the cleavage of the signal peptide by Lsp was proposed by Tjalsma *et al.*. (1999) (Tjalsma *et al.* 1999).

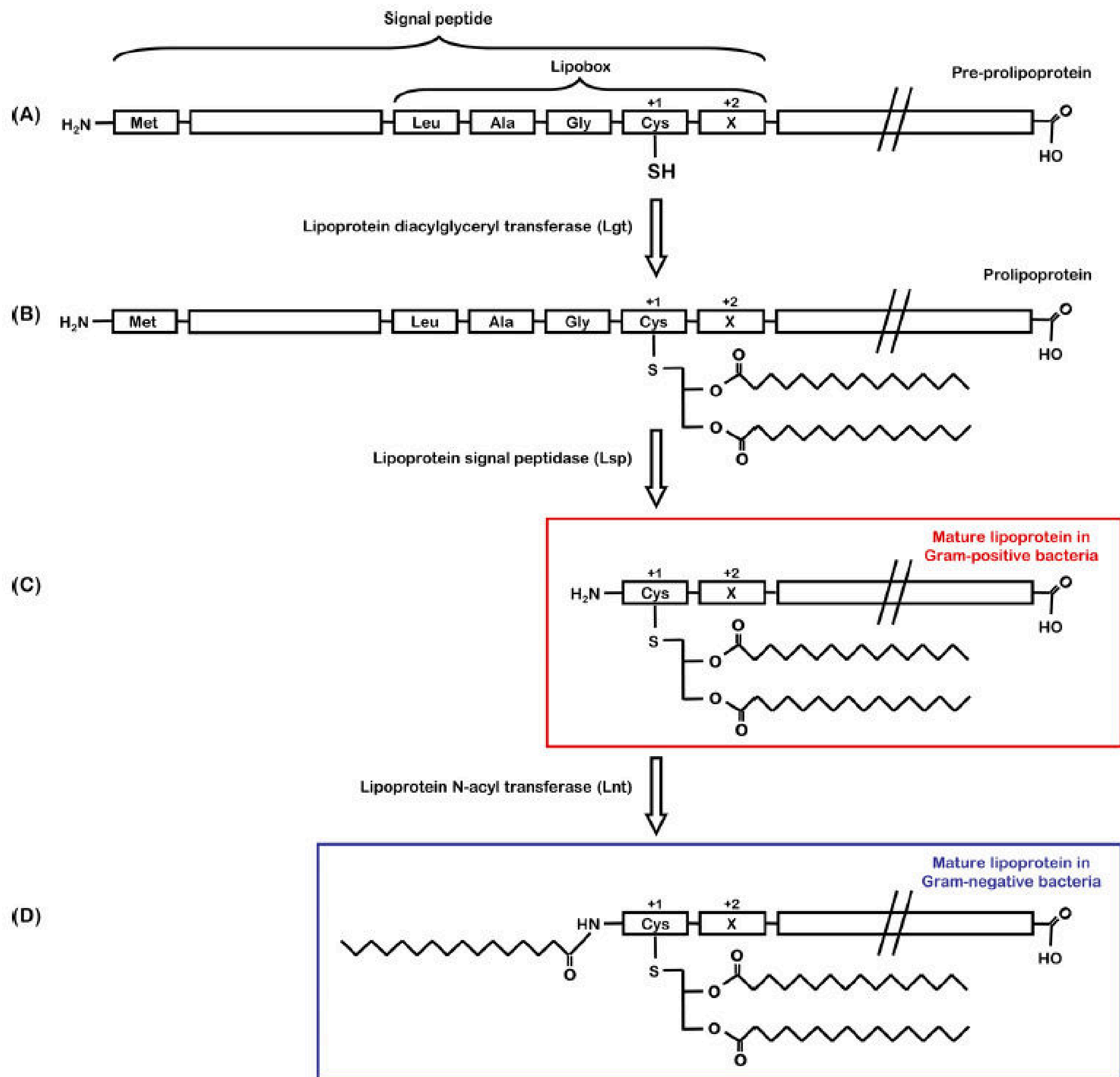


Figure 1.3. Biosynthesis of the bacterial lipoproteins. (A to C) Two-step biosynthetic pathway in Gram-positive bacteria. (A to D) Three-step biosynthetic pathway in Gram-negative bacteria. (A) Preprolipoproteins are the precursors of lipoproteins and possess an N-terminal signal peptide sequence with the characteristic lipobox sequence. (B) During lipoprotein maturation, the thiol group of the invariant cysteine in the lipobox is modified by a diacylglyceryl moiety by lipoprotein diacylglyceryl transferase (Lgt), which serves as a membrane anchor. (C) After lipidation, lipoprotein signal peptidase (Lsp) cleaves the signal peptide, leaving the cysteine as the new amino-terminal residue forming the mature lipoprotein in Gram-positive bacteria. (D) In Gram-negative and some Gram-positive bacteria, the mature lipoprotein has an additional amide-linked fatty acid at the N-terminal cysteine residue attached by lipoprotein N-acyl transferase (Lnt). Amino acid residues at position +2, +3, and +4 have a role in the localisation of Gram-negative bacterial lipoproteins in the membrane (inner or outer membrane) (Terada *et al.* 2001).

In Gram-negative bacteria, lipoproteins undergo an additional modification by the attachment of an amide-linked acyl group to the N-terminal cysteine residue by lipoprotein N-acyl transferase (Lnt) (Gupta *et al.* 1993). Similarly to the S-lipid group, the N-lipid group derives from membrane phospholipids (Hantke & Braun 1973). Indications for N-acylation in low-GC Gram-positive bacteria have also been reported (e.g., in *Bacillus subtilis* and *S. aureus*) (Hutchings *et al.* 2009, Nakayama *et al.* 2012), but there is no evidence for the presence of Lnt in this group, raising the question of which enzyme catalyses N-acylation of the proteins in those species. In contrast, *lnt* homologues have been identified in all classes of high-GC Gram-positive bacteria but Lnt activity could not be demonstrated in those species (Tschumi *et al.* 2009), except in mycobacteria (Vidal-Ingigliardi *et al.* 2007). So the previously held notion that lipoproteins of Gram-positive bacteria are diacylated and those of Gram-negative bacteria are triacylated is not as straightforward as it was thought earlier.

Finally, there is evidence for glycosylation of a number of lipoproteins (Lagoumintzis *et al.* 2003, Michell *et al.* 2003), although the role of glycosylation is not clear. It is suggested that it provides stronger anchorage of the protein to the membrane and protect against proteolytic cleavage of the protein (Herrmann *et al.* 1996).

1.5.2. Localisation

Lipoproteins are localised at various sites of the membrane (Figure 1.4). Gram-positive bacteria possess a thick and compact cell wall adjacent to the cytoplasmic membrane, which is the outermost structure of the cell and mainly composed of the peptidoglycan layer. In contrast, in Gram-negative species, the cell wall is thinner and less compact. The peptidoglycan makes up only 5 – 20 % of the cell wall and is located between the cytoplasmic membrane and an outer membrane. The space between the peptidoglycan and the inner membrane is known as the periplasmic space. The outer membrane is permeable due to the presence of porin channels.

In Gram-negative bacteria, localisation of lipoproteins is dependent on the sorting signals at the amino-terminus of the protein. Lipoproteins are either retained in the inner membrane facing to the periplasmic space or targeted to the outer membrane. Lipoproteins destined to the outer membrane are translocated by the LolCDE complex, an ATP-binding cassette (ABC) transporter, and the LolA periplasmic chaperone (LolB is an outer-membrane receptor) (Tokuda 2009). Certain amino acid sequences determine whether the lipoprotein is released from the inner membrane and transferred to the outer membrane (Terada *et al.* 2001) and aminoacylation of the protein, in general, is the prerequisite of this process (Fukuda *et al.* 2002). However, in an *lnt* mutant of *S. typhimurium*, lipoproteins without an amino-linked acyl chain are localised in the outer membrane, though how this occurs is unknown (Gupta *et al.* 1993). Nevertheless, all lipoproteins of Gram-negative bacteria possess an amino-linked acyl chain irrespective of their inner or outer-membrane localisation (Fukuda *et al.* 2002). In *E. coli,* the majority of lipoproteins are targeted to the periplasmic side of the outer membrane, while others are localised at the periplasmic side of the inner membrane (Narita *et al.* 2004). In some Gram-negative bacteria, such as spirochetes, several lipoproteins are present on the outer leaflet of the outer membrane (Figure 1.4). However, the factor determining whether the lipoprotein is directed to the outer leaflet or retained at the periplasmic side of the outer membrane is currently unknown. However, it is known that some of lipoproteins are transported across the outer membrane by the type II secretion system (T2SS) (Shi *et al.* 2008).

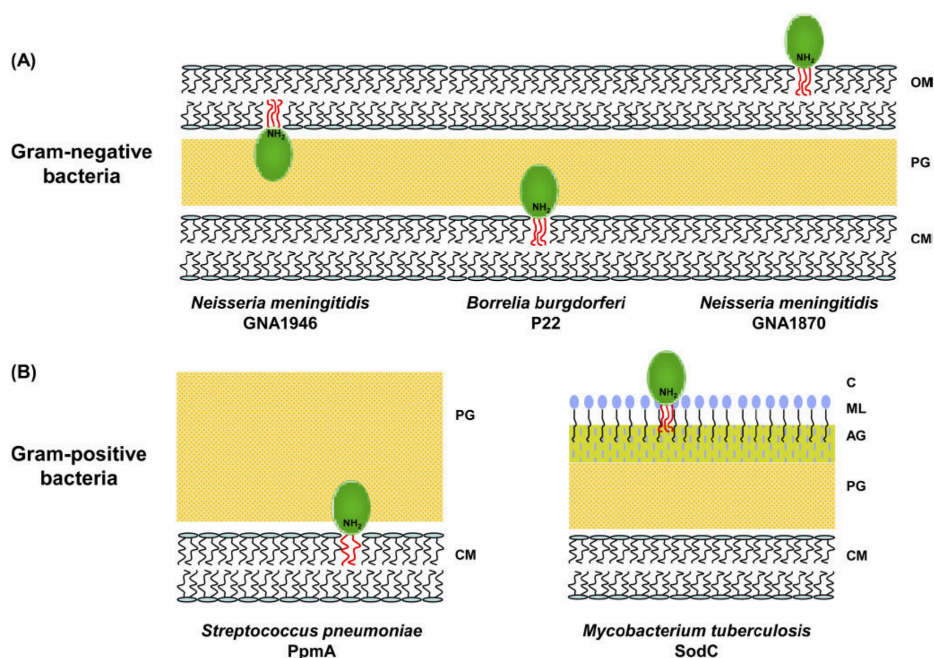


Figure 1.4. Localisation of bacterial lipoproteins. (A) In Gram-negative bacteria, lipoproteins are attached to the cytoplasmic membrane or the extracellular or peripheral side of the outer membrane. (B) In Gram-positive bacteria, lipoproteins are anchored to the extracellular surface of the cytoplasmic membrane and to the mycolic acid layer of the cell wall in mycobacteria. Lipoprotein examples are indicated below the figures. OM, outer membrane; PG, peptidoglycan; CM, cytoplasmic membrane; AG, arabino-galactan; ML, mycolic acid layer; C, capsule-like material; NH_2, N-terminal of the protein.

In Gram-positive bacteria, the issue of localisation is simpler due to the lack of the outer membrane. The preprolipoprotein precursor is translocated across the cytoplasmic membrane, directed by the N-terminal signal peptide sequence, where it is modified by the biosynthetic enzymes discussed above. The resulting lipoproteins are thus anchored to the outer leaflet of the plasma membrane and are not directed to anywhere else, except in mycobacteria. The mycobacterial cell wall contains a lipid layer at the surface of the peptidoglycan, which consists of mostly mycolic acids. Interestingly, lipoproteins have been found to be anchored to this layer in *Mycobacterium tuberculosis* (Wu *et al.* 1998).

Some lipoproteins are released from the surface of bacterial cells. Release of lipoproteins occurs after proteolytic cleavage (only part of the protein is released,

shaving) (Nielsen & Lampen 1982, Nielsen & Lampen 1983, Rothe *et al.* 1989) or the entire protein with the attached acyl chain is released (shedding) (Pearce *et al.* 1994, Perego *et al.* 1991, Sutcliffe *et al.* 1993), which enable the proteins to function away from the cell (e.g., modulate immune responses of the host) (Wiker & Harboe 1992).

1.5.3. Functions

Bacterial lipoproteins are a diverse and functionally important group of proteins (Table 1.1). Lipoproteins play important roles in maintaining essential physiological processes, especially in Gram-negative species, where most of the lipoproteins are involved in cell wall biosynthesis (Malinverni *et al.* 2006, Tanaka *et al.* 2001, Wu *et al.* 2006). Several lipoproteins of Gram-negative bacteria have strong interactions with the peptidoglycan. These lipoproteins perform essential structural functions since they stabilise the interactions between the outer membrane and the peptidoglycan layer. For example, the peptidoglycan-associated lipoprotein (Pal) and the Braun`s lipoprotein are covalently bound to the *m*-Dap residue (*meso*-diaminopimelate) of the peptidoglycan and also interact with each other, and together contribute to the integrity of the cell wall (Dramsi *et al.* 2008, Parsons *et al.* 2006). Recently, two lipoproteins of *E. coli*, LpoA and LpoB, have been described to be involved in peptidoglycan synthesis by forming complexes with penicillin-binding proteins (PBPs), which cross-link the cell wall components (Paradis-Bleau *et al.* 2010). A lipoprotein (Slp) with a putative structural function was discovered in the Gram-positive *B. subtilis* based on the homology of the amino acid sequence of the protein to those of the cell wall stabilising Pal lipoproteins from *E. coli* and *H. influenzae* (Hemila 1991). Furthermore, lipoprotein LpqW is involved in lipoarabinomannan (LAM) biosynthesis in the mycobacterial cell wall (Kovacevic *et al.* 2006).

Several Gram-positive bacterial lipoproteins reflect the functions of the periplasmic proteins of Gram-negative bacteria. The most abundant lipoproteins in Gram-positive

bacteria are the solute-binding lipoproteins of ABC-transport systems, some of which are orthologues of the soluble, periplasmic solute-binding proteins of Gram-negative species (Sutcliffe & Russell 1995). These lipoproteins have direct or indirect roles in almost all physiological processes. For example, MsmE of *Streptococcus mutans* is involved in nutrient uptake and transports all types of sugars structurally related to raffinose (Sutcliffe *et al.* 1993). PsaA of *Streptococcus pneumoniae* is a multifunctional solute-binding lipoprotein. It is recognised by the immune system and involved in the uptake of manganese. Moreover, PsaA is exposed to the cell surface and functions as an adhesin (Marra *et al.* 2002). Other lipoprotein adhesins have been described in the literature, such as SsaB from *Streptococcus sanguis.* SsaB interacts with a salivary receptor and participates in the aggregation of *S. sanguis* with other oral bacteria (e.g., *Actinomyces naeslundii*) (Ganeshkumar *et al.* 1991). Similarly, adherence to the extracellular matrix and invasion of endothelial cells by *Streptococcus agalactiae* involves a laminin-binding lipoprotein, Lmb (Spellerberg *et al.* 1999, Tenenbaum *et al.* 2007).

Bacterial lipoproteins are recognised by Toll-like receptors (TLRs) that play a central role in the innate immune system by sensing pathogen-associated molecular patterns (PAMPs) (Akira *et al.* 2006) and also contribute to the establishment of adaptive immunity (Iwasaki & Medzhitov 2010). TLRs are the major factors in the early detection of the microbes by activating signal cascades leading to the release of proinflammatory cytokines. TLR complexes are formed in the phagosome following degradation of the bacteria (or other pathogens) in the acidic environment. Diacylated lipoproteins produced by Gram-positive bacteria are ligands of the heterodimer TLR2 and TLR6 complexes, while triacylated lipoproteins of Gram-negative bacteria are recognised by the heterodimer of the TLR2 and TLR1 (Takeuchi *et al.* 2001, Takeuchi *et al.* 2002). Acyl chains of lipoproteins shed from the bacterial cells may be recognised

at early stages of infection before the bacteria itself was `processed` by immune cells, which might facilitate rapid activation of the inflammatory mechanisms and eradication of the pathogen.

Lipoproteins are as well targets of the adaptive immune system and elicit protective immune responses in the host. Antibodies produced against lipoproteins of several pathogens have been described in sera of infected patients (Jomaa *et al.* 2005, Palaniappan *et al.* 2005). Intriguingly, lipoproteins have been shown to inhibit antimicrobial responses of the immune systems and make the host unable to eradicate the pathogen during infection. An example of this is the 19-kDa lipoprotein antigen (LpqH) of *M. tuberculosis*, which inhibits gamma interferon (IFN-γ)-dependent responses, such as antigen processing, allowing the pathogen to persist in macrophages and maintain chronic infection (Pai *et al.* 2003).

Lipoproteins also have roles in the extracytoplasmic folding of secreted proteins in some bacteria. For example, the PrsA chaperone of *B. subtilis* is necessary for the secretion and stability of extracellular proteins (Jacobs *et al.* 1993). This process is required for the proteins to be able to fulfil their functions, which is supported by the fact that the *prsA* mutant of *B. subtilis* is not viable (Kontinen & Sarvas 1993, Leskela *et al.* 1999). Lipoproteins also exhibit enzymatic activities, such as hydrolysation of oligosaccharides, glycoproteins and glycolipids, for example by the sialidase lipoprotein (neuraminidase) of *Clostridium sordellii* (Rothe *et al.* 1989), and involved in the biosynthesis of hyaluronate (component of the capsule) in streptococci (hyaluronate synthetase) (Lansing *et al.* 1993). These enzymes have roles in the mobility and protection of these pathogens, respectively. Lipoproteins are involved in electron transfer processes. Several cytochrome c oxidase components (CtaC) and other small cytochromes have been described as lipoproteins in *Bacillus* and *Helicobacter* (Hutchings *et al.* 2009). Lipidation of these proteins is required for the proper

positioning of the amino-terminus of the protein. Lipid anchored penicillinases, which play a role in antibiotic resistance, have also been identified in *Bacillus licheniformis* and *S. aureus*. These enzymes exist in two forms, bound to the membrane or released by proteolytic cleavage (East & Dyke 1989, Neugebauer *et al.* 1981, Nielsen & Lampen 1983). The OppA lipoprotein of *B. subtilis* is suggested to have a role in spore formation by sensing environmental changes. Sporulation is believed to be, in part, triggered by the uptake of small peptides (degradation products derived from the peptidoglycan of dead *B. subtilis* cells) that are transported by the Opp system in *B. subtilis* (Perego *et al.* 1991). Furthermore, putative lipoproteins of *B. subtilis* involved in sporulation and germination pathways have been described. SpoIIIJ is essential in the third stage of the sporulation in *B. subtilis* and possibly necessary for the communication between the prespore and the mother cell (Errington *et al.* 1992). The GerAB and GerAC receptors are presumably temporarily bound to the membrane by their lipid anchors and have a role in sensing germinants (Corfe *et al.* 1994, Zuberi *et al.* 1987). Some lipoproteins are encoded on conjugative plasmids of *Enterococcus faecalis* (TraC and PrgZ) and *S. aureus* (TraH) and serve as receptors for sex pheromones thereby, involved in conjugation (Firth *et al.* 1993, Ruhfel *et al.* 1993, Tanimoto *et al.* 1993). Finally, the cytolethal distending toxin A (CdtA) of *Actinobacillus actinomycetemcomitans* has been identified as a lipoprotein by various assays. When exposed to CdtA, the cell cycle of eukaryotic cells are arrested before mitosis (at the G2/M stage) leading to cell death. Lipidation is necessary for further processing of CdtA by which the toxin is released from the bacterial cell (Ueno *et al.* 2006).

Taken together, lipoproteins are involved in many biological activities which are summarised in Table 1.1. These functions include various processes which are necessary for maintaining cellular processes of the bacteria, such as growth, survival or virulence. However, lipoproteins are considered to have dual functionality in

pathogenesis. They may enhance virulence of the pathogen by contributing to the fitness of the bacteria and be utilized in virulence-related processes such as interactions with or invasion of host cells. On the other hand, lipoproteins are targets for the host immune system and trigger host defence mechanisms by eliciting both innate and adaptive immune responses.

Table 1.1. Representative biological activities elicited by lipoproteins of Gram-positive bacteria.

Role or function	Lipoproteins	Strains	References
Nutrient uptake	PstS-1, FecB	*M. tuberculosis*	(Torres *et al.* 2001, Wagner *et al.* 2005)
	PsaA, Adc	*S. pneumoniae*	(Dintilhac *et al.* 1997)
	MsmE	*S. mutans*	(Sutcliffe *et al.* 1993)
Required for growth	PrsA	*B. subtilis*	(Leskela *et al.* 1999)
	SubI	*M. tuberculosis*	(Sassetti *et al.* 2003)
Conjugation	TraC, PrgZ	*E. faecalis*	(Ruhfel *et al.* 1993, Tanimoto *et al.* 1993)
	TraH	*S. aureus*	(Firth *et al.* 1993)
Sporulation	SpoIIIJ, OppA	*B. subtilis*	(Errington *et al.* 1992, Perego *et al.* 1991)
Germination	GerA, GerB	*B. subtilis*	(Zuberi *et al.* 1987)
Protein folding	PrtM	*L. lactis*	(Haandrikman *et al.* 1991)
	PrsA	*B. subtilis*	(Kontinen & Sarvas 1993)
Colonisation	SsaB	*S. sanguis*	(Ganeshkumar *et al.* 1991)
	PsaA, PpmA, SlrA	*S. pneumoniae*	(Cron *et al.* 2009, Hermans *et al.* 2006, Marra *et al.* 2002)
	Lmb	*S. agalactiae*	(Spellerberg *et al.* 1999)
Invasion	Lmb	*S. agalactiae*	(Tenenbaum *et al.* 2007)
Immunomodulation	LpqH, LprG, PstS-1	*M. tuberculosis*	(Gehring *et al.* 2004, Neufert *et al.* 2001, Sanchez *et al.* 2009, Stewart *et al.* 2005)
	PiaA, PiuA, PsaA, PspA, PspC	*S. pneumoniae*	(Jomaa *et al.* 2005, Palaniappan *et al.* 2005)
Toxin	CdtA	*A. actinomycetemcomitans*	(Ueno *et al.* 2006)
Redox processes	YpmQ, CtaC, cytochrome c_{551}	*B. subtilis*	(Hutchings *et al.* 2009)
	cytochrome c_{554}	*H. gestii*	(Hutchings *et al.* 2009)
Antibiotic resistance	β-lactamases	*S. aureus, B. licheniformis, B. cereus*	(East & Dyke 1989, Neugebauer *et al.* 1981, Nielsen & Lampen 1983)
Structural function	LpqW	*M. tuberculosis*	(Kovacevic *et al.* 2006)
	Slp	*B. subtilis*	(Hemila 1991)

1.5.4. Antimicrobial targets

The importance of lipoproteins in the physiological processes of bacteria was described in the previous section. Furthermore, lipoproteins are involved in the virulence of several bacterial pathogens (see Chapter 5 for more detail). Thereby, enzymes involved in lipoprotein biosynthesis are considered as potential targets for the development of novel antibacterial agents because there are no homologues of those enzymes in eukaryotes. Although, lipidation of some proteins is observed in eukaryotic cells, enzymes mediating these processes are distinct from the enzymes that mediate bacterial lipidation (Farazi *et al.* 2001, Linder & Deschenes 2007). Recently an inhibitor of LolA was described that may lead to a new generation of Gram-negative-specific antimicrobials (Pathania *et al.* 2009).

Not only the biosynthetic enzymes, but also numbers of lipoproteins have been proposed to be targets of countermeasures against bacterial pathogens. The first demonstration of lipopeptides eliciting protective immune responses was reported in 1994 (Nardelli *et al.* 1994). Since then, lipoproteins have been proposed as vaccine candidates from several pathogens including *Neisseria meningitidis* (GNA1870) (Masignani *et al.* 2003), Leptospira spp (LipL21, LipL34, LipL41) (Luo *et al.* 2009), *S. pneumoniae* (PsaA) (Pimenta *et al.* 2006), *M. tuberculosis* (19 kDa and 38 kDa antigen) (Rao *et al.* 2003) and *P. aeruginosa* (OprI) (Weimer *et al.* 2009).

1.6. This study

1.6.1. Objectives

Although *C. difficile* is the major cause of hospital-acquired diarrhoea around the world, the molecular basis of its virulence is poorly understood. It is now evident that the pathogenicity of CDI is mainly mediated by the two toxins (TcdA and TcdB) produced by the vegetative cells, but other factors undoubtedly contribute to the virulence of *C.*

difficile, particularly at the initial colonization process. Lipoproteins have been shown to be necessary for the virulence of many Gram-positive pathogens, and therefore represent a potential group of proteins that might have a role in the pathogenesis of CDI. This study has been carried out to investigate whether lipoproteins play a role in the virulence of *C. difficile*. For this, the erythromycin sensitive derivative of the *C. difficile* 630 strain (*C. difficile* 630Δ*erm*) was chosen as it was suitable for mutagenesis studies. *C. difficile* 630 is a fully virulent, highly transmissible, drug resistant strain and was isolated from a hospital patient with severe pseudomembraneous colitis in Zurich in 1985. Based on the previous knowledge of other pathogens, lipoproteins are considered to be involved in the establishment of several phenotypes of *C. difficile*, some of which were studied in this work.

1.6.2. Thesis overview

Firstly, I investigated lipoprotein processing in *C. difficile* by inactivating enzymes of the lipoprotein biosynthetic pathway (Lgt, LspA, LspB) using the ClosTron mutagenesis system. In the second part, I studied the roles of *C. difficile* lipoproteins in adaptive immunity. The third part analysed the effects of Lgt inactivation on the physiology and virulence of *C. difficile*. Finally, involvement of the CD0873 lipoprotein in the adherence of *C. difficile* was investigated and discussed.

Chapter Two

Materials and Methods

2.1. General materials

Unless otherwise indicated, all chemicals and biochemicals were purchased from Sigma-Aldrich, bacterial growth media were supplied by Sigma-Aldrich or Oxoid, molecular biology enzymes and their buffers were supplied by New England Biolabs or Promega. All solutions were prepared in ultrapure water. All media were sterilised by autoclaving at 121°C for 15 minutes.

C. difficile 630Δ*erm*, *fliC* and *spo0A* mutants of C. *difficile* 630Δ*erm*, *E. coli* CA434 bacterial strains and the pMTL007C-E2, pMTL84151 vectors were obtained from Nigel Minton at the University of Nottingham. *E. coli* C43(DE3) was provided by John Walker from the Medical Research Council, Cambridge. The *tetM* mutant of C. *difficile* 630Δ*erm* was obtained from Zoe Seager and the pRPF144 vector was obtained from Robert Fagan at the Imperial College London. The *C. difficile* clinical isolates were provided by Ray Sheridan from the Royal Devon and Exeter Hospital. The following materials were obtained from the University Exeter: *E. coli* DH5α was available in the BPRG (Bacterial Pathogenesis Research Group) strain collection, pNIC28-Bsa4 vector was a gift from Nic Harmer at the Biocatalysis Centre, and the recombinant ε prototoxin of *C. perfringens* was provided by Monika Bokori-Brown. Caco-2 human colorectal epithelial cell line (ECACC # 86010202) was purchased from the European Collection of Cell Cultures. Bacterial strains were stored in 16 % glycerol at -80°C, Caco-2 cells were stored in 90 % FBS and 10 % DMSO in liquid nitrogen.
Hamster sera were obtained from Gill Douce at the University of Glasgow. Human sera from patients diagnosed with *C. difficile* infection were obtained from Stephen Lewis at the Derriford Hospital, Plymouth. Control human serum samples were provided by David Strain from the Royal Devon & Exeter Hospital.

2.2. Bacterial strains and growth conditions

Unless otherwise stated, *C. difficile* strains were grown at 37°C in an anaerobic workstation (Don Whitley, United Kingdom) in an atmosphere of 10 % CO_2, 10 % H_2 and 80 % N_2 on prereduced BHI (brain heart infusion) agar, Braziers agar containing 4% (v/v) egg yolk, cycloserine/cefoxitin selective supplement (BioConnections, UK) and 2 % (v/v) defibrinated horse blood or SMC (90 g of peptone, 5 g of proteose peptone, 1 g of $(NH_4)_2SO_4$, 1.5 g of Tris in 1 liter) agar, and cultured in BHI broth or chemically defined medium (CDM, Table 2.1) (Karasawa *et al.* 1995, Karlsson *et al.* 1999). *E. coli* strains were routinely grown on LB agar, and cultured in Luria-Bertani (LB) broth or 2x YT medium (16 g of enzymatic digest of casein, 10 g of yeast extract and 5 g of NaCl in 1 liter ultrapure water) at 37℃, with 200 rpm shaking for liquid cultures unless otherwise indicated. CDM was sterilised by membrane filtration (0.2 µm pore size, Nalgene), all other media were sterilised by autoclaving at 121°C for 15 minutes.

E. coli DH5α was used as a recipient for all cloning procedures, *E. coli* C43(DE3) (Miroux & Walker 1996) was used for recombinant protein production and *E. coli* CA434 (HB101 carrying R702) (Purdy *et al.* 2002) was used as donor strain for conjugation of plasmids into *C. difficile*. When appropriate, sterile solutions of antibiotics were added to the medium at the following concentrations: kanamycin, 50µg/ml; tetracycline, 10 µg/ml; chloramphenicol, 12.5 µg/ml (broth) and 25 µg/ml (agar); cycloserine, 250 µg/ml; cefoxitin, 8 µg/ml; thiamphenicol, 15 µg/ml; erythromycin, 5 µg/ml.

Table 2.1. Composition of the chemically defined medium (CDM).

Amino acids	Concentration (mg/l)	Minerals, carbohydrates, vitamins	Concentration (mg/l)
Try (W)	100	**$CoCl_2$ x 6 H_2O**	1
Met (M)	200	**$MnCl_2$ x 4 H_2O**	10
Iso (I)	300	**$MgCl_2$ x 6 H_2O**	20
Pro (P)	600	**$CaCl_2$ x 2 H_2O**	26
Val (V)	300	**$(NH_4)_2SO_4$**	40
Leu (L)	400	**KH_2PO_4**	300
Cys (C)	500	**NaCl**	900
Gly (G)	100	**$NaHCO_3$**	5000
Thr (T)	200	**Na_2HPO_4**	1500
His (H)	100	**$FeSO_4$ x 7 H_2O**	4
Tyr (Y)	100		
Ala (A)	200	**Glucose**	2000
Arg (R)	200		
Asp (D)	200	**D-Biotin**	0.012
Phe (F)	300	**Calcium D-pantothenate**	1
Lys (K)	300	**Pyridoxine**	1
Ser (S)	300		
Glu (E)	900		

Table 2.2. Strains used in this study.

Strains	Description	Reference
C. difficile		
Cdi::WT	*C. difficile* 630Δ*erm* wild-type strain	(Hussain *et al.* 2005)
Cdi::*lgt*	*C. difficile lgt* mutant, group II inserted between 477|478s; Ery^r	this study
Complemented Cdi::*lgt*	Cdi::*lgt* carrying pMTL84151::Cdi-*lgt*; Ery^r, Thi^r	this study
Cdi::*lspA*	*C. difficile lspA* mutant, group II inserted between 321|322s; Ery^r	this study
Cdi::*lspB*	*C. difficile lspB* mutant, group II inserted between 317¦318s; Ery^r	this study
Cdi::CD0873	*C. difficile* CD0873 mutant, group II inserted between 317¦318a; Ery^r	this study
Complemented Cdi::CD0873	Cdi::CD0873 carrying pMTL960::Cdi-0873; Ery^r, Thi^r	this study
Cdi::*tetM*	*C. difficile tetM* mutant, group II inserted between 936|937; Ery^r	ICL
Cdi-fliC-260a	*C. difficile fliC* mutant, group II inserted between 260|261a; Ery^r	(Dingle *et al.* 2011)
spo0A mutant	*C. difficile spo0A* mutant, group II inserted between 178|179a; Ery^r	(Heap *et al.* 2007)
FA07003754,	*C. difficile* clinical isolate	Royal Devon and Exeter Hospital
FA07007522	*C. difficile* clinical isolate	
FA07004080	*C. difficile* clinical isolate	
E. coli		
DH5α	Cloning host	Invitrogen
CA434	conjugation donor; Kan^r, Tet^r	(Purdy *et al.* 2002)
C43(DE3)	expression host	(Miroux & Walker 1996)
C43(DE3)_CD0855	C43(DE3) expressing CD0855	this study
C43(DE3)_CD0873	C43(DE3) expressing CD0873	this study
C43(DE3)_CD1653	C43(DE3) expressing CD1653	this study
C43(DE3)_CD2029	C43(DE3) expressing CD2029	this study
C43(DE3)_CD2672	C43(DE3) expressing CD2672	this study

2.3. Preparation and manipulation of DNA and RNA

2.3.1. Plasmid design

Plasmid maps were designed and sequence data were analysed using the Clone Manager software.

Table 2.3. Plasmids used in this study.

Plasmids	Description	Reference
pMTL007C-E2	Clostridium shuttle vector; Chl^r, Thi^r	(Heap *et al.* 2010)
pMTL007C-E2::Cdi-*lgt*-477|478s	pMTL007C-E2 carrying the re-targeted intron for *lgt* at 477|478s	this study
pMTL007C-E2::Cdi-*lspA*-321|322s	pMTL007C-E2 carrying the re-targeted intron for *lspA* at 321|322s site	this study
pMTL007C-E2::Cdi-*lspB*-317|318s	pMTL007C-E2 carrying the re-targeted intron for *lspB* at 317¦318 site	this study
pMTL007C-E2::Cdi-0873-317|318a	pMTL007C-E2 carrying the re-targeted intron for CD0873 at 317¦318 site	this study
pMTL84151	Clostridium modular vector, Chl^r, Thi^r	(Heap *et al.* 2009)
pMTL84151::Cdi-*lgt*	pMTL84151 carrying *lgt*	this study
pRPF144 (modified pMTL960)	Clostridium modular vector, Chl^r, Thi^r	(Fagan & Fairweather 2011)
pRPF144::Cdi-0873	pRPF144 carrying CD0873	this study
pNIC28-Bsa4	His-tag protein expression vector; Kan^r	(Stols *et al.* 2002)
pNIC-CD0855	pNIC28 carrying CD0855	this study
pNIC-CD0873	pNIC28 carrying CD0873	this study
pNIC-CD1653	pNIC28 carrying CD1653	this study
pNIC-CD2029	pNIC28 carrying CD2029	this study
pNIC-CD2672	pNIC28 carrying CD2672	this study
pGEM®-T Easy	TA cloning vector	Promega

2.3.2. Oligonucleotide design

Oligonucleotides for PCR primers were designed and analysed (hairpin, dimer, stability, melting temperature etc.) using the Clone Manager software. Primers were synthesised by Eurofins MWG Operon, Germany

Table 2.4. List of oligonucleotides used in this study.

Primers	Sequence (5`->3`)
Intron re-targeting	
Cdi-*lgt*-477|478s-EBS2	TGAACGCAAGTTTCTAATTTCGGTTACCTTCCGATAGAGGAAAGTGTCT
Cdi-*lgt*-477|478s-EBSld	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCATCCAACTAACTTACCTTTCTTTGT
Cdi-*lgt*-477|478s-IBS	AAAAAAGCTTATAATTATCCTTAAAGGTCCATCCAGTGCGCCCAGATAGGGTG
Cdi-*lspA*-321|322s-EBS2	TGAACGCAAGTTTCTAATTTCGGTTAATCGTCGATAGAGGAAAGTGTCT
Cdi-*lspA*-321|322s-EBSld	CAGATTGTACAAATGTGGTGATAACAGATAAGTCGGTTTTGTTAACTTACCTTTCTTTGT
Cdi-*lspA*-321|322s-IBS	AAAAAAGCTTATAATTATCCTTACGATTCGGTTTTGTGCGCCCAGATAGGGTG
Cdi-*lspB*-317|318s-EBS2	TGAACGCAAGTTTCTAATTTCGATTGGACTTCGATAGAGGAAAGTGTCT
Cdi-*lspB*-317|318s-EBSld	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCAAGACATTAACTTACCTTTCTTTGT
Cdi-*lspB*-317|318s-IBS	AAAAAAGCTTATAATTATCCTTAAGTCCCCAAGACGTGCGCCCAGATAGGGTG
Cdi-0873-317|318a-EBS2	TGAACGCAAGTTTCTAATTTCGGTTATATTCCGATAGAGGAAAGTGTCT
Cdi-0873-317|318a-EBSld	CAGATTGTACAAATGTGGTGATAACAGATAAGTCAAGTCCTTTAACTTACCTTTCTTTGT
Cdi-0873-317|318a-IBS	AAAAAAGCTTATAATTATCCTTAAATATCAAGTCCGTGCGCCCAGATAGGGTG

Mutant screening	
lgt F	TAATACAAAGAGGTGACGTTATGG
lgt R	ACTGATACATGATGAAATTCCATAG
lspA F	TAGAGGTGCAGCATTTGG
lspA R	CCAGGAGCAGGATGGACAAC
lspB F	AATGCGAGGTCTGGTAGC
lspB R	TGTACCAGCAAGTCCACAAG
CD0873 F	CAGCATTAGATGCCACTAGG
CD0873 R	GCAGCAACAGGGTCACTCAC
*erm*RAM F	ACGCGTTATATTGATAAAAATAATAATAGTGGG
*erm*RAM R	ACGCGTGCGACTCATAGAATTATTTCCTCCCG
intron F	GTCGCGAAGTTCCTATTCTC
Complementation	
*lgt Eco*RI F	CTGGAATTCTAATAAGAATAACTAATTAAG
*lgt Bam*HI R	TCGGATCCCTTAAATTCACTTTTTAAACTA
pMTL84151 R	GCTCTCATGTTATATGCACAACTAC
CD0873 *Sac*I F	GAGCTCTTTTTGAGGGGGATATAAAAATG
CD0873 *Bam*HI R	GGATCCCTATTCTTGTTTAGTCTTTAC
RT-PCR	
maa-RT F	TGTTGTCTGGGAAAGGATAC
maa-RT R	TGGATTTCCAACAGCTACAG
CD0873-RT F	GCTGGTGCTCTTAGTATATC
CD0873-RT R	GATGTAGCCACAGGCATATC
CD0874-RT F	GGAACTTGCCCTTCTATGAC
CD0874-RT R	GGGTCAAGTGCTGCTGTATG
ftsW-RT F	CTGAATTTGATGGAGTAGTG
ftsW-RT R	TACACCTGTAGCTGGCATTG
murF-RT F	AGATAGCAGGGAGGCAAATG
murF-RT R	AGTATCGCTACCCTTCTACC
mraW-RT F	AAAGCCAGATGGCGTGTATG
mraW-RT R	TGCTGTGGACATATACAATC
lgt-RT F	TTACACTGTTTGGCATAGAC
lgt-RT R	GCAATTATGCCACCAATAAC
CD2660-RT F	GCAACCTGATGGAGGAGATG
CD2660-RT R	CATCCCTACAAGTATATCAG

Recombinant protein production	
CD0855-pNIC F	TACTTCCAATCCATGGCTTCTAGTGGAGGAGACAAAGA
CD0855-pNIC R	TATCCACCTTTACTGTCATTCAATTGTCCAGTCAGCTA
CD0873-pNIC F	TACTTCCAATCCATGGCTTCACAAGGAGGAGACTCTGG
CD0873-pNIC R	TATCCACCTTTACTGTCATTCTTGTTTAGTCTTTACAT
CD0999-pNIC F	TACTTCCAATCCATGGCTTCACCAGGAAAAGATAACCC
CD0999-pNIC R	TATCCACCTTTACTGTCATTTCTCAACAAAGTATATTG
CD2029-pNIC F	TACTTCCAATCCATGGCTTCAAGTGCAAAACCAGAAGA
CD2029-pNIC R	TATCCACCTTTACTGTCATTCAAGTGTATTCATAGAGT
CD1653-pNIC F	TACTTCCAATCCATGGCTTCTAAAGCTAAAGATGATAA
CD1653-pNIC R	TATCCACCTTTACTGTCAAAAAGTAGGAATCACAATAC
CD2672-pNIC F	TACTTCCAATCCATGGCTGCTTCCAATGATAAAGACAA
CD2672-pNIC R	TATCCACCTTTACTGTCATTCTACATACAGTTTAGACC
CD1992-pNIC F	TACTTCCAATCCATGGCTTCCCCAAGAGAAAGTGCTAG
CD1992-pNIC R	TATCCACCTTTACTGTCAGTTTAGATGTTTTTTTATTG
CD1546-pNIC F	TACTTCCAATCCATGGCTTTATCTCTTTTAGCAACTAT
CD1546-pNIC R	TATCCACCTTTACTGTCATAAAACATACAATAAAACTG
LIC F	TGTGAGCGGATAACAATTCC
LIC R	AGCAGCCAACTCAGCTTCC

2.3.3. DNA extraction and purification

For cloning purposes, chromosomal DNA of the *C. difficile* 630Δ*erm* was extracted and purified using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer`s recommendations. For PCR screening, *C. difficile* chromosomal DNA was obtained using Chelex 100 (Fenicia *et al.* 2007). Briefly, 1 ml of bacterial culture was centrifuged (10,000g, 10 minutes, 4℃), the pellet was resuspended in 200 µl of 5% Chelex 100 by vortexing and then incubated at 100°C for 10 minutes. Following centrifugation at 10,000*g* for 10 minutes at 4℃, the supernatant containing purified chromosomal DNA was retained and stored at -20°C.

Plasmid DNA of *E. coli* and *C. difficile* was extracted and purified using the QIAprep Spin Miniprep kit (Qiagen) in accordance with the manufacturer`s instructions. For screening of the *E. coli* transformants, colonies were picked, inoculated into 100 µl of nuclease-free water (Ambion), boiled at 100℃ for 10 minutes and analysed.

2.3.4. Quantification of DNA

The concentration and the purity of the DNA samples were determined using a nanodrop ND-1000 spectrophotometer at 260 nm (NanoDrop 1000 Spectrophotometer, Thermo Fisher Scientific Inc.).

2.3.5. Polimerase Chain Reaction (PCR)

DNA amplification was performed using the Qiagen HotStar HiFidelity Polymerase kit for cloning and the Qiagen HotStarTaq DNA Polymerase kit for screening by following the manufacturer`s protocol.

2.3.6. Restriction enzyme digestion of DNA

Plasmid DNA was cut with restriction endonucleases using appropriate enzymes according to the manufacturer`s instructions.

2.3.7. Agarose gelelectrophoresis

PCR products, restriction fragments, plasmid and chromosomal DNA were separated by electrophoresis at 100-120 V for 30 minutes through 0.8 - 1% (w/v) agarose gels in TAE (Tris-acetate and EDTA) buffer containing SYBR® Safe DNA Gel Stain (Life Technologies). Stained DNA was subsequently visualized and analysed using the Bio-Rad Gel Doc imaging system (Bio-Rad Laboratories Ltd.) under UV light.

2.3.8. Gel extraction of DNA

Desired DNA fragments (PCR products, cleaved vectors) were extracted and purified with the QIAquick Gel Extraction kit (Qiagen) according to the manufacturer`s recommendations.

2.3.9. DNA ligation

PCR products were cloned into the pGEM®-T Easy Vector (Promega) following the manufacturer`s protocol. Cleaved DNA fragments (plasmids and inserts) with compatible ends were ligated together using T4 DNA ligase in accordance with the manufacturer`s instructions.

In ligation-independent cloning (LIC), PCR products were treated with T4 DNA polymerase in the presence of dCTP, while treatment of pNIC-28-BsaI previously digested with *BsaI* was carried out in the presence of dGTP (Stols *et al.* 2002). Treated PCR products were mixed with the treated plasmid DNA in a ratio of 3:1, incubated for 10 minutes at room temperature then the ligation reaction was transformed into *E. coli.*

2.3.10. DNA sequencing

DNA nucleotides (plasmids, PCR products) were prepared as required and sequenced by Geneservice Ltd, Oxford or Geneservice Ltd, Cambridge.

2.3.11. Southern blot

DNA probes were labelled using the BrightStar® Psoralen-Biotin Nonisotopic Labeling Kit (Ambion, Texas, USA) according to the manufacturer's instructions. Labelled probes for the DNA size ladder were also prepared. Genomic DNA (3 µg) of each strain and plasmid DNA (50 ng) were digested with *HindIII* overnight at 37°C and separated by electrophoresis at 100 V for 2 hours through thin (~0.5 cm) 0.8 % (w/v) agarose gel. Following separation, gels were photographed under UV light to ensure that complete digestion had occurred. DNA was then transferred onto a positively charged nylon membrane (BrightStar®-Plus Membrane, Ambion, Texas, USA) by the Invitrogen iBlot Dry Blotting System (Invitrogen) with the manufactures' instructions. After disassembling the blotting stack, membranes were denatured in 0.4 M NaOH for 10 minutes on a rotary shaker, air dried and DNA was UV fixed to the membrane at 254nm wavelength treated with 5 mJ/cm^2 for 2 minutes (BLX-254 UV chamber, BDH Laboratories, England). Labelled probes were hybridized to the blots as follows: blots were prehybridized in ULTRAhyb® Hybridization Buffer (Ambion, Texas, USA) for 30 minutes at 42°C prior to overnight hybridisation of denatured probes to DNA blots. Membranes were subsequently washed in Ambion NorthernMax® Low Stringency Wash Buffer then in NorthernMax® High Stringency Wash Buffer (2 x 10 and 2 x 30 minutes, respectively). Hybridized probes were detected as described in the manual of the BrightStar® BioDetect™ Kit (Ambion, Texas, USA) and chemiluminescent signals were visualized using the Bio-Rad ChemiDoc system (Bio-Rad Laboratories Ltd.).

2.3.12. Reverse transcriptase PCR (RT-PCR)

Total RNA was extracted using the FastRNA® Pro Blue Kit (Fisher Scientific Ltd, UK) according to the manufacturer's recommendations. Contaminating DNA was removed by DNase I (Ambion) digestion following the manufacturer`s protocol. For cDNA synthesis, 4 µg of total RNA (in 9 µl) was mixed with 3 µl of random primers (3 µg/µl) (Invitrogen) and 1 µl of a dNTP mixture (10 mM each) (Promega). After primer annealing at 65°C for 5 minutes, a mix of 5x first-strand buffer (4 µl), 0.1 M of DTT (1µl), 40 U of RNase OUT recombinant RNase inhibitor (1 µl) (Invitrogen), and 200 U of Superscript III reverse transcriptase (1 µl) (Invitrogen) was added to the samples according to the manufacturer's recommendations. cDNA synthesis was performed at 50°C for 60 minutes, followed by heat inactivation at 70°C for 15 minutes. RNA was hydrolysed by RNAse H (Invitrogen) and cDNA was precipitated in ethanol overnight at -20°C. cDNA was pelleted by centrifugation, washed with 70% ethanol, air dried for 5 minutes at room temperature, resuspended in nuclease-free water and used for PCR amplification. As a control for DNA contamination, PCRs were performed using total RNA without any reverse transcription reaction.

2.4. Manipulation of *E. coli*

2.4.1. Transformation of *E. coli*

2.4.1.1. Preparation of $CaCl_2$-competent *E. coli* cells

A fresh overnight culture of *E. coli* DH5α, CA434 or C43(DE3) was inoculated in 1/200 into 250 ml LB medium previously warmed to 37°C and supplemented with antibiotics when appropriate (CA434). To provide good aeration, 1 liter conical flasks were used for culturing. Cultures were grown at 37°C with 200 rpm shaking until the optical density at 590 nm (OD_{590}) of 0.6 - 0.7 was achieved indicating the exponential growth phase. Bacterial cultures were poured into 50 ml falcon tubes, immediately placed onto

ice and kept chilled for 30 minutes before centrifugation at 3,000 g for 10 minutes at 4°C. Cells were then resuspended in 30 ml of sterile, ice-cold 0.1 M $CaCl_2$ by gentle pipetting, pooled and incubated on ice for 2.5 hours. Following centrifugation as described above, cell pellets were resuspended in 2.5 ml of sterile, ice-cold 0.1 M $CaCl_2$, 15% glycerol solution and stored at -80°C in 200 µl aliquots.

2.4.1.2. Transformation of $CaCl_2$-competent *E. coli*

200 µl aliquots of $CaCl_2$-competent *E. coli* cells was thawed on ice then plasmid DNA or ligation sample and the appropriate controls of the transformation were added by gentle mixing and incubated on ice for 30 minutes. Following incubation, the sample was placed into a 42°C water bath on a floater for 60 seconds then immediately transferred back to the ice for 10 minutes. 1 ml LB broth was added to the mixture and the entire volume was transferred into a sterile universal tube and incubated at 37°C with 200 rpm shaking for 1 h to allow expression of antibiotic resistance genes. 200 µl and 100 µl of the samples in triplicate were then plated onto LB agar supplemented with the appropriate antibiotics and incubated overnight at 37°C.

2.4.2. Expression of recombinant proteins in *E. coli* C43(DE3)

Expression of the recombinant proteins fused with a histidine tag was performed under the T7 promoter using the pNIC vectors listed in Table 2.2 as follows: fresh overnight cultures of each expression clone grown in 2x YT medium were diluted in fresh 2x YT medium previously warmed to 37°C and supplemented with kanamycin, to maintain the plasmid, to an OD_{590} of 0.1. Cultures were grown at 37°C with 200 rpm shaking until the optical density of 0.5 - 0.6 at 590 nm (OD_{590}) was achieved (~ 2 hours), indicative of the exponential growth phase. Protein expression was induced by adding sterile IPTG to

the bacterial cultures at final concentration of 0.5 mM, then cultures were transferred to 18°C and grown for 20 hours.

2.5. Generation of *C. difficile* mutants

2.5.1. ClosTron system

Target genes of *C. difficile* were inactivated by the ClosTron system as previously described (Heap *et al.* 2010, Heap *et al.* 2007), using the second generation modular plasmid, pMTL007C-E2 (Heap *et al.* 2010) (Figure 2.1). The ClosTron technology incorporates a Retro-transposition-Activated Marker (RAM) based on the *ermB* gene. RAM elements comprise an inactive erythromycin resistant gene (*ermB*) which becomes activated when the group I intron, located upstream of the *ermB* gene, self-splice out and, after insertion of the group II intron into its target site occurred, leads to resistance to erythromycin. The basic principle of the system is to make specific changes to the group II intron in such a way that it inserts into the DNA region of interest. Detailed mechanism of the mutagenesis is the following (Cartman *et al.* 2010): expression from the *thlA* promoter yields an RNA transcript of the group I intron and the *ermB* gene, but the intron I RNA does not splice out as it is in antisense orientation, thus, ErmB protein is not expressed, which results in the lack of erythromycin resistance. Expression from the *fdx* promoter yields an RNA transcript of both the group II and the group I intron which binds to the LtrA protein forming a ribonuclear protein (RNP) complex. In this case, the group I intron is able to splice out, as it is in the correct orientation, which results in expression of the ErmB after the group II intron integrates into the the desired target site within the chromosome. This is directed by the RNP complex. Both strands of the DNA are cut (RNP has DNA endonuclease activity) (Matsuura *et al.* 1997) and the intron RNA is inserted. LtrA reverse transcribes the intron RNA and host repair mechanisms complete the integration process to give double

stranded DNA insertion. Recognition of the target site is the combination of interactions between LtrA and certain nucleotides, and between the target sequence and the modified sequences of the intron RNA (Mohr *et al.* 2000).

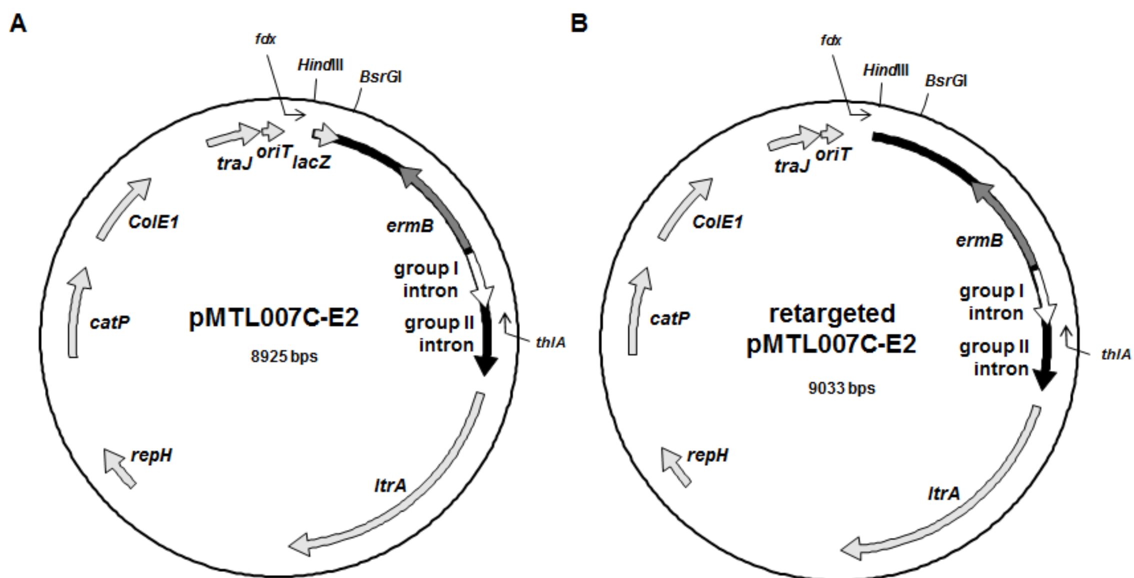


Figure 2.1. Map of the pMTL007C-E2 ClosTron plasmid. A) The modular pMTL007C-E2 plasmid. B) Retargeted pMTL007C-E2 vector. *LacZ* is replaced by the fragment determining the insertion site of the group II intron following restriction endonuclease digestion of both the plasmid and the fragment with *Hind*III and *Bsr*GI. LacZ, β-galactosidase α subunit; LtrA, group II intron-encoded protein; RepH, plasmid replicon protein; CatP, chloramphenicol acetyltransferase (conferring chloramphenicol resistance in *E. coli* and thiamphenicol -methyl-sulfonyl analogue of chloramphenicol- in *C. difficile*); ColE1, Gram-negative replicon; TraJ, conjugal transfer protein. Strong *fdx* and *thlA* promoters drive the expression of the group II intron and the *ermB* gene, respectively.

In this study, target sites within four genes of *C. difficile* 630Δ*erm* (CD2659, CD2597, CD1903, CD0873) were identified and intron re-targeting PCR primers were designed using a computer algorithm (Perutka *et al.* 2004) available at http://www.clostron.com. Targeted fragments were generated by Splicing by Overlap Extension (SOE) PCR using a special template (Sigma-Aldrich TargeTron kit), and three primers designed through the intron re-targeting algorithm (IBS, EBS2, EBS1d) and a universal primer (EBS

Universal). Targeted fragments consisting of the 5' end of the intron and the region of the intron conferring target specificity were introduced into the pMTL007C-E2 vector between the *Bsr*GI and *Hind*III restriction sites, which replaces the *lacZ* fragment. This allows for the re-targeted pMTL007C-E2 to be analysed by blue/white screening on agar supplemented with X-Gal (5-bromo-4 chloro-3-indol-β-D-galactopyranoside). Vector pMTL007C-E2 re-targeted for the *lgt* (CD2659) gene was constructed in-house, *lspA* (CD2597), *lspB* (CD1903) and CD0873 specific vectors were synthesised commercially by DNA2.0, USA.

2.5.2. Conjugation

The retargeted plasmids were first transformed into *E. coli* CA434 and then introduced into *C. difficile* by conjugation as previously described (Purdy *et al.* 2002). Briefly, 1 ml of overnight cultures of the donor strain (*E. coli* CA434 re-targeted pMTL007C-E2) were pelleted by centrifugation (1,500g, 1.5 minutes), washed with phosphate-buffered saline (PBS) and transferred into the anaerobe cabinet. Donor cells were then resuspended in mid-exponential phase culture (200 µl) of the recipient strain (*C. difficile* 630Δ*erm*), the suspension was inoculated onto BHI agar in 20 µl spots and incubated anaerobically at 37°C overnight allowing sufficient time for conjugation to occur. Donor only and recipient only samples were used as negative controls. Following incubation, cells were scraped off from the agar and resuspended in 350 µl of PBS. 100 µl of the neat and 100 µl of 1:10 dilution of the suspension in triplicate was then plated onto BHI agar containing cycloserine (250 µg/ml), cefoxitin (8 µg/ml) and thiamphenicol (15 µg/ml) to counter select against growth of *E. coli* donor strains and to positively select for *C. difficile* transfconjugants. Plates were incubated in the anaerobe cabinet at 37°C until colonies appeared (2 - 3 days) and 12 single colonies were restreaked on the two following days to obtain pure clones.

2.5.3. Isolation of the integrants (mutants)

One full loop from each transconjugant was resuspended in 120 µl PBS then 100 µl of the neat and 100 µl of 1:10 dilution of the suspension was plated onto BHI agar supplemented with erythromycin (5 µg/ml) to select for integration of the intron. Plates were incubated anaerobically at 37°C until colonies appeared (1 - 2 days) and 12 single colonies were restreaked on the two following days to obtain pure clones. Single colonies were inoculated into liquid media and cultures were grown overnight. Genomic DNA was then extracted and used in PCR to amplify the intron-exon junction.

2.6. Protein techniques

2.6.1. Preparation of the cellular and extracellular protein fractions of *C. difficile*

Whole cell lysates of *C. difficile* used for the experiments described in Chapter 3 were prepared by following a protocol described earlier (Molloy 2008). Bacterial cells were harvested by centrifugation at 3,200g for 10 minutes at 4°C, washed once and the pellets were resuspended in PBS. Cell suspension was probe-sonicated on ice in glass bottles, 15 times for 30 seconds with 30 seconds intervals using a soniprep 150 (MSE Ltd) at 6 amplitude microns. Cell debris was removed by centrifugation at 3,200g for 10 minutes at 4°C and the supernatant containing soluble proteins was filtered through a ministart 0.2 µm filter and frozen at -20°C.

Due to its simplicity, whole cell lysates of *C. difficile* used for the experiments described in Chapter 6 were prepared by following a recently described protocol (Fagan & Fairweather 2011). Briefly, bacterial cells were harvested by centrifugation at 5,000g for 15 minutes at 4°C, washed once in PBS and the cell pellets were frozen at -20°C. Cells were then thawed, resuspended to an OD_{590} of 20 in warmed PBS containing 1mg/ml lysosyme and 10 kU/ml DNAse I (Thermo Scientific) and the cell suspension

was incubated at 37°C for 1 hour. Following the incubation, samples were centrifuged at 5,000g for 15 minutes at 4°C to remove cell debris.

Subcellular protein fractions from total cell lysates were extracted using the following protocols:

Membranes of *C. difficile* were isolated by ultracentrifugation (115,000g, 1 hour, 4°C). Crude membrane pellet was then washed once and resuspended in PBS.

Membranes enriched for the integral and the tightly attached membrane proteins were extracted by carbonate extraction. In this method, ice-cold sodium carbonate solution was added to the cell lysate to a final concentration of 100 mM and stirred for 1 hour at 4°C prior to the ultracentrifugation (Molloy 2008). Isolated proteins were washed once and resuspended in PBS.

Hydophilic and hydrophobic protein fractions were obtained by Triton X-114 extraction (Bordier 1981). Triton X-114 was previously purified by precondensation. Briefly, 1 ml of Triton X-114 was dissolved in 100 ml of ice-cold PBS with continuous mixing at 4°C for 1 hour. The solution was then incubated at 30°C overnight until the two phases separated. The upper aqueous phase was removed and replaced with an equal volume of ice-cold PBS. The condensation procedure was repeated two more times. The final detergent phase had a Triton X-114 concentration of about ~ 10 % (w/v). Precondensed Triton X-114 was added to the total cell lysate or to the crude membrane extract to a final concentration of ~2 % (w/v), vortexed vigorously and stirred at 4°C for 1.5 hour to obtain the extract in a single phase. Insoluble matter was removed by centrifugation at 16,000g at 4°C for 15 minutes then the suspension was layered over an equal volume of warm sucrose cushion (0.06% Triton X-l 14 in PBS containing 6% (w/v) sucrose) and placed into a 37°C water bath for 10 minutes to separate the phases. The solution was centrifuged at 500g for 3 minutes at room temperature. The upper aqueous-rich layer

was carefully separated from the lower detergent-rich layer by pipetting. Both phases were washed as follows: 5x volume of PBS was added to the detergent-rich phase, while precondensed Triton X-114 to a final concentration of 1 % (w/v) was added to the aqueous-rich phase. Both solutions were mixed for 10 minutes at 4°C followed by 10 minutes of incubation at 37°C and centrifugation at 500g for 3 minutes at room temperature. The detergent and aqueous phases were retained. 9 x volume of ice-cold acetone was added to the samples to precipitate the extracted proteins and incubated at -20°C overnight. Precipitated proteins were pelleted at 16,000g for 15 minutes at 4°C, washed once with 80 % (v/v) acetone containing 10 mM DTT, air dried and resuspended in PBS.

Extracellular proteins were extracted from the culture supernatant. Bacterial cultures were centrifuged as described above and the supernatant containing the extracellular proteins was retained. Proteins were precipitated with 2x volume of 10 % (wt/vol) trichloroacetic acid (TCA) in acetone overnight at -20°C. Precipitated proteins were pelleted at 16,000g for 15 minutes at 4°C, washed once with 80 % acetone containing 10 mM DTT, air dried and resuspended in PBS.

2.6.2. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Extracted proteins were separated by SDS-PAGE with 4-12% Bis-Tris gel in an XCell SureLock Mini-Cell System (Invitrogen) at 160 V for 60 minutes according to the manufacturer’s recommendations. Separated proteins were subsequently stained with SimplyBlue™ SafeStain (Invitrogen) following the fast microwave protocol and scanned using the Bio-Rad Gel Doc system (Bio-Rad Laboratories Ltd.).

2.6.3. Western blotting

Proteins separated by SDS-PAGE were transferred onto nitrocellulose membranes (iBlot Dry Blotting System, Invitrogen) and stained with Ponceau dye to ensure that proteins were correctly transferred; this was followed by blocking the membranes overnight in TBS-T (0.1 % (v/v) TWEEN-20 in TBS) supplemented with 5 or 7 % (wt/vol) skimmed milk powder. Membranes were then washed 3 x 15 minutes in TBS-T prior to incubation with primary antibodies. After three washing steps, membranes were incubated with the secondary antibodies and subsequently washed with TBS-T. Blots were developed using LumiGLO chemiluminescent substrate (Cell Signaling Technology, Inc.) when secondary antibodies were conjugated to horseradish peroxidise (Cell Signaling Technology, Inc.) and visualized using the Bio-Rad ChemiDoc system (Bio-Rad Laboratories Ltd.). When fluorescence secondary antibodies were used, blots were visualised by measuring the fluorescence intensities using an Odyssey CLx infrared scanner (LI-COR). Depending on the visualisation method, biotinylated (Bio-Rad) or fluorescence (LI-COR) protein ladders were used as protein size markers.

2.6.4. Two-dimensional gel electrophoresis (2DGE)

Hydrophobic proteins were analysed by two-dimensional gel electrophoresis (2DGE). Dried detergent phase proteins were resuspended in distilled water, lyophilised, weighted and resolved in a solubilasation buffer (7 M urea, 2 M thiourea, 3 % CHAPS, 2 % ASB-14, 0.2 % carrier ampholytes 4 - 7, 0.0002 % bromophenol blue and 50 mM DTT) to give a final protein concentration of 2 mg/ml. Solubilised proteins were stored in 200 µl aliquots (400 µg of protein) at - 20°C until use.

An aliquot of the protein extracts was thawed and used for passive rehydration of 11 cm IPG strips with pH ranging from 4 to 7 (Bio-Rad) overnight at room temperature (Gorg

et al. 2000) before separating the proteins by isoelectric point in the first dimension. Isoelectric focusing (IEF) was performed at 20°C in an IEFcell (Bio-Rad Laboratories Ltd.) using the following six-step program: (a) 0–150 V in 1 hour; (b) 150 V constant for 30 minutes; (c) 150–300 V in 1 hour; (d) 300 V constant for 1 hour; (e) 300–3500 V in 4.5 hours; and (f) 3500 V constant until 32 kVh.

Prior to the running of the second dimension (SDS-PAGE), IPG strips were equilibrated sequentially in equilibration buffer I (6 M urea, 2% SDS, 0.375 M, Tris–HCl, pH 8.8, 20 % glycerol, 130 mM DTT) and equilibration buffer II (6 M urea, 2% SDS, 0.375 M, Tris–HCl, pH 8.8, 20 % glycerol, 135 mM iodoacetamide). Each strip was then loaded on top of a vertical SDS–polyacrylamide gel (Bio-Rad Laboratories Ltd.) and sealed in place with 1 % (w/v) agarose (Bio-Rad Laboratories Ltd.). Molecular mass markers were loaded into a separate well at the side of the gel. Electrophoresis was performed in a Criterion system (Bio-Rad Laboratories Ltd.) at a constant voltage of 175 V until the indicator dye (bromophenol blue) approached the bottom edge of the gels (~ 1 hour). Proteins were stained with SimplyBlue™ SafeStain (Invitrogen) and scanned using the Bio-Rad Gel Doc system (Bio-Rad Laboratories Ltd.).

2.6.5. Mass spectrometric analysis

Protein fractions were first subjected to in-solution tryptic digest as follows: 4 µl of 10mM DTT was added to 10 µl sample and incubated at 50°C for 20 minutes. After addition of 4 µl of 50 mM iodoacetamide and 2 µl of 500 mM ammonium bicarbonate, the mixture was incubated at room temperature in dark for 20 minutes. 0.1 µg of trypsin was then added and the samples were incubated at 37°C overnight. The reaction was stopped with 1 µl of 1% formic acid. Tryptic digests were loaded onto a large capacity micro C_{18} reverse phase analytical column (Agilent Protein Identification Chip). Analysis of the samples were carried out using a QTOF 6520 (Agilent) coupled to a

1200 series HPLC-Chip interface system by Hannah Florance at the University of Exeter, Mass spectrometry facility. Spectrometry results were compared with the known sequence of *C. difficile* 630 strain using Protein Prospector and Spectrum Mill MS Proteomics Workbench software (Agilent). Protein quantification was carried out using Progenesis LC-MS software (Non-Linear Dynamics).

2.6.6. Chemical biology experiment (\`Click\` chemistry)

This experiment was carried out by Thomas Charlton at Imperial College London. \`Click\` chemistry was based on alkyne tagged lipid analogues that are taken up by the bacterial cells and used like normal fatty acids. *C. difficile* was grown in the presence of a myristic acid analogue (YnC12; myristic acid was used as a negative control). Following cell lysis, a fluorophore reagent was attached to the alkyne group of the lipid analogue and fluorescence intensity was measured by in-gel fluorescence imaging (\`Click\` chemistry). The click reaction was carried out on 100 µl of a 1 mg/ml protein solution in Click Buffer (0.2 % SDS in 1x PBS). Click solution was prepared such that the final concentration when added to the protein solution would be as the following: 100 µM capture reagent (azide), 1 mM $CuSO_4$, 1 mM tris-(2-carboxyethyl)phosphine (TCEP), 100 µM tris-(benzyltriazolylmethyl)amine (TBTA). The solution was vortexed after the addition of each reagent. Following the addition of the TCEP, the solution was left to stand for 2 minutes to allow enough time for reduction of the copper. The click solution was then added to the samples to give the desired final concentrations and the reaction was vortexed at room temperature for 1 hour. The click reaction was quenched by the addition of EDTA to a final concentration of 10 mM. Proteins were recovered by methanol/chloroform precipitation. Briefly, the sample was diluted with 4x volume of ice cold methanol, 1x volume of chloroform and x volume of water, and vortexed after addition of each component. Proteins were precipitated to the interface by centrifugation

(17,000g, room temperature, 5 minutes) and the aqueous/methanol was layer removed. A further 4x volume of methanol were then added to the sample, vortexed and the proteins were pelleted by centrifugation at 17,000g for 5 minutes at room temperature. The supernatant was carefully removed, the pellet (precipitated proteins) was air dried and the proteins were resuspended in 2 % SDS and 10 mM EDTA in PBS by sonication to a concentration of 10 mg/ml and diluted with 1x PBS to 2 mg/ml. 10 μg of each protein sample was separated by SDS-PAGE. Gels were imaged by in-gel fluorescence and Coomassie staining. Prior to imaging, gels were fixed for 30 minutes in fixing solution (45 % (v/v) methanol, 10 % (v/v) acetic acid) and rehydrated for at least 45 minutes in 10 % (v/v) acetic acid solution. Visualisation by fluorescence was carried out using an Ettan™ DIGE Imager (GE Healthcare). For detection of all proteins, gels were covered in Coomassie Stain solution (0.25 % (w/v) Coomassie Brilliant Blue R-250, 45% (v/v) methanol, 10 % (v/v) acetic acid) and incubated for at least an hour with gentle agitation. Gels were then washed and covered with Coomassie Destain solution (45 % (v/v) methanol, 10 % (v/v) acetic acid) and incubated until the background staining had been reduced to an acceptable level. Gels were rehydrated with 10 % (v/v) acetic acid for 45 minutes prior to imaging.

2.6.7. Purification of recombinant proteins from *E. coli* C43(DE3)

Following induction of protein expression (see 2.4.2), *E. coli* cell lysates were prepared using BugBuster Protein Extraction Reagent (Novagen) according to the manufacturer`s protocol and expression of the proteins was examined by SDS-PAGE. Recombinant proteins were purified using the Novagen His-Bind kit (Novagen) then samples were loaded onto PD-10 desalting columns (Novagen) to exchange the buffer to PBS. Protein purity was assessed by separation by SDS-PAGE (Life Technologies). The N-terminal His-tag was cleaved by TEV protease (prepared by Nic Harmer as a histidine-tagged

recombinant protein) where indicated. The reaction was stopped by removing the protease using the Novagen His-Bind kit (Novagen) which, at the same time, removed the His-tag from the sample. Concentration of the protein preparation was determined by spectrophotometer at 280 nm (NanoDrop 1000 Spectrophotometer, Thermo Fisher Scientific Inc.) and adjusted to the desired concentration either by concentrating the samples in concentration tubes (Millipore, 3kDa cut off) or by diluting in PBS.

2.6.8. Differential scanning fluorometry (DSF)

Metal ions and amino acids were prepared in 10x final concentration and distributed row-wise in a 96-well mother plate with water only as a control. After distributing 18 µl of CD0873 (0.1 mg/ml, ~2.7 mM) and SYPRO Orange (Sigma) mixture into the wells of a 96-well thin wall PCR plate (Fisher Scientific), metal ions or amino acids (2 µl) were transferred from the mother plate to the PCR plate with the final concentrations ranging from 0.01 to 10 mM. PCR plates were sealed with optically transparent sealing tape, centrifuged at 300g for 2 minutes to ensure good mixing and heated in a Step-One Real-Time PCR Systems (Applied Biosystems) at intervals of 1°C from 25°C to 99°C with a ramping rate of 1 % per 30 seconds. Fluorescence changes in the wells of the plate were monitored simultaneously with a charge-coupled device (CCD) camera. Wavelengths for excitation and emission were 490 and 575 nm, respectively. Melting temperatures (T_{m},) of the protein was determined by StepOne software.

2.6.9. Native PAGE

Reaction mixtures of 12 µl samples containing the non-histidine tagged recombinant CD0873 protein at 5 µM and metal ions at various concentrations ranging from 0.02 to 50 mM were incubated for 5 minutes at room temperature. Protein without addition of metal ions was used as a control for the experiments. Protein samples were separated by

Native PAGE through 3-12% Bis-Tris gels (Life Technologies) using Dark Blue Cathode Buffer (Life Technologies) which allows fast staining of the gels. After electrophoresis, gels were fixed in a solution of 40 % (v/v) of methanol and 10 % (v/v) of acetic acid for 45 seconds in a microwave and then for 15 minutes at room temperature followed by destaining in 8 % acetic acid for 45 seconds in a microwave. Gels were shaked in destaining solution until the background reduced to an acceptable level and scanned using the Bio-Rad Gel Doc system (Bio-Rad Laboratories Ltd.).

2.6.10. Production of antibodies to the CD0873 protein

Antisera raised against the CD0873 protein were generated commercially (ENZO Life Sciences (UK) Ltd.). Three New Zealand White SPF rabbits were immunized with 350µg of the recombinant CD0873, which was emulsified in Freund complete adjuvant. Animals were boosted seven times at 2-week intervals with 50 µg of protein each time. A week after the last immunization, blood samples were collected, serum samples were prepared, pooled and used for further experiments as polyclonal antibodies to the CD0873 protein of *C. difficile*. Pre-immune serum samples were also prepared from each animal prior to the commencement of immunisation.

2.6.11. Protein microarray

This experiment was carried out by Manuele Biazzo and myself at Novartis Vaccines and Diagnostics, Siena, Italy. Purified recombinant proteins were printed (0.1 ng protein per spot, spot diameter: ~ 90-100 µm) onto nitrocellulose coated glass FAST slides (Whatman) using an ArrayJet Marathon microarray printer (ArrayJet Ltd.). All proteins were spotted in 8 replicates on each slide. Each chip was also printed with control spots consisting of the purified N-terminal histidine tag (without fusion to recombinant proteins), PBS and serial dilutions of purified human IgGs and BSA-Cy5 (bovine serum

albumin conjugated to the fluorescence dye N,N'-(dipropyl) tetramethylindodicarbocyanine). Slides were stored at 4℃ in dark. Unless otherwise mentioned, serum samples were diluted to 1:200 in protein array blocking buffer (Whatman). Slides were washed with PBS-T (0.1 % (v/v) Tween-20 in PBS) for 1 minute and rehydrated in protein array blocking buffer at room temperature for 1 h in dark with shaking before probed with the sera for 1 h at room temperature in dark with constant agitation. Following washing with PBS-T (room temperature, three times 5 minutes), array slides were incubated with anti-human IgG (DyLight 649-conjugated goat anti-IgGfcγ, Jackson Immuno Research) secondary antibodies diluted in protein array blocking buffer (1:800) and then washed three times in PBS-T. Slides were subsequently washed once in water and air dried before scanned using Tecan PowerScanner (Tecan Group Ltd., Switzerland) at 10 μm resolution. Microarray spot intensities were quantified using ImaGene 9.0 software.

2.7. Phenotypic assays

2.7.1. Determination of the growth rate of *C. difficile*

10 ml of prereduced liquid medium was inoculated with a fresh single colony and grown for 12 hours at 37℃. Fresh liquid medium was then inoculated with the exponential phase cultures. To monitor the growth rates, an aliquot of 1 ml was removed from the bacterial cultures at different time points and the OD at 590 nm (OD_{590}) was measured in cuvettes with a Spectrophotometer (Fisher Scientific). In parallel, viable cell numbers were measured as Colony Forming Units (CFU) for which an aliquot of the culture was 10-fold serially diluted in BHI broth and a known volume of each dilution was spread onto BHI agar, supplemented with antibiotics where appropriate. Colonies were counted after an overnight incubation at 37°C.

2.7.2. Scanning electronmicroscopy (SEM)

Morphology of bacterial cells was examined by scanning electronmicroscopy at the University of Exeter Bioimaging Centre.

2.7.3. Recovering *C. difficile* spores

Sporulation cultures were set up as follows to ensure that no spores were present when the sporulation medium was inoculated. 10 ml of prereduced BHI medium was inoculated with a fresh single colony and grown overnight at 37°C. Overnight cultures were diluted 1:100 in fresh liquid medium and incubated until the OD_{590} was between 0.2 and 0.5 (starter culture). Starter cultures were then diluted 1:100 in fresh medium (sporulation culture) and sporulation cultures were grown for six days. An aliquot of 100 µl was removed from the sporulation medium every 24 hours and heated at 69°C for 25 minutes to kill the vegetative cells but not the spores. Vegetative outgrowth was measured as Colony Forming Units (CFU) for which the heat-treated samples were 10-fold serially diluted in BHI broth and a known volume of each dilution was spread onto BHI agar supplemented with 0.1 % (w/v) taurocholate. Colonies were counted after an overnight incubation at 37°C.

2.7.4. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) were determined by preparing stock solutions at a 2x concentration as the first concentration of the assay and 2-fold serially diluted in a 96-well micro-titer plate (100 µl of each concentration). OD_{590} of fresh exponential phase cultures of *C. difficile* was adjusted to 0.1 and diluted 1:100 in prereduced BHI liquid medium which corresponds to approximately 1 x 10^{6} CFU/ml. 100 µl of the bacterial suspension was added to each well of the 96-well micro-titer plate already containing 100 µl solution by which bacterial suspension was diluted 2-

fold resulting in 5 x 10^5 CFU/ml in the wells. Wells without either solutions or bacteria were also set up as positive and negative controls, respectively. Plates were incubated anaerobically at 37°C for 20 hours and the OD_{570} was measured using a Bio-Rad plate reader. Readings were normalized to the solution only controls.

2.7.5. Caco-2 cell adherence assays

Adherence assays were performed with the Caco-2 human intestinal epithelial cell line (ECACC, UK). Caco-2 cells were used between passages 54 and 69. Cells were cultured in Eagle`s minimal essential medium (EMEM; PromoCell GmbH, Heidelberg, Germany) supplemented with 10 % of non-heat treated fetal bovine serum (FBS; SAFC, Sigma-Aldrich) and 1 % of MEM nonessential amino acids (NEAA; Life Technologies). Cells were regularly tested for *Mycoplasma* contamination (e-MycoTM plus Mycoplasma PCR Detection Kit, Chembio Ltd). Caco-2 cells were incubated in a humidified 5 % CO_2 atmosphere at 37°C. For the adherence experiments, Caco-2 cells were seeded at a cell density of 7 x 10^4 cells per well in 24-well tissue culture plates. Confluency occurred after 2 days of incubation. Monolayers were used 8 days after seeding at a half differentiated state. Culture medium was changed every other day and replaced with fresh, non-supplemented EMEM 24 hours before infection. To determine the number of Caco-2 cells per well, monolayers were collected by trypsinisation and counted under microscope using a haemocytometer.

2.7.5.1. Bacterial binding assays

Bacterial suspension was prepared in prereduced EMEM using cultures in mid exponential phase. Adherence experiments were conducted under anaerobe conditions. Caco-2 monolayers were washed with PBS and infected in triplicate with 1 ml of bacterial suspension to give a multiplicity of infection (MOI) of 1:5. After 2 hours of

incubation, nonadherent bacteria were removed by pipetting and cells were washed three times with PBS. After this step, adherence of *C. difficile* was determined by three methods:

(i) CFU counts; washed Caco-2 cells were disrupted by vigorous pipetting, resuspended in 1 ml of BHI broth and the number of adherent bacteria was determined by plating 10-fold dilutions of this sample onto BHI agar.

(ii) On-cell western assay; following the washing steps, Caco-2 cells with the adhered bacteria were fixed with 5 % (v/v) paraformaldehyde (Fisher Scientific) for 25 minutes at room temperature. Cells were then washed five times with PBS and blocked overnight at 4°C with Odyssey blocking buffer (LI-COR). After washing the wells five times, cells were then incubated with rabbit sera raised against *C. difficile* cells (1:2,500; gift from Neil Fairweather, Imperial College London) for 1 hour at room temperature. After five washing steps, IRdye 680LT goat anti-rabbit IgG (1:2,000; LI-COR) secondary antibodies were added to the wells and cells were incubated in dark for 1 h at room temperature. Wells were then washed five times with PBS and fluorescence intensity was measured by the Odyssey CLx infrared scanner (LI-COR).

(iii) Immuno-fluorescence microscopy; this experiment was carried out by Magdalena Kasendra at Novartis Vaccines and Diagnostics, Siena, Italy. In this method, Caco-2 cells were seeded on collagen I coated chamber slides and infected with an MOI of 1:100 followed by centrifugation at 60g for 5 minutes. After removal of the non-adherent bacteria, Caco-2 cells were washed, and fixed with 4 % of paraformaldehyde for 15 minutes at 37°C. Cells were then again washed and permeabilized with 1 % of saponin, 0.1 % of Triton X-100 in PBS for 20 minutes at room temperature. After washing with PBS, cells were blocked with 3% of BSA in PBS overnight at 4°C. Following the blocking step, rabbit anti-*C. difficile* primary antibodies (1:1,000) were added to the slides and incubated for 1 hour at room temperature. Cells were then

washed and incubated with Alexa Fluor 568-phalloidin and Alexa Flluor 488 goat anti-rabbit IgG antibodies (1:1,000 and 1:400, respectively) (Molecular Probes) for 1 hour at room temperature. After successive washes with PBS, cells were incubated with ProLong® Gold antifade reagent containing 6-diamidino-2-phenylindole (DAPI) (Molecular Probes), dried and analyzed with Zeiss LSM710 confocal microscope.

2.7.5.2. Protein binding assays

These experiments have been carried out under aerobic condition. After removal of the EMEM and washing with PBS, Caco-2 cells were incubated with 1 ml of EMEM containing either 100 μg of recombinant protein (non-His tagged CD0873 or His-tagged ε prototoxin of *C. perfringens* as a control) or 1 mg of *C. difficile* whole cell lysates. After three washes with PBS, cells were disrupted with 200 μl of lysis buffer (0.8 % SDS in PBS) and boiled for 10 minutes. 15 μl of the lysate was then subjected to Western blot analysis using anti-CD0873 and anti-His antibodies.

2.7.6. Cell viability assay

Viabilty of the Caco-2 cells was evaluated by MTT assay as previously described (Mosmann 1983). The assay is dependent on the cellular reduction of MTT by the mitochondrial dehydrogenase of viable cells to a blue solubilised formazan product which can be measured spectrophotometrically. Optical density is directly correlated with cell quantity. Media were substituted with 1 ml media + 100 μl sterile MTT solution (5 mg/ml in PBS). After incubation at 37°C for 4 h, media were discarded and the produced formazan was solubilized in dimethyl sulfoxide (DMSO) (100 μl per well). Absorbance at 570 nm and 630 nm (background) was measured using a plate reader (Bio-Rad). Optical density measured at 630 nm was subtracted from the optical

density measured at 570 nm to neutralize the effect of the cell debris and the precipitated proteins which may be produced when DMSO was added to the sample.

2.7.7. Mouse CDI model

This experiment was carried out by Zoe Seager and myself at Imperial College London. 6-8 weeks old black C57b/6 mice were co-infected with equal numbers of wild-type (Cdi::*tetM*) and Cdi::*lgt* spores. A *tetM* mutant of the *C. difficile* 630Δ*erm* (Cdi::*tetM*) which is sensitive to tetracycline was used as wild-type strain to be able to select for *C. difficile* with erythromycin and distinguish between the recovered challenge strains in the faecal and tissue samples. The Cdi::*tetM* strain was generated by Zoe Seager at the Imperial College London using the ClosTron system. To prepare *C. difficile* spores for challenge, *C. difficile* was grown on SMC agar for 7 days. Spores were then scraped from the plates, resuspended in PBS and washed five times in ice-cold water (8,000g, 1 minute). Following the washing steps, spores were resuspended in 20 % (w/v) HistoDenz, layered on top of 50 % (w/v) HistoDenz and subsequently centrifuged at 15,000g for 15 minutes at room temperature. HistoDenz was then removed, spores were washed a further five times in ice-cold water and resuspended in PBS. Samples were diluted to 5 x 10^7 spores/ml after counting under phase-contrast microscopy and the Cdi::*tetM* and the Cdi::*lgt* strains were mixed 1:1. Mice were initially treated with a single dose of clindamycin (1 mg in 200 µl PBS). On the next day, mice (n = 10, kept separately in two cages - 5 mice in each cage) were orally challenged with 200 µl of spore suspension (0.5 x 10^7 Cdi::*tetM* control spores and 0.5 x 10^7 Cdi::*lgt* spores). A group of mice (n = 5) was challenged with PBS and served as a control. Faecal samples were collected at different time points from each mouse (see Figure 5.9). Faeces were weighed and diluted in PBS in order to obtain a concentration of 100 mg/ml. Numbers of vegetative cells present in the faeces were determined by spreading known volumes

of 10-fold serially diluted samples onto Braziers agar supplemented with the appropriate antibiotics. The number of the Cdi::*tetM* cells was calculated by subtracting the number of CFU grown on tetracycline-containing agar (Cdi::*lgt* only) from the number of CFU grown on non-tetracycline agar (both Cdi::*tetM* and Cdi::*lgt*). Spores were recovered by plating heat-treated samples (60℃, 10 minutes) onto Braziers agar supplemented with the appropriate antibiotics and 0.1 % (v/v) of taurocholate. On day 11 and day 53, after the first and second peak of bacterial shedding, respectively, when no bacteria were detected in the faeces, drinking water was replaced with 250 mg/l of clindamycin in water for 5 days to induce infection.

4 and 16 days after *C. difficile* challenge, during the first and second peak of bacterial shedding, respectively, two mice (one of each infected group) were sacrificed and dissected in order to obtain the colon, caecum and caecal content. Following homogenisation, the number of vegetative cells and spores of each strain in the tissues was determined as described above.

2.7.8. Immunofluorescence microscopy

This experiment was carried out by Rosanna Leuzzi at Novartis Vaccines and Diagnostics, Siena, Italy. To verify the presence of the CD0873 protein on the surface of *C. difficile*, bacteria were grown until exponential phase, washed with PBS and fixed in 3.7 % (v/v) paraformaldehyde. After multiple washings, bacteria were spread on polylisine-coated slides and blocked with PBS + 3 % (w/v) Bovine Serum Albumin (BSA) for 1 h at room temperature. After incubation, samples were washed and incubated with rabbit polyclonal serum against recombinant CD0873 (1:500) for 1 h at room temperature. Bacteria were washed several times with PBS and incubated with Alexa Fluor 568 goat anti-rabbit IgG (1:1,000) (Molecular Probes). Labeled samples were mounted with ProLong® Gold antifade reagent with DAPI (Molecular Probes) and analysed with Zeiss LSM710 confocal microscope.

2.8. Bioinformatical methods

2.8.1. Identification of putative lipoproteins

Putative lipoproteins of *C. difficile* were identified as follows: predicted protein sequences of *C. difficile* 630 (RefSeq: NC_009089) were downloaded from NCBI's FTP site and the following criteria were applied to identify potential lipoproteins: (1) all amino acid sequences were matched against the PROSITE pattern PS00013/PDOC00013, implemented as the PERL regular expression: [ACFGHILMNOPQSTUVXYZ]{6}[LIVMFWSTAG]{2}[LIVMFYSTAGCQ][AGS]C / (Sigrist *et al.* 2010); (2) position of the cysteine residue is between the 15th and 35th amino acids of the sequence; (3) at least one lysine or one arginine is found in the first seven positions of the sequence. These rules localise the lipidation pattern of the N-terminal sequences. Presence of the signal peptide was checked using SignalP 3.0 (Bendtsen *et al.* 2004). This analysis was carried out by David Studtholme. Annotations of amino acid sequences were obtained from the database provided by NCBI available at http://www.ncbi.nlm.nih.gov.

2.8.2. Other bioinformatical methods

Protein searches were carried out through RCSB Protein Data Bank (PDB) (www.rcsb.org) or using the Basic Local Alignment Search Tool (BLAST) available at http://www.ncbi.nlm.nih.gov.

Alignments of two or more amino acid sequences were routinely performed by the Clone Manager software using the BLOSUM62 scoring matrix.

Molecular weight calculations were performed using online tools at the http://www.sciencegateway.org/tools/proteinmw.htm website.

Results were statistically analysed in the GraphPad Prism software using t test for individual comparisons, one-way analysis of variance (ANOVA) and two-way ANOVA for multiple comparisons.

Chapter Three

Investigation of lipoprotein biosynthesis in *C. difficile*

3.1. Introduction

Lipoprotein biosynthesis has not previously been studied in *C. difficile*, possibly because of the lack of methods for the directed mutagenesis in Clostridia. Therefore, current knowledge of the lipoprotein biosynthetic pathway in *C. difficile* is poor. However, the recent development of clostridial mutagenesis methods now makes it possible to investigate lipoprotein biogenesis, which is considered to be potential target for drug development in several pathogens.

3.1.1. Identification of lipoproteins by bioinformatics

Bacterial lipoproteins can be identified based on the presence of the N-terminal signal peptide sequence possessing a c-region containing the recognition motif (lipoprotein box) of the type II signal peptidase. Secretion of lipoproteins is directed by the signal peptide, while the lipobox sequence is necessary for the lipidation of lipoproteins. The characteristic sequence of the lipobox is [LVI][ASTVI][GAS]C. The lipobox contains an invariant lipid-modifiable Cys at position +1. In 75.9 % of the cases, the -3 position is Leu, followed by Val (7.7 %) and I (3.7 %). The amino acid at position -2 is more flexible and can accommodate uncharged, polar, and nonpolar residues, such as Ala (29.8 %), Ser (25.4%), Thr (12.2 %), Val (12.1 %) and Ile (9.8 %). Position -1 is occupied equally by Gly (44.7 %) and Ala (40.5 %), and Ser is present in 14.8 % of the sequences (Babu *et al.* 2006).

The number of predicted lipoproteins is different in the different bacterial species, and there is no correlation between the predicted proteome size and the size of the predicted proteome coding for lipoproteins (Babu *et al.* 2006). In genomes with more than 1,000 ORFs, the numbers of lipoproteins range from as many as 223 (*Bacteroides thetaiotaomicron*) to as few as nine (*Aquifex aeolicus*). In the case of smaller genomes, two species of *Buchnera* have no predicted lipoproteins and a third has only one.

Rhodopirellula baltica has one of the biggest genomes (7,325 ORFs) but contains only 46 lipoproteins (Babu *et al.* 2006).

3.1.2. Lipoprotein processing in Gram-positive bacterial species

In Gram-negative bacteria, two lipoprotein biosynthetic enzymes (Lgt and Lsp) appear to be essential for viability (Gan *et al.* 1993, Gupta *et al.* 1993, Paitan *et al.* 1999, Tjalsma *et al.* 1999). Consequently, inactivation of those enzymes causes lethal phenotype in Gram-negative species. In contrast, Lgt and Lsp are not essential in the majority of Gram-positive bacteria. Although, the *lgt* gene is found in one copy in most of the bacterial genomes, two putative *lgt* paralogues are encoded in some bacteria, such as *Coxiella burnetti*, *B. cereus*, *C. perfringens* or *Streptomyces coelicolor* (Hutchings *et al.* 2009). The reason why multiple Lgt enzymes are present in those species is unknown. Similarly to *lgt*, only a single *lsp* gene is present in most of the bacteria, although some species possess two putative *lsp* paralogues (for example, *Staphylococcus epidermidis*, *Listeria monocytogenes* and *Nocardia farcinica*). However, some of these paralogues does not seem to function as signal peptidases (Hutchings *et al.* 2009, Reglier-Poupet *et al.* 2003). Other peptidases cleaving the signal peptides of lipoproteins also exist, such as the Eep (enhanced expression of pheromone) of *E. faecalis* (An *et al.* 1999) and *Streptococcus uberis* (Denham et al. 2008).

In Gram-negative bacteria, the lipoprotein biosynthetic pathway is strictly ordered (Lgt, Lsp, Lnt), while it seems to be less stringent in Gram-positives. It was believed for a long time that Lsp is able to cleave only lipid-modified precursors. However, in some species, it has been demonstrated that lipidation is not always a prerequisite for the activity of Lsp. Lgt-independent Lsp cleavage was first reported in *L. monocytogenes* (Baumgartner *et al.* 2007). In later studies, processing of non-lipidated lipoproteins by

Lsp has been shown in *Mycobacterium smegmatis* (Tschumi *et al.* 2012), *S. agalactiae* (Bray *et al.* 2009, Henneke *et al.* 2008) and *Streptococcus uberis* (Denham *et al.* 2009).

3.1.3. Aim of this study

This study aimed to identify lipoproteins and lipoprotein biosynthetic enzymes in *C. difficile*, and investigate how lipoprotein localisation and biogenesis is affected when the enzymes of the lipoprotein biosynthetic pathway are inactivated.

3.2. Results

3.2.1. Identification of the putative lipoproteins of *C. difficile*

Unique features of lipoproteins allow identification of putative lipoproteins within the bacterial proteomes. Lipoproteins of *C. difficile* were predicted using bioinformatic tools. Briefly, amino acid sequences of *C. difficle* 630 (CD0001-CD3798) were downloaded from the NCBI FTP site and matched against the criteria which allow recognition of the lipoprotein box at the N-terminal of the protein (PROSITE pattern PS00013/PDOC00013) (Sigrist *et al.* 2010). Sequences retained in the above filter were then screened by SignalP 3.0 searching for the presence of the signal peptide. This analysis identified a total of 86 lipoproteins (Appendix I), indicating that lipoproteins represent approximately 2.2 % of the predicted proteome in the *C. difficile* 630 strain.

Since little is known about lipoproteins in *C. difficile*, annotation of the putative lipoproteins is mainly based on theoretical considerations. These annotations are available on the NCBI website which is shown in Appendix I and listed by functional groups in Table 3.1. Similarly to other bacterial species, numbers of the predicted *C. difficile* lipoproteins have a role in molecule transport, majority of those functioning as solute binding proteins (SBP) of ABC transport systems. These SBPs have a role in the

binding of diverse substrates, such as minerals, sugars, amino acids or peptides, suggesting the importance of lipoproteins in nutrient uptake. Some lipoproteins are predicted to be involved in signalling, redox processes or protein folding. The role and function of 33 lipoproteins of *C. difficile* is not currently assigned.

Table 3.1. Predicted roles of the lipoproteins of *C. difficile*.

Role	Numbers	%
Molecule transport (mainly substrate binding for ABC transport systems)	32	37.2
Signalling	4	4.6
Redox processes	2	2.3
Protein folding	2	2.3
Other roles	13	15.2
Unknown functions	33	38.4

3.2.2. Disruption of the lipoprotein biogenesis in *C. difficile*

Three lipoprotein biosynthetic enzymes are encoded in the genome of *C. difficile* 630 strain: the CD2659 open reading frame was annotated as prolipoprotein diacylglyceryl transferase (*lgt*), and CD2597 and CD1903 as lipoprotein signal peptidases (*lsp*). In common with most other bacteria, the *C. difficile* 630 genome encodes a single Lgt (CD2659) enzyme but two putative Lsp enzymes, named LspA (CD2597) and LspB (CD1903). The Lgt protein consists of 248 amino acids with the predicted molecular weight of 27.5 kDa, exhibits 44 % (118/267) amino acid sequence identity with the Lgt enzyme of *B. subtilis* (Leskela *et al.* 1999), and high degree of amino acid identity (98 to 100 %) to the predicted Lgt enzymes of several other *C. difficile* strains. CD2597 encodes a 148 amino acid polypeptide with the predicted molecular mass of 17 kDa, CD1903 encodes a protein consisting of 178 amino acids predicted to be 20.4 kDa. LspA shows 38 % (59/154) and LspB exhibits 17 % (32/185) amino acid sequence identity with the SPase II of *B. subtilis* (Pragai *et al.* 1997). LspA and LspB share low

amino acid sequence identity with each other (20 %, 37/181). Similarly to Lgt, LspA and LspB proteins have homologues with high amino acid sequence identity (97 to 100 % for both enzymes) in a numbers of *C. difficile* strains suggesting that lipoprotein biosynthetic enzymes are conserved at least in some *C. difficile* strains.

In this study, the CD2659 gene (proposed *lgt* gene) was inactivated by following the protocol of the ClosTron insertional mutagenesis system (Heap *et al.* 2010, Heap *et al.* 2007) (Figure 3.1).

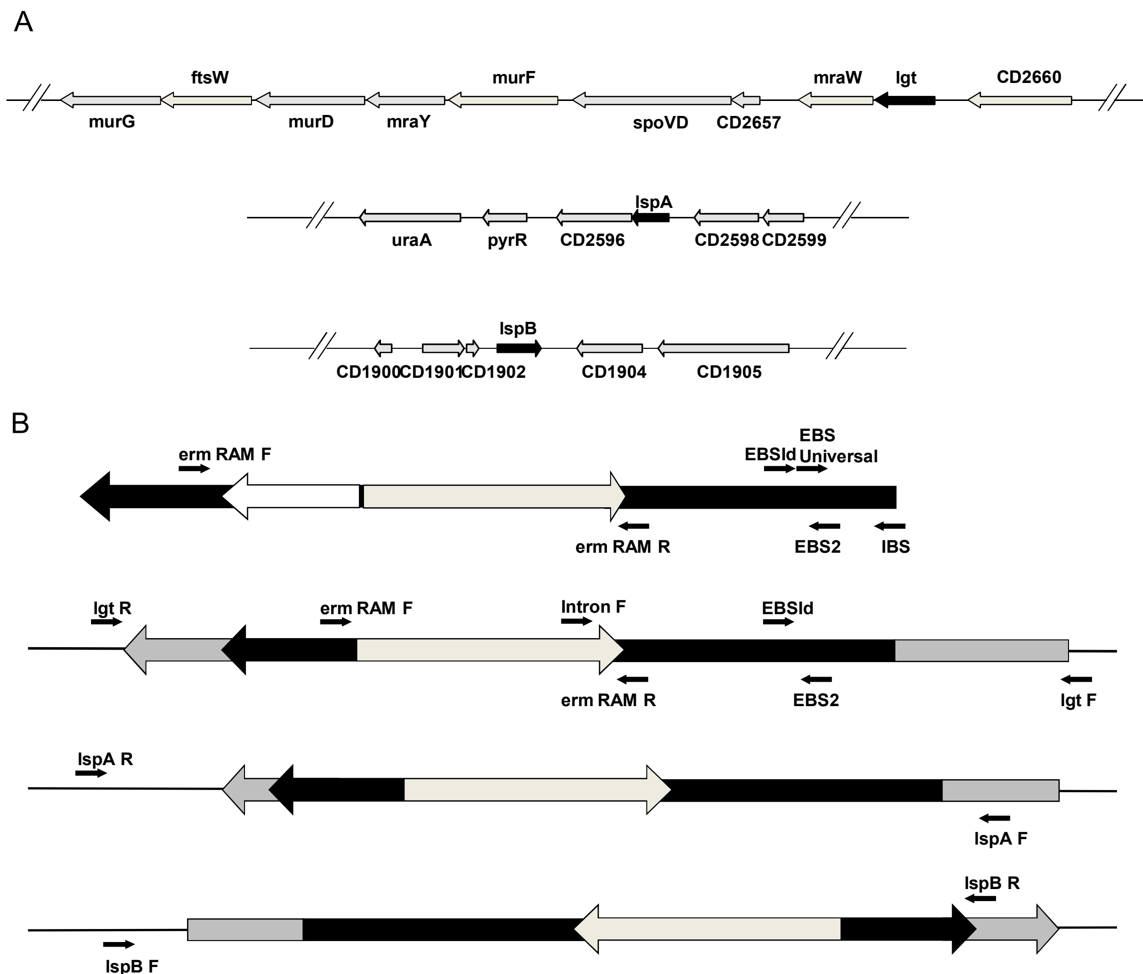


Figure 3.1. Overview of the inactivation of the *lgt*, *lspA* and *lspB* genes of *C. difficile* 630Δ*erm*. (A) Schematic representation of the *lgt*, *lspA* and *lspB* region on the chromosome of *C. difficile* 630Δ*erm*. (B) Schematic representation of the ClosTron system. The group II intron (black arrow) contains an erythromycin-resistance gene, ermRAM (light grey arrow), of which transcription is interrupted by a group I intron (white array) (top image). Group I intron splices out after the group II intron integrates into the target gene (dark grey), here *lgt*, *lspA* and *lspB* (from the top to the bottom, respectively). Primers amplifying the target genes around the integration site are indicated on the diagram.

Briefly, the pMTL007C-E2::Cdi-*lgt*-477|478s (Figure 2.1.B) was generated by SOE PCR in order to disrupt the *lgt* gene by inserting the group II intron containing the erythromycin-resistance gene between the base pairs 477 and 478 (Figure 3.1.B). The retargeted vector was designed in such a way that the intron inserts into the gene in the sense orientation. Mutation of the *lgt* gene at the 430|431 target site using pMTL007C-E2::Cdi-*lgt*-430|431s was also attempted but was not possible. To select for the *lgt* mutant (Cdi::*lgt*), erythromycin-resistance colonies were picked, genomic DNA was extracted and used as a template in PCR to amplify the *lgt* gene with forward and reverse gene-specific primer pairs (*lgt* F, *lgt* R). Correct size of the PCR products obtained with the *lgt* F and *lgt* R primers confirmed that the integration of the intron had occurred (Figure 3.2.A): the 819 bp amplicon obtained in the wild-type (Cdi::WT) indicates the size of the intact *lgt* gene, while the larger, 2668 bp DNA fragment amplified in Cdi::*lgt* shows disruption of the gene. PCR analysis amplifying the intron-exon junction using gene-specific primers (*lgt* F, *lgt* R) against intron-specific primers (EBS2, EBSId) further confirmed that the integration occurred into the desired site.

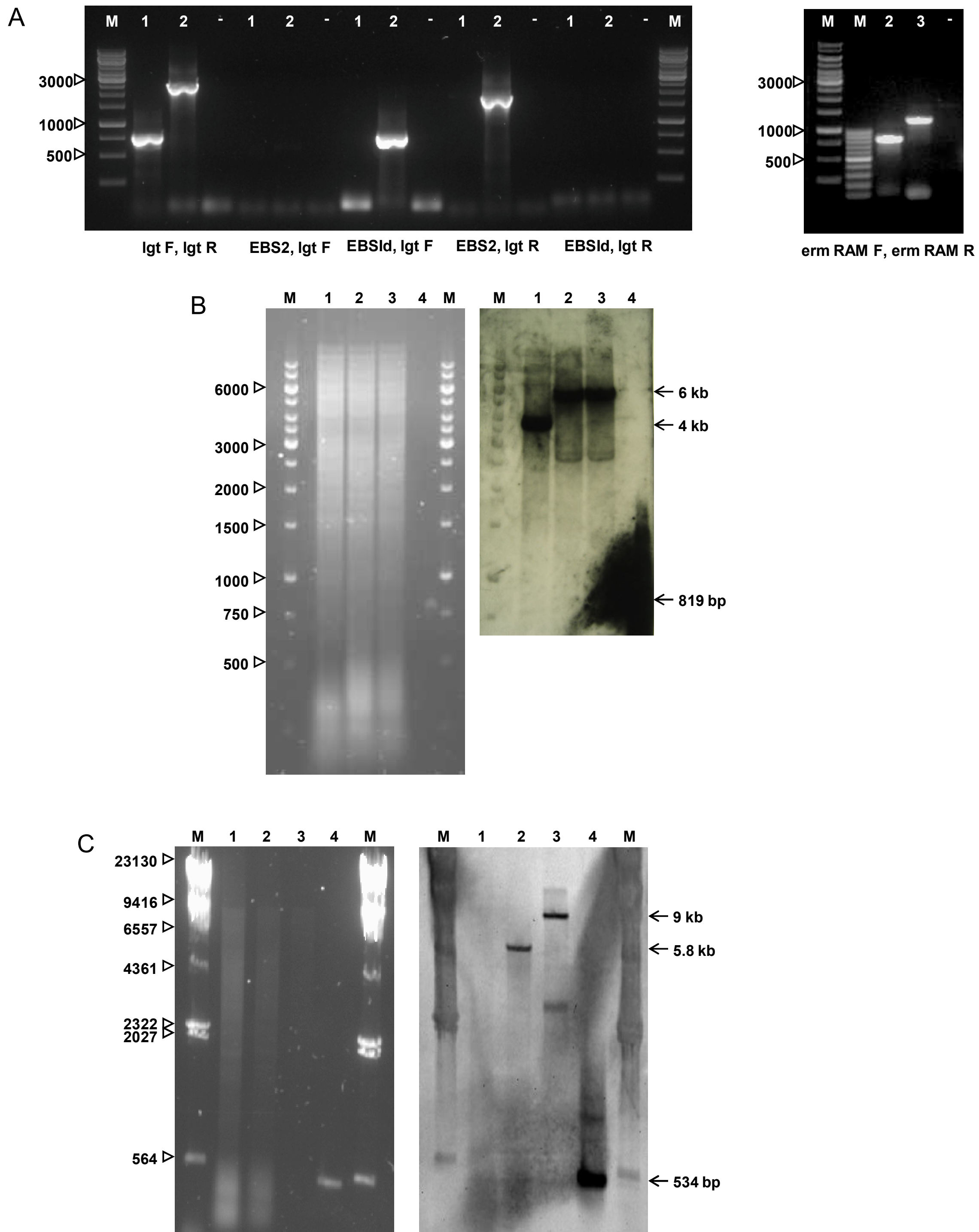


Figure 3.2. Confirmation of the *lgt* mutant *C. difficile* 630Δ*erm* by PCR and Southern blot analysis. (A) Confirmation of the Cdi::*lgt* by PCR using primer pairs shown below the images. Lane M, DNA ladder (bp); Lane 1, Cdi::WT; Lane 2, Cdi::*lgt;* Lane 3, pMTL007C-E2::Cdi-*lgt*-477|478s; -, water (negative) control. Sizes of the PCR products: *lgt* F, R: Cdi::WT, 819 bp, Cdi::*lgt*: 2668 bp; EBSId, *lgt* F: Cdi::*lgt*: 815bp; EBS2, *lgt* R: Cdi::*lgt*: 1704 bp. (B) Confirmation of the Cdi::*lgt* by Southern blot using a probe specific for the *lgt* gene. Lane M, DNA ladder (bp); Lane 1, Cdi::WT; Lane 2, 3, Cdi::*lgt;* Lane 4, unlabelled *lgt* probe (positive control). Fragment sizes: Cdi::WT: 4046 bp; Cdi::*lgt*: 5896 bp. (C) Confirmation of the single intron insertion into the chromosome of Cdi::*lgt* by Southern blot using an intron-specific probe. Lane M, DNA ladder (bp); Lane 1, Cdi::WT; Lane 2, Cdi::*lgt;* Lane 3, pMTL007C-E2::Cdi-*lgt*-477|478s (positive control); Lane 4, unlabelled intron probe (positive control). Fragment sizes: Cdi::*lgt*: 5896 bp; pMTL007C-E2::Cdi-*lgt*-477|478s: 9033 bp.

To further confirm the integration of the intron and that the isolated mutant contains only a single intron insertion, DNA of the putative Cdi::*lgt* strains were subjected to Southern blot analysis. Probes specific for the *lgt* gene and for the group II intron were prepared in a PCR using Cdi::WT and Cdi::*lgt* chromosomal DNA as templates, respectively, using *lgt* F, *lgt* R and EBS2(lgt), intron F (anneals within the intron upstream of the *ermB*) primer pairs, respectively. Figure 3.2.B shows the analysis using *lgt* specific probe. In Cdi::*lgt*, the probe bound to a DNA fragment which is ~ 1.8 kb larger than the fragment reacted in Cdi::WT, further confirming disruption of the *lgt* gene. The intron specific probe reacted with a single DNA fragment in the Cdi::*lgt* strain indicating that only a single intron insertion occurred in the isolated mutant (Figure 3.2.C).

For complementation of the Cdi::*lgt* strain, the *lgt* gene and 367-bp 5` noncoding region immediately upstream of the open reading frame presumed to contain the promoter was amplified using wild-type genomic DNA as template and *lgt EcoRI* F and *lgt BamHI* R oligonucleotides as primers. Primers were designed to allow subsequent cleavage of the fragment with *Eco*RI at the 5` end and *Bam*HI at the 3` end. PCR product was ligated into pGEM holding vector and then transformed into *E. coli* DH5α. Correct sequence of the amplified DNA fragment was confirmed by sequencing the DNA region cloned into pGEM. The vector was then cut with *Eco*RI and *Bam*HI and the cleaved PCR fragment was ligated into the modular plasmid pMTL84151 which was previously cut with *Eco*RI and *Bam*HI. This ligation yielded the pMTL84151::Cdi-*lgt* complementation plasmid (Figure 3.3). Ligation reaction was transformed into *E. coli* CA434. Subsequently, pMTL84151::Cdi-*lgt* was transferred from *E. coli* CA434 into Cdi:: *lgt* by conjugation. Positive complements (thiamphenicol resistant colonies) were screened by PCR. Stability of the complementation plasmid was confirmed by restreaking 12

single colonies on BHI agar ten times and then on BHI agar supplemented with thiamphenicol.

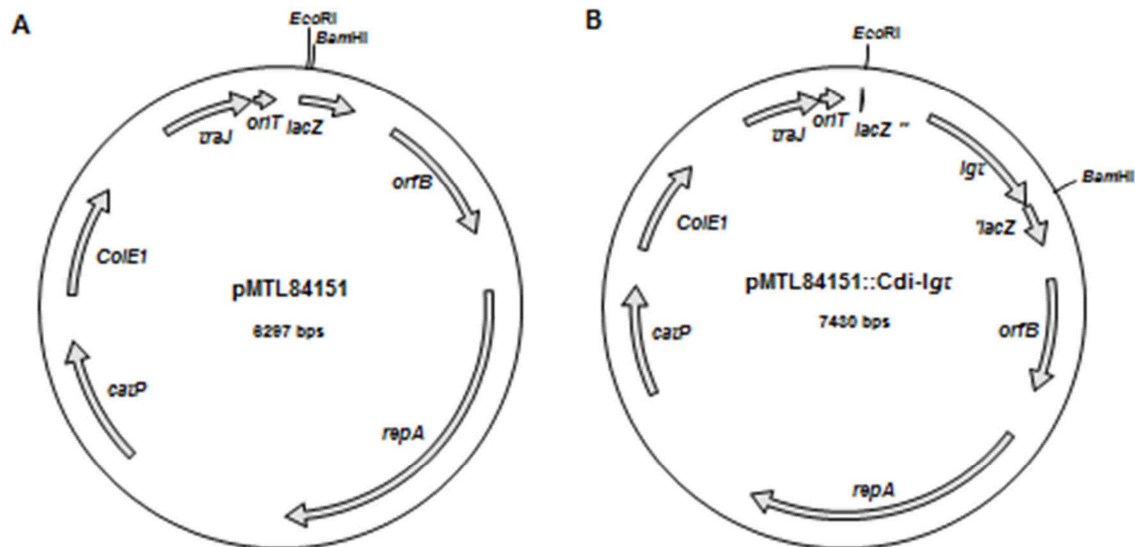
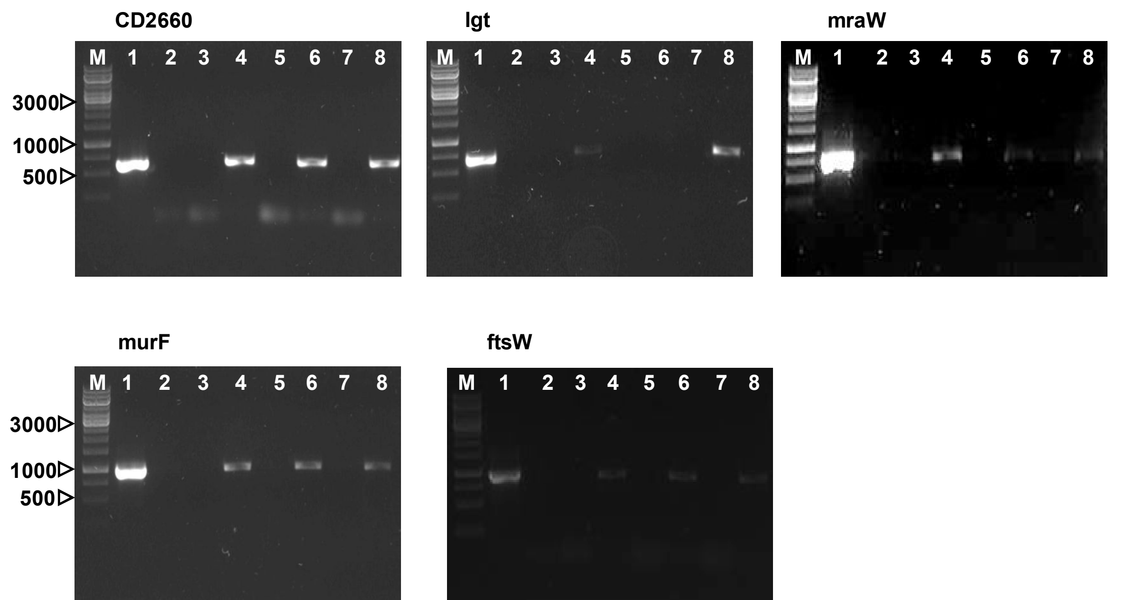


Figure 3.3. Map of the pMTL84151 modular plasmid. A) Modular plasmid pMTL84151. B) Plasmid pMTL84151 consisting the *lgt* structural gene and its native promoter. A 1,154 bp fragment consisting of the *lgt* gene and 367-bp 5` noncoding region presumed to contain its promoter was cloned into the plasmid using *Eco*RI and *Bam*HI restriction enzymes. LacZ, β-galactosidase α subunit; repA and orfB, Gram-positive replicon, CatP, chloramphenicol acetyltransferase (conferring chloramphenicol resistance in *E. coli* and thiamphenicol -methyl-sulfonyl analogue of chloramphenicol- in *C. difficile*); ColE1, Gram-negative replicon; TraJ, conjugal transfer protein.

The Cdi::*lgt* and the complemented Cdi::*lgt* strains were further characterised by reverse transcriptase PCR (RT-PCR) to ensure that the *lgt* gene is no longer expressed and to check whether the expression of the genes upstream and downstream of the *lgt* gene are affected by *lgt* mutation (Figure 3.4). RNA was extracted from the strains at early logarithmic phase as that was the only time point when transcription from the *lgt* gene was observed in the wild-type *C. difficile* (no detectable *lgt* transcription was observed in the mid and late logarithmic phase in the wild-type *C. difficile*). RT-PCR analysis showed continued transcription of the genes upstream and downstream of the

lgt locus, lack of *lgt* transcription in the mutant and restored transcription from *lgt* in the complemented strain.

Taken together, PCR, Southern blot and RT-PCR analyses indicated that the *lgt* gene was successfully disrupted and inactivated in the Cdi::*lgt* strain.



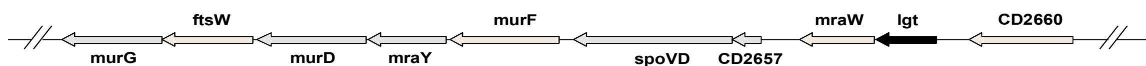


Figure 3.4. Transcriptional analysis of the *lgt* gene, and the genes upstream and downstream of the *lgt* gene in the *lgt* mutant and the complemented *lgt* mutant of *C. difficile*. Transcriptional analysis of the CD2660, *lgt*, *mraW*, *murF* and *ftsW* genes in the Cdi::WT, Cdi::*lgt* and complemented Cdi::*lgt* strains were performed by RT-PCR using primer pairs listed in Table 2.4. Lanes: 1, Cdi::WT DNA (positive) control; 2, water (negative) control; 3, 5, 7, DNAse treated RNA; 4, 6, 8, cDNA; 3,4, Cdi::WT; 5,6, Cdi::*lgt*; 7,8, complemented Cdi::*lgt.* Sizes of the RT-PCR products: CD2660-RT F, R: 643 bp; lgt-RT F, R: 682 bp; *mraW*-RT F, R: 744 bp; *murF*-RT F, R: 966 bp; *ftsW*-RT F, R: 985 bp.

Two *lsp* mutants, Cdi::*lspA* and Cdi::*lspB* were generated by the ClosTron system by inserting the group II intron between the base pairs 321|322 and 317|318 of the *lspA* and *lspB* genes, respectively (Figure 3.1.B). Retargeted pMTL007C-E2 plasmids (pMTL007C-E2::Cdi-*lspA*321|322s, pMTL007C-E2::Cdi-*lspB*-317|318s) were designed in such a way that the intron inserts into the target genes in the sense

orientation. Following isolation of the erythromycin-resistant clones, genomic DNA was extracted from the putative mutants and used as a template in PCR amplifying the insertion site using forward and reverse screening primer pairs (*lspA* F, *lspA* R and *lspB* F, *lspB* R) to confirm the insertion of the intron. As shown in Figure 3.5, PCR products obtained from the mutants were ~1.8 kb larger than in the wild-type indicating that disruption of the *lspA* and *lspB* genes was successful. DNA sequencing of the PCR products amplified from the mutants revealed that the intron was inserted in sense orientation into the desired sites of the *lsp* genes (Figure 3.1.B).

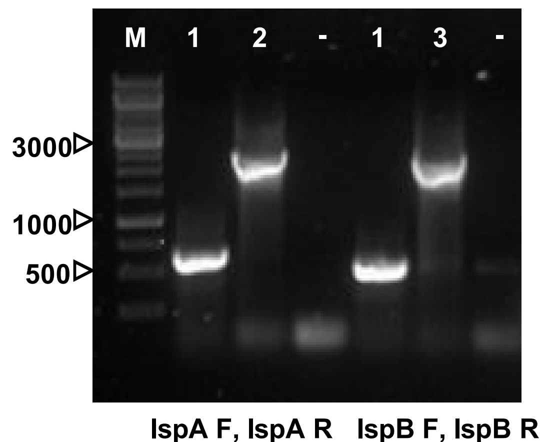


Figure 3.5. Confirmation of the *lspA and lspB* mutants of *C. difficile* 630Δ*erm* by PCR analysis. Primer pairs used for amplification are indicated below the image. Lane M, DNA ladder (bp); Lane 1, Cdi::WT; Lane 2, Cdi::*lspA;* Lane 3, Cdi::*lspB*; -, water (negative) control. Sizes of the PCR products: *lspA* F, R: Cdi::WT, 633 bp, Cdi::*lspA*: 2482 bp; *lspB* F, R: Cdi::WT, 539 bp, Cdi::*lspB*: 2388 bp. Sequencing analysis of PCR products obtained from the mutants confirmed that the group II intron insertion occurred at the expected sites.

3.2.3. Lipoproteins are differentially distributed in the *lgt* mutant of *C. difficile*

To investigate the effects of *lgt* inactivation on the protein profiles of *C. difficile*, membrane fractions likely to contain lipoproteins were isolated from the *C. difficile* wild-type and *lgt* mutant strains grown in BHI liquid medium. Proteins were isolated by carbonate extraction which enriches for the integral membrane proteins and the tightly

attached lipoproteins, while the loosely associated proteins (e.g., ribosomal proteins, elongation factors) are removed from the membranes. Phase partitioning using the non-ionic detergent Triton X-114 extracts lipoproteins into the detergent phase (due to the attached diacylglycerol group) and hydrophilic proteins into the aqueous phase. The extracellular proteome was also analysed since lipoproteins could be shed from the cell surface. Protein fractions isolated from the Cdi::WT, Cdi::*lgt* and complemented Cdi::*lgt* strains were separated by SDS-PAGE. Comparison of the membrane protein profiles showed obvious differences: some protein bands were absent or reduced in the membrane of Cdi::*lgt* (Figure 3.6) suggesting that *lgt* mutation cause a reduction in the membrane protein content of *C. difficile*.

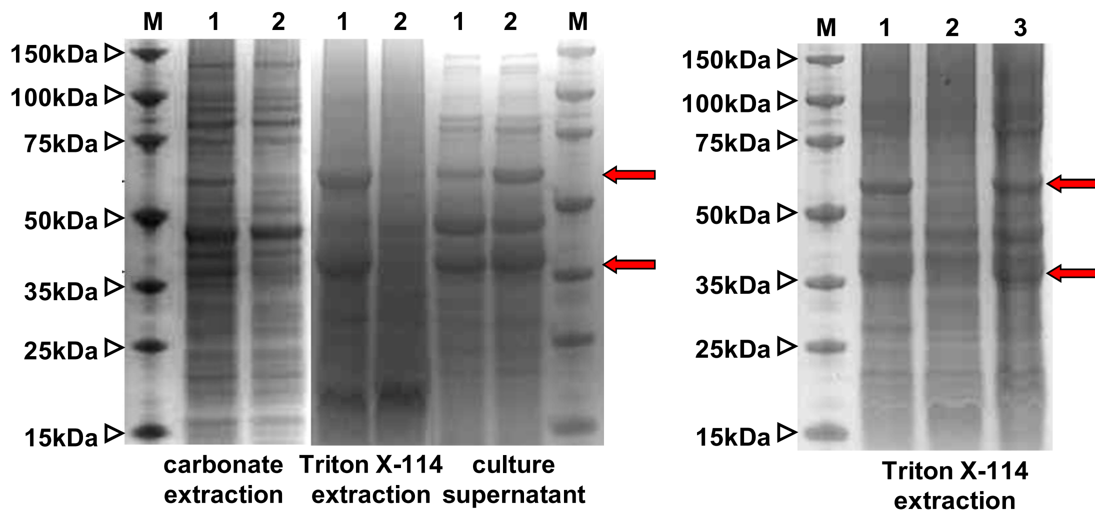


Figure 3.6. Effect of *lgt* mutation on the extracellular and membrane proteome of *C. difficile*. Proteins were isolated by carbonate and Triton X-114 extraction from the bacterial cells and by precipitation from the growth medium of the Cdi::WT (Lane 1) and Cdi::*lgt* (Lane 2) strains. Proteins were extracted by Triton X-114 extraction from the complemented Cdi::*lgt* (Lane 3). Proteins were separated by SDS-PAGE and stained with SimplyBlue™ SafeStain. Lane M, protein ladder. These images show clear differences between the protein profiles of the Cdi::WT and Cdi::*lgt* strains: the intensity of some protein bands are reduced in the membrane extracts of the Cdi::*lgt*, while the intensity of those bands are increased in the culture supernatant of the Cdi::*lgt* relative to the Cdi::WT (indicated by red arrows). The protein profile was restored by complementation.

Triton X-114 extracted proteins of the Cdi::WT and Cdi::*lgt* resolved by 2D gel electrophoresis also indicated that some proteins were missing and the amount of some other proteins was reduced in the Cdi::*lgt* strain (Figure 3.7, indicated by green, yellow, orange, red and blue arrows and oval shape). Interestingly, some proteins were present in the Cdi::*lgt* strain but not in the Cdi::WT strain (indicated by purple, pink, brown and black arrows in Figure 3.7). It might be that these proteins are not lipoproteins, suggesting that inactivation of the *lgt* gene perhaps has an indirect effect on the accumulation of non-lipoproteins in the membrane. Changes in the localisation of non-lipoproteins by Lgt inactivation have also been previously observed in *B. subtilis* (some non-lipoproteins that were not released from the wild-type cells were released from the *lgt* mutant cells) (Antelmann *et al.* 2001).

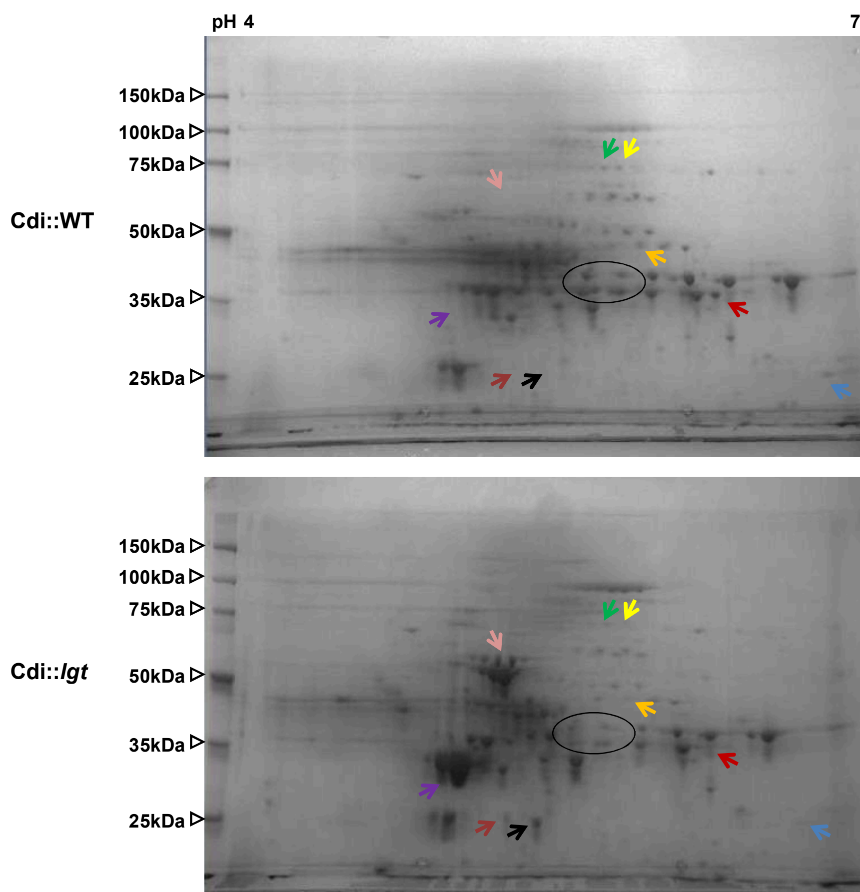


Figure 3.7. Comparison of the detergent phase proteins extracted from the wild-type and *lgt* mutant *C. difficile* by 2DGE. Proteins were extracted by Triton X-114 extraction from the Cdi::WT (top panel) and the Cdi::*lgt* (bottom panel) strains, separated by 2DGE between pH range of 4 and 7 and stained with SimplyBlue™ SafeStain. Arrows indicate the differences between the protein profiles. Arrows outlined with same colours are pointing to corresponding areas on the gels. Green, yellow, orange, red and blue arrows and the oval shape show that some spots are missing or reduced in Cdi::*lgt*. Purple, pink, brown and black arrows show that some spots appeared in the extract of the Cdi::*lgt* which were not present in the Cdi::WT.

1D pattern of the proteins in the culture supernatant was also clearly different in the Cdi::*lgt* strain (Figure 3.6). In contrast to the observations in the membrane extract, the intensity of the protein bands corresponding to the less intense bands in the membrane of the mutant was higher in the culture filtrate of Cdi::*lgt* than in the culture filtrate of Cdi::WT.

In order to show that the observed protein profiles are specific consequences of the mutation of the *lgt* gene, lipoproteome of the complemented Cdi::*lgt* strain was also

analysed. Figure 3.6. shows that complementation restored the protein profile of Cdi::*lgt* in the detergent phase.

To investigate whether the differences in the protein profiles caused by Lgt inactivation are due to an altered lipoproteome, protein fractions of the Cdi::WT and Cdi::*lgt* strains were subjected to mass spectrometric analysis. In addition to the membrane fractions and culture filtrate proteins analysed on 1D gel (Figure 3.6), crude membrane extracts isolated by ultracentrifugation and the detergent phase of the crude membrane extracts using Triton X-114 were also analysed by mass spectromertry. Protein fractions were subjected to tryptic digest and then mass spectrometric analysis using a QTOF 6520 instrument coupled to a 1200 series HPLC-Chip interface system as described in Chapter 2. Peptides of the protein fractions were compared with the known sequences of *C. difficile* 630 using Protein Prospector search engine and Spectrum Mill MS Proteomics Workbench software. The two programmes generated slightly different data sets possibly due to the different algorithms used for peptide identification (Table 3.2).

Table 3.2. Distribution of lipoproteins in the Cdi::WT and Cdi::*lgt* strains. Peptides of tryptic digested protein fractions were loaded onto a QTOF 6520 instrument coupled to a 1200 series HPLC-Chip interface system. Identification of the peptides was carried out by comparing the amino acid sequences with the known amino acid sequences of *C. difficile* 630 strain using Protein Prospector search engine ( ) and Spectrum Mill MS Proteomics Workbench software ( ); lipoproteins detected by both search engines are indicated by ( ). Ult - ultracentrifuged (crude membrane), CE - carbonate extracted, TX Tot - Triton X-114 extracted fraction of total cell lysate, TX Ult - Triton X-114 extracted fraction of ultracentrifuged (crude membrane) fraction, CF - culture filtrate.

	Cdi::WT					**Cdi::*lgt***				
	Ult	**CE**	**TX Tot**	**TX Ult**	**CF**	**Ult**	**CE**	**TX Tot**	**TX Ult**	**CF**
CD0545										
CD0750										
CD0855										
CD0873										
CD0999										
CD1058										
CD1156										
CD1653										
CD1687										
CD1979										
CD2029										
CD2174										
CD2177										
CD2365										
CD2645										
CD2672										
CD2701										
CD2999										
CD3500										
CD3525										
CD3528										
CD3669										

Table 3.2 shows that 21 lipoproteins were detected in the cellular extracts (Ult, CE, TX Tot, TX Ult) of Cdi::WT. In contrast, only 10 of these lipoproteins were present in the Cdi::*lgt* cells (Ult, CE, TX Tot, TX Ult fractions). 10 lipoproteins were found in the culture filtrate (CF) of Cdi::*lgt*, while only three lipoproteins were released into the extracellular milieu from the Cdi::WT cells. These findings clearly indicate that inactivation of the *lgt* gene causes the release of some lipoproteins from the membrane of *C. difficile*. Interestingly, CD1156 was detected exclusively in the culture medium and CD2645 was not detected in the Cdi::WT but was present in the culture filtrate of Cdi::*lgt*. Eight putative lipoproteins (CD0545, CD0750, CD1687, CD2029, CD2701, CD3500, CD3525, CD3528) were present in the carbonate extracted fraction containing wide range of membrane proteins but were not detected in the detergent fraction (Triton X-114 extracted) which is expected to be enriched for lipoproteins. This could be due to that these lipoproteins did not partition in the expected way or they are not true lipoproteins.

To further investigate the effect of *lgt* inactivation on the lipoproteome of *C. difficile*, lipoproteins in the protein extracts were quantified. Progenesis LC-MS software was used for quantification which generated data only for 14 lipoproteins and detected these lipoproteins in all the analysed fractions (Appendix II). This is possibly because of the algorithm applied by Progenesis LC-MS software has different criteria for detection than the Protein Prospector and the Spectrum Mill MS Proteomics Workbench softwares. As the detergent phase is considered to be enriched for lipoproteins, I compared abundances of each protein relative to the abundances measured in the detergent fraction of the total proteins extracted from Cdi::WT. On the basis of the protein profiles and the results of the previous mass spectrometric analyses, amount and/or numbers of lipoproteins are suggested to be decreased in the bacterial cells and

increased in the culture medium by *lgt* inactivation. However, the results obtained using the Progenesis LC-MS software are contradictory with the expected quantities in the case of several proteins. However, quantification of other lipoproteins, such as CD0873 or CD2672 reflects the expected pattern of lipoprotein distribution both within and between the strains.

As the three software packages used for the analysis of the mass spectrometric results generated three different data sets, it was not possible to draw strong conclusions from these results for the effect of *lgt* inactivation on localisation of lipoproteins. However, the results overlapped with the application of all three softwares for some of the lipoproteins (e.g., CD0873, CD2177, CD2672, CD2999, CD3525, CD3669) and showed that the amount of those lipoproteins decreased in the membrane and increased in the culture supernatant of the Cdi::*lgt* relative to those of the Cdi::WT. This is in correspondence with the protein profiles observed on 1D gel and clearly suggest that lipoprotein biosynthesis is disrupted in Cdi::*lgt* and causes the release of some lipoproteins from the bacterial cells.

3.2.4. Investigation of the lipoprotein processing in *C. difficile*

To investigate whether Lgt inactivation abolishes lipidation of proteins, metabolic labelling experiments were performed using a `Click` chemistry technique. Cdi::WT, Cdi::*lgt*, and complemented Cdi::*lgt* strains were grown in the presence of alkyne tagged lipid analogues which are taken up by the bacterial cells and used like normal fatty acids. Following cell lysis, a fluorophore molecule was added to the samples. These molecules attach to the alkyne group of the lipid analogues previously incorporated into the bacterial cells (`click` reaction). Same amount of the recovered

proteins from each strain was separated by SDS-PAGE and in-gel fluorescence was detected (Figure 3.8).

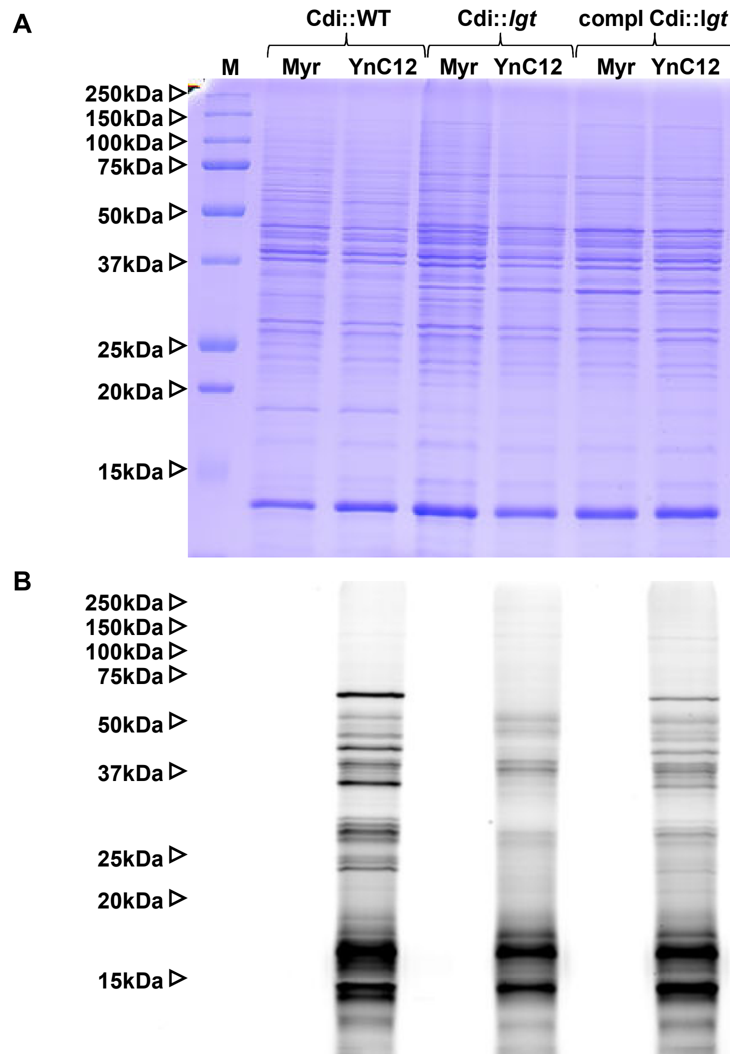


Figure 3.8. Protein lipidation is reduced in the *lgt* mutant *C. difficile*. Total proteins were extracted from the Cdi::WT, Cdi::*lgt*, and complemented Cdi::*lgt* strains grown in the presence of YnC12 (alkyne-tagged myristic acid (Myr) analogue). Lipid analogues incorporated into the bacterial cells were `clicked`, same amount of proteins from each strain was separated by SDS-PAGE followed by Coomassie staining (A) and detecteion of the fluorescence intensity (B). Protein molecular weight marker is indicated on the left. This image shows clear reduction in protein lipidation in the Cdi::*lgt* strain which is partially restored by complementation.

As expected, multiple labelled proteins were clearly evident in the Cdi::WT extracts, whereas these bands were almost absent in the Cdi::*lgt* strain. Although, there were

some bands remaining at low molecular weight, these are suggested to be either lipidated metabolites (e.g. lipoteichoic acids) or products of an uncharacterised lipidation process. To ascertain that the loss of lipidation is the specific consequence of the *lgt* inactivation, protein extracts of the complemented Cdi::*lgt* strain was also analysed. In-gel fluorescence revealed that most of the bands lost in the mutant strain appeared in the complementated strain. However, the intensity of the signal was lower in the complemented Cdi::*lgt* strain than in the parental strain which show only partial restoration of the observed phenotype. These observations demonstrate that Lgt is responsible for lipidation of proteins in *C. difficile*. However, low fluorescence signal intensity in the Cdi::*lgt* suggests that lipidation is not exclusively performed by Lgt.

In order to study the effects of *lgt*, *lspA* and *lspB* mutation on the cleavage of the signal peptide, Western blotting using antibodies recognising a specific lipoprotein was performed. The CD0873 lipoprotein was selected for antibody production because, based on its cellular distribution, CD0873 is most likely a lipoprotein, and it was detected with the highest abundance in all the protein fractions (Appendix II). Immunoblot using the CD0873 antibodies further confirmed lipidation of the CD0873 protein since the protein was detected in the detergent phase enriched for lipoproteins but not in the aqueous phase (Figure 3.9).

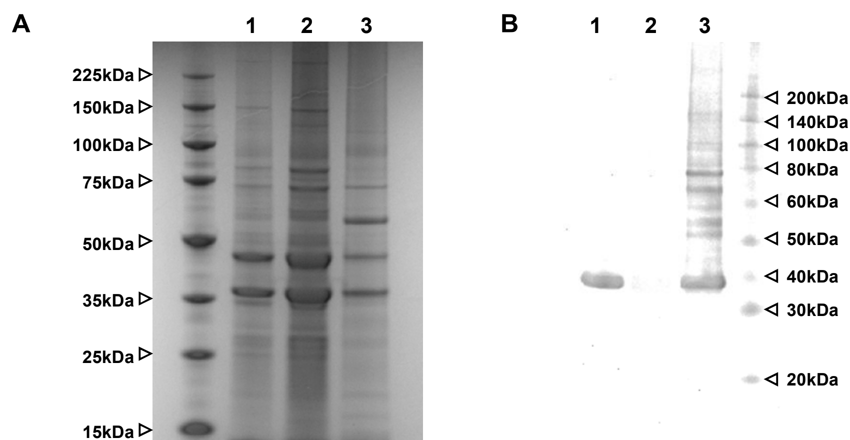


Figure 3.9. Detection of the CD0873 lipoprotein in the hydrophobic fraction of the wild-type *C. difficile* cell lysate. Detergent phase proteins of the Cdi::WT were extracted by Triton X-114 extraction, separated by SDS-PAGE followed by staining with SimplyBlue™ SafeStain (A). Total (Lane 1), aqueous (Lane 2) and detergent (Lane 3) phase proteins of the Cdi::WT were probed with rabbit polyclonal anti-CD0873 antibodies (1:2500) (B). Protein molecular weight markers are indicated on the sides. Western blot analysis indicates that the CD0873 protein partition into the detergent phase suggesting attachment of a hydrophobic lipid group to the protein.

Total cell lysates of the wild-type, mutants (Cdi::*lgt*, Cdi::*lspA*, Cdi::*lspB*) and complemented (complemented Cdi::*lgt*) strains were prepared using a protocol recently described (Fagan & Fairweather 2011) and analysed by Western blotting using CD0873 antisera (Figure 3.10). Lack of the signal when whole cell lysates were blotted with the pre-immune sera confirmed the specificity of the antisera (image is not shown). The recombinant CD0873 protein (with an additional mass of ~2.6 kDa corresponding to the polyhistidine tag) served as a positive control in the Western blots using the immune sera. In the Cdi::WT, a single cross-reacting band of ~37 kDa was seen corresponding to the approximate mass of the lipidated and cleaved protein (no N-terminal signal peptide). In the Cdi::*lgt*, the CD0873 protein has an increased molecular mass. This suggests that the CD0873 lipoprotein is not proteolytically processed in Cdi::*lgt* which might be the consequence of the abolished lipidation of the protein. Moreover, intensity of the detected signal against the CD0873 protein seems to be lower in the Cdi::*lgt* than

in the Cdi::WT, indicating lower amount of the protein in the mutant cells which is in a good agreement with the previous finding of that some lipoproteins are released from the bacterial cells when the *lgt* gene is inactivated.

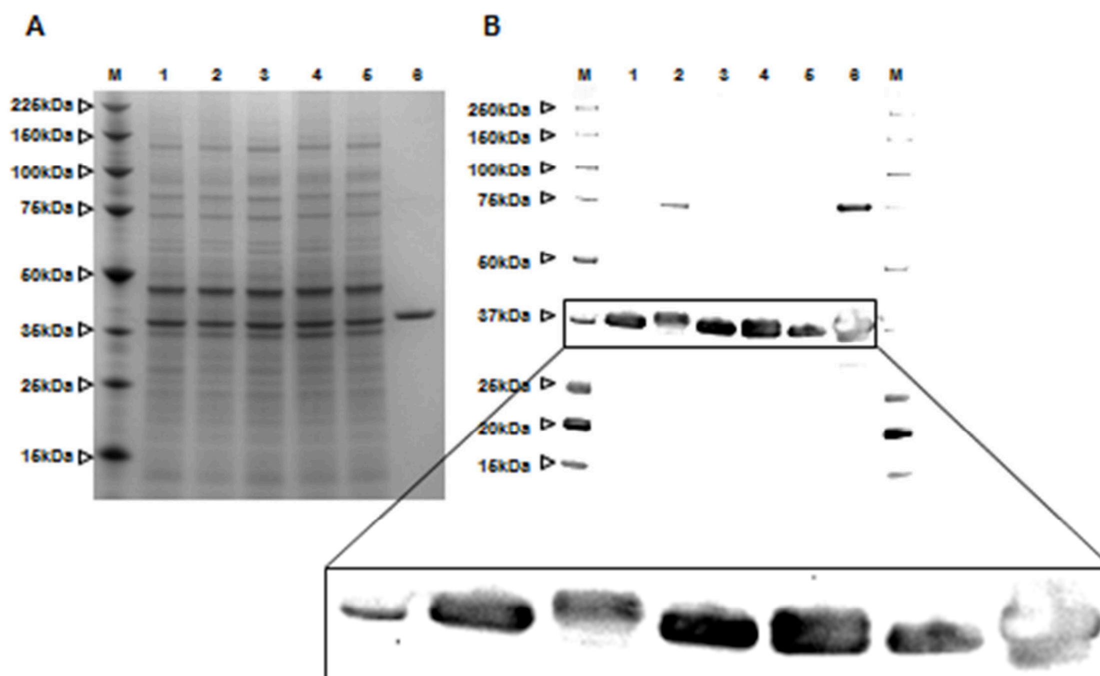


Figure 3.10. Processing of the CD0873 lipoprotein is affected in the *lgt* and *lspA* mutant *C. difficile*. Total proteins were extracted from the Cdi::WT (Lane 1), Cdi::*lgt* (Lane 2), complemented Cdi::*lgt* (Lane 3), Cdi::*lspA* (Lane 4) and Cdi::*lspB* (Lane 5) strains, separated by SDS-PAGE and stained with SimplyBlue™ SafeStain (A). Whole cell lysates were probed with rabbit polyclonal anti-CD0873 antibodies (1:2500) (B). His-tagged recombinant CD0873 protein (Lane 6) served as a positive control for the Western blot. The signal detected at ~75 kDa might represent the dimer form of the protein. Protein molecular weight marker is shown on lanes M. Western blot analysis indicates that only a larger (presumably uncleaved and non-lipidated) form of the CD0873 protein is present in the Cdi::*lgt* and two forms of the CD0873 protein, a larger (presumably uncleaved) and a smaller (presumably cleaved) one, are present in the Cdi::*lspA* strain.

To ascertain that the effects detailed above are mediated by Lgt, processing of the CD0873 protein was examined in the complemented Cdi::*lgt* strain. Using Western blot analysis, the same apparent single band was detected in the complemented Cdi::*lgt* as it was in the Cdi::WT. This shows that it was possible to complement the disrupted processing of the CD0873 protein, indicating that the observed forms of the protein were due solely to the inactivation of the *lgt* gene.

Next, the role of the two putative lipoprotein signal peptidases in the processing of the CD0873 lipoprotein was investigated by immunobloting of whole cell lysates of the Cdi::*lspA* and Cdi::*lspB* strains with CD0873 antibodies (Figure 3.10). In *lsp* mutants, removal of the signal peptide from the N-terminal of lipoproteins is inhibited and bacteria accumulate uncleaved, lipidated lipoproteins in the cell membrane (Sankaran & Wu 1995, Venema *et al.* 2003). In the Cdi::*lspA* strain, two cross-reacting bands were seen on the Western blot. One of the two bands appeared at the same position as the protein observed in the Cdi::WT, whilst the other band was seen at higher molecular mass. The approximately 2-2.5 kDa difference in the size of the two bands likely corresponds to the mass of the signal peptide. This indicates that cleavage of the signal peptide occurred only on some of the protein precursors supporting the prediction of that LspA is a lipoprotein signal peptidase. The larger form of the protein in Cdi::*lspA* also indicates that the CD0873 protein is processed by an alternative signal peptidase. In contrast, in the Cdi::*lspB* strain, where LspA is functional, CD0873 appeared only at the size of its mature form. The single band in Cdi::*lspB* and the double band in Cdi::*lspA* raise the question whether LspB acts as a signal peptidase and addresses two possibilities: existence of signal peptidase(s) other than LspB in *C. difficile* and functionality of LspB under certain conditions.

To further study whether LspA and LspB are responsible for cleaving off the signal peptide from the amino-terminus of lipoproteins, Cdi::WT, Cdi::*lspA* and Cdi::*lspB* were subjected to the `Click` chemistry experiment (Figure 3.11). In Cdi::*lspA*, several protein bands shifted to higher molecular mass indicating that the signal peptide is not cleaved off from those lipoproteins (indicated by purple, yellow, blue, grey, red and black arrows in Figure 3.11). Other bands appeared at the same position in the Cdi::*lspA* strain as in the Cdi::WT strain, however some of those bands were fainter in Cdi::*lspA* and an additional, also fainter band is seen at higher molecular weight

(indicated by brown, green and black arrows in Figure 3.11). This suggests that the signal peptide is removed from some of the lipoproteins while it is still present on other lipoproteins with similar molecular mass. It is also possible that some lipoproteins are only partially processed by LspA which correlates with the results obtained by CD0873 immunoblot. In Cdi::*lspB*, shifting of the protein bands were not clearly seen, but it is apparent that the signal in general was lower relative to the two other strains. This could be due to that lack of LspB disrupts in some way the whole lipidation mechanism.

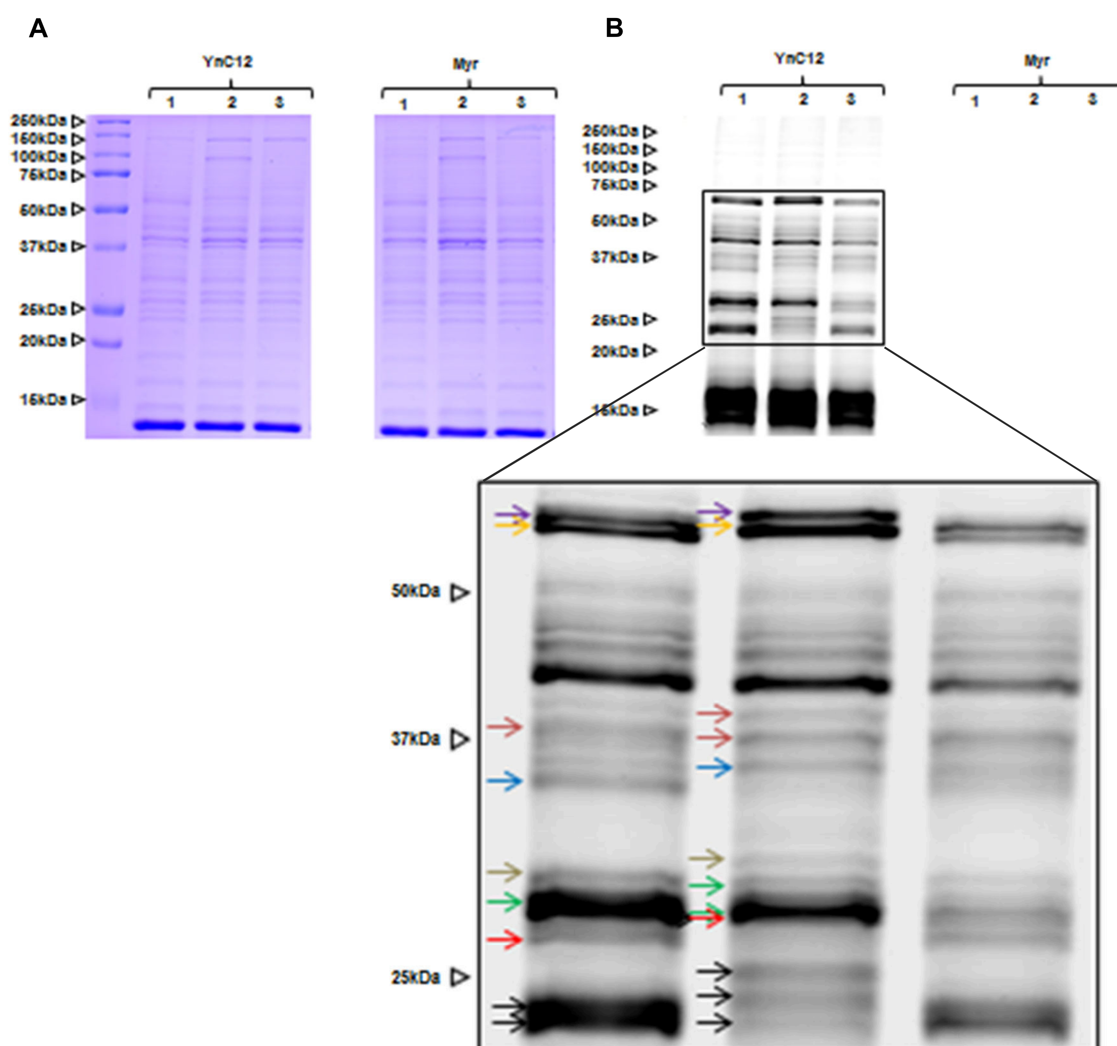


Figure 3.11. Effect of the *lspA* and *lspB* mutations on the cleavage of the lipoprotein signal peptides in *C. difficile*. Total proteins were extracted from the Cdi::WT, Cdi::*lspA* and Cdi::*lspB* strains grown in the presence of YnC12 (alkyne-tagged myristic acid (Myr) analogue). Lipid analogues incorporated into the bacterial cells were `clicked`, same amount of proteins from each strain was separated by SDS-PAGE followed by Coomassie staining (A) and then in-gel fluorescence was detected (B). Protein molecular weight marker is indicated on the left. This image shows that the molecular weight of the majority of lipoproteins is affected by mutation of the *lspA* gene but not by *lspB* mutation. Reduction in the fluorescence signal was detected in Cdi::*lspB*.

Taken together, increased molecular mass of the lipoproteins in Cdi::*lspA* observed in the `Click` chemistry experiment and by Western blot analysis strongly suggest that LspA acts as a lipoprotein signal peptidase in *C. difficile* and responsible for cleaving the signal peptide off from the majority of lipoproteins. However, the role of LspB in lipoprotein processing is not straightforward. LspB appears to have only a few lipoprotein substrates and seems to disrupt the whole lipidation mechanism.

3.3. Discussion

Since lipoproteins play important roles in the physiology and virulence of many pathogens, identification of the lipoproteome may contribute to understanding the pathogenesis of diseases. In this study, 86 putative lipoproteins of the *C. difficile* 630Δ*erm* have been identified by bioinformatics. 22 of these proteins have been detected in the *C. difficile* 630Δ*erm* membrane and extracellular protein extracts, 16 of those in the detergent phase which strongly suggests attachment of a lipid group to those proteins. The reason for that the other predicted lipoproteins have not been detected could be that those proteins may be present in low amount below the detection limit, or expressed either in different growth phases or under certain conditions.

Lipoprotein biosynthetic pathway is an important virulence factor in several pathogens (see Chapter 5). However, no study has so far focused on either lipoprotein processing or its role in virulence of *C. difficile.* Three genes encoding lipoprotein biosynthetic enzymes, *lgt, lspA* and *lspB*, were mutated in this study in order to investigate how lipoprotein localisation and biogenesis is affected when the lipoprotein biosynthetis pathway is disrupted. `Click` chemistry experiments indicated that the majority of lipoproteins in the Cdi::*lgt* strain lack the lipid residue demonstrating that Lgt is responsible for protein lipidation. However, low fluorescence signal in the Cdi::*lgt* strain suggests that Lgt might not be the only enzyme that lipidate proteins in *C.*

difficile. It could be that lipidation is performed by an unidentified Lnt or another lipidating enzyme (e.g., Lgt paralogue). Although, Lnt orthologues are generally absent from Firmicutes (low-GC Gram positive bacteria, such as Clostridia), there is evidence for N-acylation of lipoproteins in low-GC Gram-positive bacteria, such as *B. subtilis* (Hayashi et al. 1985) and *S. aureus* (Navarre et al. 1996, Kurokawa et al. 2009). On the other hand, Lnt orthologues are encoded in several high-GC Gram-positive bacteria, including Streptomyces, Nocardia, Corynebacteria and Mycobacteria. However, Lnt activity of those homologues could not be demonstrated except in *M. smegmatis* (Tschumi et al. 2009).

The release of lipoproteins from the bacterial cell surface could be due to inefficient membrane retention of the acyl chain attached to the protein (shedding). In this study, some lipoproteins were detected in the extracellular medium of Cdi::WT. Similarly, secretome analysis of *B. subtilis* revealed that seven lipoproteins were shed into the culture medium by the wild type (Antelmann *et al.* 2001). Following translocation, lipoproteins are attached to the outer side of the cytoplasmic membrane by their signal peptides (Ichihara *et al.* 1982) which is generally cleaved after lipidation occurrs at the amino terminus of the protein. Therefore, in *lgt* mutants, non-lipidated lipoproteins are anchored to the membrane by the signal peptide, unless Lsp is active on non-lipidated lipoproteins and cleaves the signal peptide. In this case, lipoproteins are no longer attached to the cells (shaving). 1D gel protein profiles and some of the mass spectrometry results indicate that more lipoproteins are released from the surface of *C. difficile* when *lgt* is inactivated. Similar observations have been reported in *B. subtilis* (24 lipoproteins were released from the *lgt* mutant) (Antelmann *et al.* 2001). It suggests that some lipoproteins are shaved from the cells in both species. However, this is only an unproven theory in *C. difficile* which could have been confirmed by determination of

whether lipoproteins are lipidated and whether the signal peptide is present at the amino terminus of the lipoproteins in the culture supernatant of Cdi::*lgt*. Cleavage of the signal peptide from the amino terminus of lipoproteins independently from lipidation has been demonstrated, for example, in *L. monocytogenes, Streptococcus* species and *M. smegmatis* (Baumgartner *et al.* 2007, De Greeff *et al.* 2003, Denham *et al.* 2009, Henneke *et al.* 2008, Tjalsma *et al.* 1999, Tschumi *et al.* 2012).

In contrast to the findings detailed above, Western blot analysis demonstrated that removal of the signal peptide did not occur from the CD0873 lipoprotein in the Cdi::*lgt* strain. This supports the original hypothesis that lipidation is the prerequisite for the activity of Lsp. However, lack of CD0873 lipidation is again only assumed in Cdi::*lgt* and has not been proved. This could have been investigated by CD0873 immunoblot of the detergent and the aqueous phase of the Cdi::*lgt* cell lysate but limitation of time prevented this analysis.

It was suggested in this study that LspA and a signal peptidase(s) other than LspB cleave the signal peptide of the CD0873 lipoprotein, and that lipoproteins are mainly cleaved by LspA. It was also shown that a single lipoprotein can be processed by more than one signal peptidase in *C. difficile* which is/are most likely different from the LspA enzyme in terms of substrate specificity and lipidation dependency. Thus, in Cdi::*lgt*, some non-lipidated lipoproteins are possibly cleaved, while others are not, depending on which enzyme is responsible for processing the protein.

Chapter Four

Immunoreactive lipoproteins of *C. difficile*

4.1. Introduction

Immunogenic proteins of pathogens elicit protective immune responses in the host during infection. Therefore, the identification of immunogenic proteins is of great importance to better understand the pathogenesis of diseases and for the design of prophylactic and therapeutic agents, and to improve diagnostic tests. Several studies have shown that recognition of *C. difficile* toxins by the adaptive immune system plays a major role in the host defence mechanisms in response to CDI (Aronsson *et al.* 1985, Johnson *et al.* 1992, Kyne *et al.* 2000, Mulligan *et al.* 1993, Warny *et al.* 1994). However, not only the toxins, but also the nontoxin surface proteins of *C. difficile* are able to elicit antibody responses. As lipoproteins are important in the adaptive immunity against several pathogens, lipoproteins of *C. difficile* may also be potential targets of the adaptive immune system during CDI.

4.1.1. Toxin and non-toxin antigens of *C. difficile*

Immune responses of the host are crucial in determining the clinical manifestations of diseases. It is believed that eliciting adaptive immune responses during CDI is one of the key factors in conferring protection against *C. difficile* (Aronsson *et al.* 1985, Johnson *et al.* 1992, Mulligan *et al.* 1993). It has been reported that both local (mucosal) and systemic immune responses are developed by the host to *C. difficile* toxins during infection (Aronsson *et al.* 1983, Johnson *et al.* 1992) and these responses involve both binding and neutralizing antibodies (Aronsson *et al.* 1985, Johnson *et al.* 1995). However, it is considered that antibodies against non-toxin antigens of *C. difficile* are also produced during CDI, and it is suggested that defence mechanisms triggered when antibodies bind to non-toxin antigens are as important as those of to the toxins (Drudy *et al.* 2004, Pechine *et al.* 2007). Pantosti *et al.*. (1989) reported that surface proteins of *C. difficile* are recognized by human IgG antibodies (Pantosti *et al.* 1989). Several other

authors have studied the immune responses directed to non-toxic antigens. Pechine *et al.*. (2005) examined the production of antibodies against surface-associated virulence factors and found that many CDI patients developed antibodies to FliC, FliD, Cwp66 and Cwp84 (Pechine *et al.* 2005). Drudy *et al.*. (2004) and Sanchez-Hurtado *et al.*. (2008) have demonstrated that IgA and IgM antibodies against *C. difficile* surface-layer proteins (SLPs) were present in human serum as shown by their reactivity with patient sera (Drudy *et al.* 2004, Sanchez-Hurtado *et al.* 2008).

4.1.2. Lipoprotein antigens of bacterial pathogens

Several lipoproteins of pathogenic bacteria elicit immune responses during infection and play important roles in the host defence mechanisms. For example, LpqH (19kDa antigen) is one of the most studied lipoprotein antigens of *M. tuberculosis* (Grange 2000). PstS-1 (38-kDa antigen) is also a lipoprotein that induces strong antibody responses during tuberculosis (Abebe *et al.* 2007). In *S. pneumoniae*, the multifuncional PsaA lipoprotein is considered to be a major antigen and is a promising candidate for vaccine development (Rajam *et al.* 2008). Lipoproteins PiaA and PiuA of *S. pneumoniae* have also been reported to be immunogenic (Jomaa et al. 2005, Palaniappan et al. 2005). In addition, at least two lipoproteins of *B. burgdorferi* (VlsE, Osp) are involved in eliciting adaptive immune responses during Lyme disease (Bankhead & Chaconas 2007, Edelman *et al.* 1999). Lipoproteins of *S. aureus* are also recognised by the adaptive immune system as evaluated by their reactivity with sera collected from rabbits previously infected with the pathogen (Brady *et al.* 2006). Furthermore, the immunogenic lipoprotein GNA1870 of *N. meningitidis* is currently in Phase III clinical trials as a vaccine component (Giuliani *et al.* 2006, Mascioni *et al.* 2009, Masignani *et al.* 2003).

4.1.3. Aim of this study

To better understand antibody-mediated immune responses to *C. difficile*, this study aimed to determine the antibody reactivity of *C. difficile* lipoproteins using hamster sera, and human sera taken from healthy individuals and from patients with CDI.

4.2. Results

4.2.1. Hamster antibody responses to *C. difficile* proteins

The Golden Syrian hamster is considered the most relevant small animal model of CDI as it resembles the infection in humans (e.g., bacterial colonisation and sporulation, diarrhoea, histological damage). However, hamsters are very sensitive to CDI and infection results in death in few days (Razaq *et al.* 2007). In order to determine the immuno-reactivity of membrane proteins and ultimately the immuno-reactivity of lipoproteins of *C. difficile*, hamster serum samples were prepared. Hamster sera were provided by Gill Douce from the University of Glasgow. Six animals were vaccinated with the C-terminal domain of both the toxin A and toxin B in order to prolong the time to death and allow enough time for the immune system to develop a strong immune responses. Animals were then challenged with spores of the *C. difficile* 630Δ*erm* strain. Hamsters survived up to 14 days post-challenge and sera were collected at that point. Colonisation by *C. difficile* was confirmed at the time of sera collection. Control serum samples were collected from the hamsters prior to the infection. Serum samples were pooled by group and used in Western blot analysis in order to determine whether serum IgG antibodies were produced against *C. difficile*, and in subsequent experiments, to identify immunogenic lipoproteins during CDI of hamsters.

Whole cell lysates and crude membrane extracts of *C. difficile* 630Δ*erm* were isolated and probed with naive and convalescent hamster sera (Figure 4.1). Serum samples collected from the infected animals reacted with some high-molecular weight proteins

which are presumably S-layer protein (SLP) complexes. Immuno-reactivity of SLPs has previously been shown in humans (Wright *et al.* 2008). Although, the signal was very weak, some other proteins in the lower molecular weight range also reacted with the antisera. However, those proteins were recognised by the control uninfected sera, too, which suggests unspecific binding of the convalescent sera to those protens.

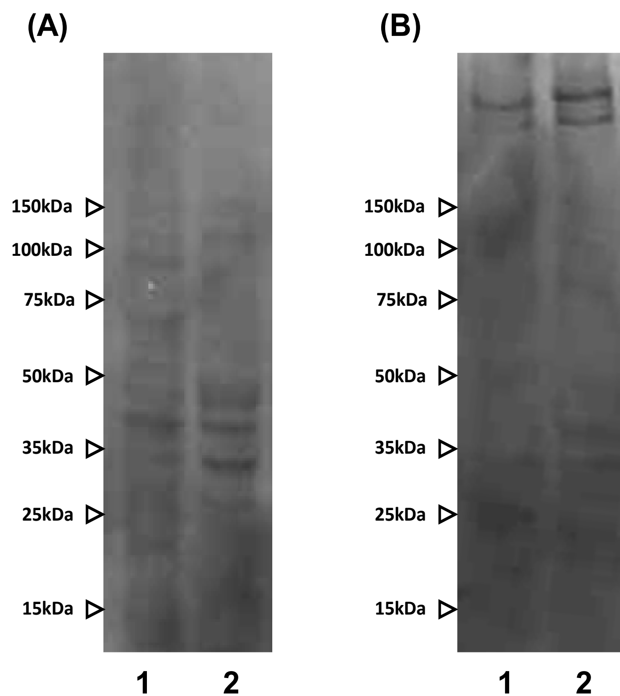


Figure 4.1. Immunoblot of *C. difficile* proteins using hamster sera. Total proteins were extracted from the wild-type *C. difficile* (Lane 2) and fractionated by ultracentrifugation to obtain the crude membrane extract (Lane 1). Extracted proteins were then probed with naive hamster sera (A) and with sera collected from hamsters infected with *C. difficile* spores (B) to see the proteins recognised by hamster IgG antibodies produced during CDI.

Regrettably, blots could not be improved to reduce the high background and better visualize the recognition signal. Therefore, apart from the high molecular weight protein complexes, specific recognition of *C. difficile* proteins by convalescent hamster sera could not be observed and no conclusions could be drawn from these experiments.

4.2.2. Building of a protein microarray

To further investigate the immuno-reactivity of *C. difficile* lipoproteins, a protein microarray was used. Eight histidine-tagged recombinant lipoproteins were prepared by myself and nine lipoproteins were purified by Novartis Vaccines and Diagnostics, Siena, Italy. Lipoproteins for recombinant protein production were selected from the lipoproteins detected by mass spectrometry in the membrane fractions of *C. difficile* (Chapter 3) and by reverse vaccinology. Expression clones constructed by myself were generated by PCR and ligation-independent cloning (LIC) into pNIC28-BsaI vector (Figure 4.2) (Stols *et al.* 2002) as described in Chapter 2 (section 2.3.9).

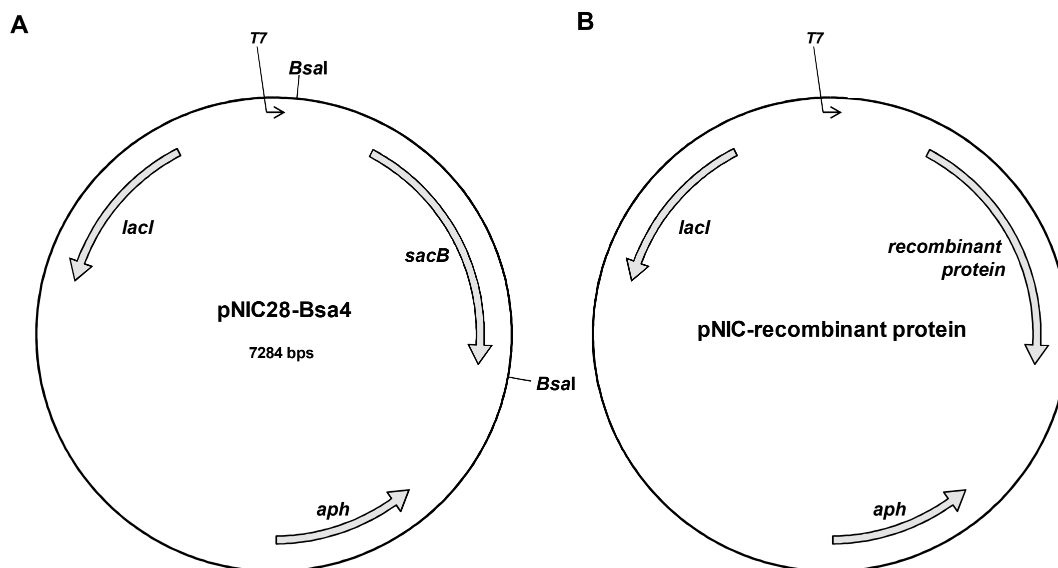


Figure 4.2. Map of the pNIC28-Bsa4 LIC vector. (A) Plasmid pNIC28-Bsa4. (B) The pNIC28-Bsa4 plasmid encompassing genes of protein for recombinant protein production. The pNIC28-Bsa4 vector incorporates an N-terminal His(6x)-tag in a 22-amino acid fusion peptide followed by a TEV (Tobacco Etch Virus) protease recognition site and a *BsaI* restriction site used for LIC. SacB converts sucrose to a toxic product allowing negative selection for the plasmid on plates containing 5 % sucrose. SacB, negative selection gene from *B. subtilis*; Aph, kanamycin resistance protein from *Corynebacterium diphtheria*; LacI, lac repressor; T7, promoter.

Briefly, *C. difficile* 630 lipoproteins were amplified using gene specific primers which were engineered according to the ligation independent cloning protocol (Stols *et al.* 2002). In order to facilitate solubility of the recombinant protein, the amplicon lacked the sequence of the N-terminal leader peptide and the invariant cysteine in the lipobox. All PCR reactions were confirmed by gel electrophoresis for correct insert size prior to cloning into pNIC-28-Bsa4 (Figure 4.3).

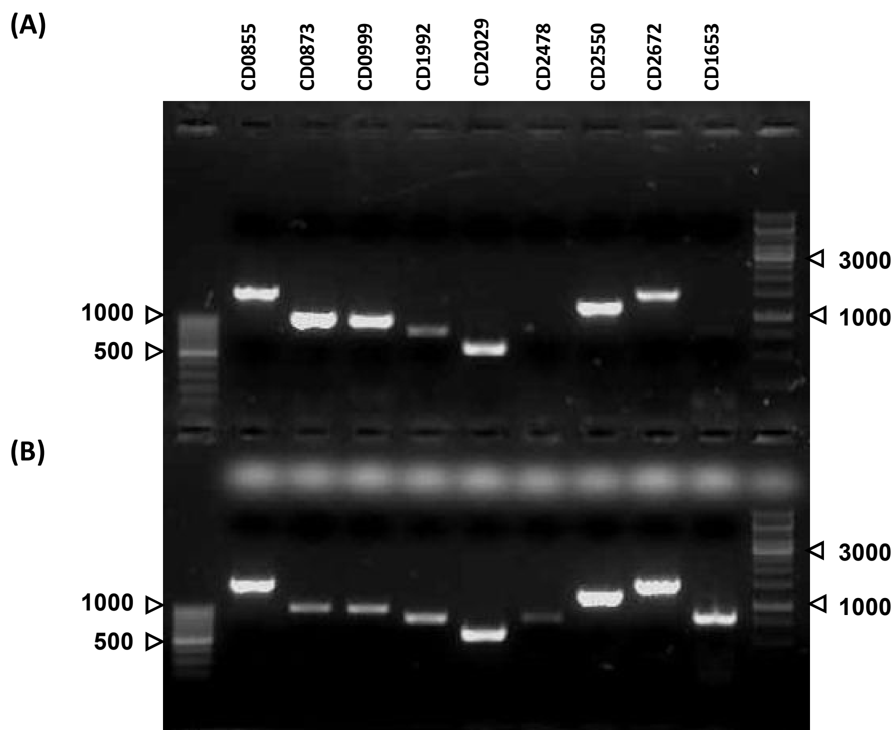


Figure 4.3. PCR amplification of the genes of *C. difficile* lipoproteins for recombinant protein production. Genomic DNA of the *C. difficile* 630Δ*erm* strain was extracted and used as template for amplification of the selected ORFs using gene-specific primers that were engineered according to the protocol of ligation independent cloning into pNIC28-Bsa4. PCR was performed by ramp programs between temperature range of (A) 43-53°C and (B) 53-63°C. Molecular weight standards are indicated on the sides in bps. Sizes of the PCR products: CD0855, 1539 bp; CD0873, 984 bp; CD0999, 966 bp; CD1992, 813 bp; CD2029, 591 bp; CD2478, 813 bp; CD2550, 1239 bp; CD2672, 1527 bp; CD1653, 765 bp.

PCR products and the pNIC28-Bsa4 vector previously digested with *BsaI* were treated with T4 DNA polymerase in the presence of dCTP or dGTP, respectively to generate cohesive ends prior to the ligation. Ligation reactions were transformed into *E. coli* C43(DE3) (Miroux & Walker 1996), the resultant plasmids were purified from positive transformant clones (ligation of the CD2478 gene into the pNIC28-Bsa4 vector was not successful) and inserts were completely sequenced on both strands using LIC F and LIC R primer pairs to ensure against the accidental introduction of mutations during the cloning processes.

Expression of the histidine-tagged recombinant proteins were induced by IPTG and the proteins were purified using a histidine-binding column (Figure 4.4). Two out of the eight recombinant lipoproteins (CD0999 and CD2550) I aimed to purify were not expressed of which investigation was not progressed further. In addition, purification of the CD1992 protein was also not possible since the protein was not expressed after resuscitating the *E. coli* strain from the freezer stock.

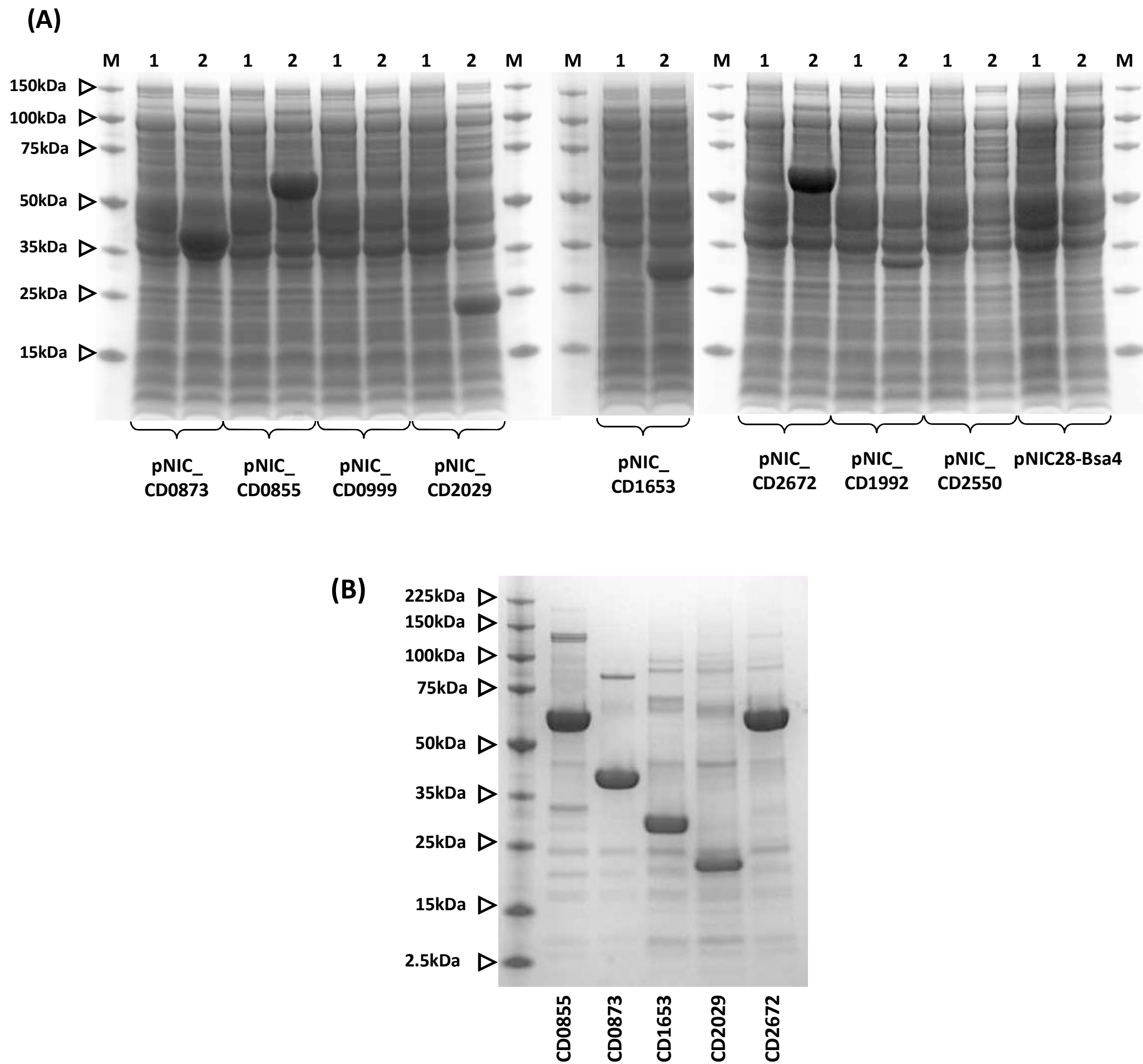


Figure 4.4. Expression and purification of the recombinant lipoproteins of *C. difficile* in *E. coli*. Expression of the recombinant proteins from pNIC28-Bsa4 constructs was carried out in *E. coli* C43(DE3) by IPTG induction (0.5 mM). *E. coli* C43(DE3) cell lysates were prepared, proteins in the soluble fractions were separated by SDS-PAGE and stained with SimplyBlue™ SafeStain (A). Lanes: 1, uninduced control; 2, protein expression induced with IPTG; M, protein molecular weight (Mw) marker. Histidine-tagged recombinant proteins were purified by loading the *E. coli* C43(DE3) lystes onto a His-binding column. After exchanging the buffer to PBS, purified histidine-tagged recombinant proteins were subjected to SDS-PAGE and stained with SimplyBlue™ SafeStain (B) to see if the purification was successful. Lane M, protein molecular weight marker. Mw of the expressed proteins: CD0873, 36 kDa; CD0855, 58.5 kDa; CD0999, 36 kDa; CD2029, 23 kDa; CD1653, 30 kDa; CD2672, 58 kDa; CD1992, 33 kDa; CD2550, 47 kDa.

Finally, 14 recombinant lipoproteins of *C. difficile* (five – CD0855, CD0873, CD1653, CD2029, CD2672 – by myself and nine – CD0300, CD0440, CD0999, CD1119, CD1131, CD1774, CD2177, CD2645, CD2365 – by Novartis Vaccines and Diagnostics, Siena, Italy) were purified in order to assess their reactivity with human sera using a protein microarray.

Purified recombinant proteins along with the experimental controls (purified N-terminal histidine tag without fusion protein, PBS and serial dilutions of purified human IgGs and BSA-Cy5) were printed onto nitrocellulose coated glass slides at Novartis by Manuele Biazzo. Protein probing was carried out at Novartis by Manuele and myself.

4.2.3. Serum samples and experimental controls of the protein microarray

Human sera from seven CDI patients (4 male and 3 females) aged 71 - 88 years were obtained from Derriford Hospital, Plymouth (Table 4.1). Diagnosis of the *C. difficile* disease was confirmed by various diagnostic tests with supportive clinical symptoms (e.g., diarrhoea). Serum samples were collected at different times after treatment of the disease started with administration of metronidazole (Table 4.2). Sera from seven age matched (71 - 88 years) individuals (4 male and 3 females) without exhibiting symptoms of CDI were provided by Royal Devon & Exeter Hospital (Table 4.3). However, the lack of *C. difficile* colonisation was not confirmed in these patients.

Table 4.1. CDI patient information.

Patients	Age	Sex	Hospital administration (days before MTZ treatment started)	Diarrhoea started (days before MTZ treatment started)	End of diarrhoea (days after MTZ treatment started)	Length of diarrhoea	Hospital discharge (days after end of diarrhoea)	Total hospital stay (days)	Diagnostics (*Clostridium difficile*)			
									GDH	toxin PCR	Ribotype	Note
A	87	M	9	9	8	17	36	53	+	+	Type 070	
B	85	M	0	1	38	39	12	50	ND	ND	Type 072	
C	77	M	0	5	8	13	7	15	+	+	Type 020	
D	79	F	1	1	6	7	7	14	+	+	ND	Toxin negative
E	88	F	ND	ND		ND	ND	ND	+	+	Type 002	
F	88	F	22	22	3	25	20	45	+	+	Type 014	
G	71	M	41	2	8	10	2	51	+	+	Type 016	

M = Male
F = Female
MTZ = metronidazole
GDH = Glutamate dehydrogenase
ND = no data

Table 4.2. Details of the serum samples collected from the CDI patients. Numbers refer to the numbers of days passed after metronidazole treatment started and indicate the time of sample collection (Day 1 = metronidazole treatment started). Letters indicate patients listed in Table 4.1.

Days												
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**30**
	A2					**A7**					**A12**	**A30**
	B2		**B4**									
	C2	**C3**	**C4**									
		D3	**D4**			**D7**						
	E2											
			F4	**F5**								
	G2	**G3**							**G10**	**G11**		

Table 4.3. Details of the control group patients (asymptomatic for CDI).

Patient ID	Age	Sex
1	71	M
2	77	M
3	88	M
4	84	M
5	87	F
6	86	F
7	83	F

M = Male
F = Female

The histidine tag, attached to the N-terminal of the recombinant proteins, was expressed on its own, purified, probed with the serum samples and the mean fluorescence intensity (MFI) was measured (Figure 4.5). MFI measured with the probed histidine-tagged recombinant proteins were normalised to that of the purified histidine-tag with each serum. Normalisation was considered to be accurate if the measured MFI for the tag was between 50 and 1000. The detected MFI was in the accepted range when serum samples were used 1:200 dilution, except with the A2, A7, A12, A30 serum samples where this dilution gave higher values for the His tag (MFI of 2102 – 2618). Therefore,

these serum samples were used at a higher dilution to decrease the signal (1:1,500). However, seroreactivity of the A12 serum was still slightly above the upper threshold when used at 1:1,500 dilution.

Human IgG and BSA conjugated to a fluorophore molecule (BSA-Cy5) were also printed onto the nitrocellulose membranes and served as experimental controls. Dose-dependent signal was observed with serial dilutions of these positive controls indicating specific human IgG recognition and specific signal detection (data not shown). MFIs detected when the recombinant proteins were probed with anti-human IgG secondary antibodies only were also in the accepted low range (MFIs between 12 and 131).

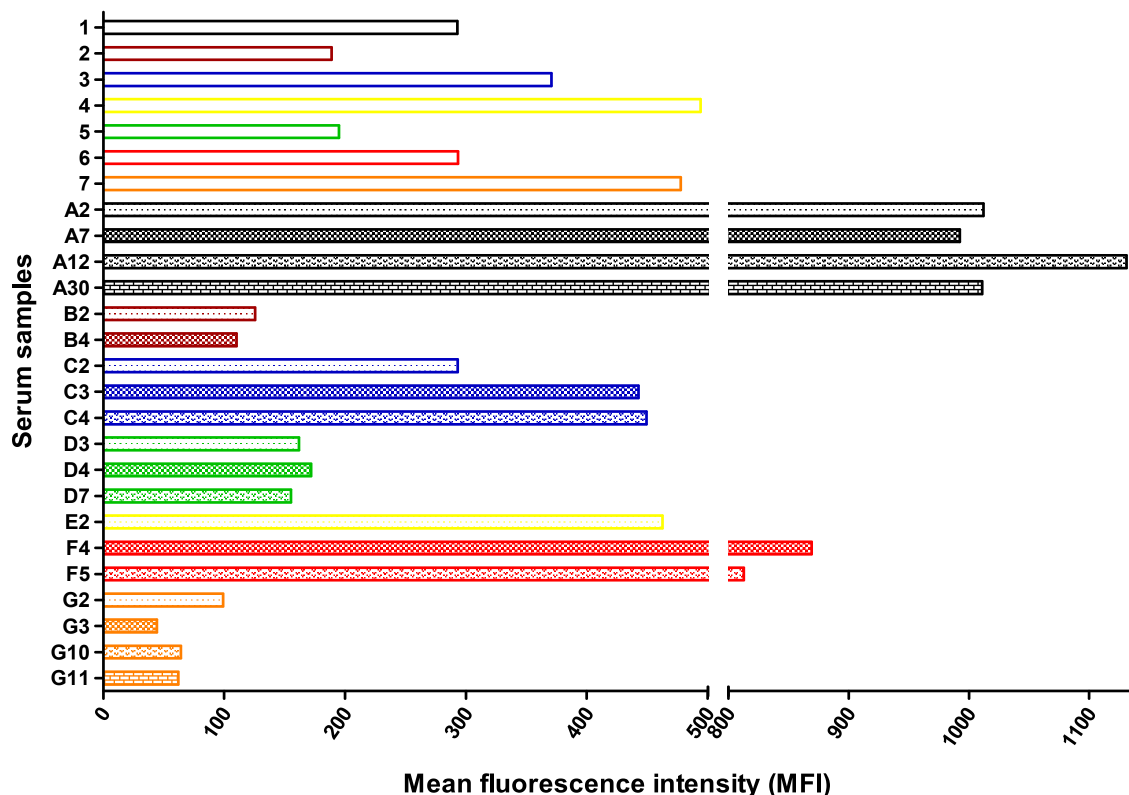


Figure 4.5. IgG seroreactivity of the purified histidine tag with human serum samples. IgG seroreactivity of the proteins of interest was normalized to the MFI signal detected with the purified His-tag when probed with the serum samples. MFI detected for the His-tag is recommended to be used for normalization only if it falls between 50 and 1000. His-tag was probed with serum samples diluted 1:200 except with the `A` samples which were diluted 1:1,500. This graph shows that the seroreactivity of the His-tag was in the accepted range when probed with the serum samples in the dilutions described above, except with the A12 sample, of which reactivity was slightly above the upper threshold.

4.2.4. Human antibody responses to *C. difficile* lipoproteins

In order to determine the IgG seroreactivity of 14 lipoproteins of *C. difficile*, recombinant proteins printed onto nitrocellulose membrane were probed with serum samples obtained from patients known to have been infected with *C. difficile* and from asymptomatic control patients. Proteins with seroreactivity five times or more greater than that of the purified histidine tag were considered reactive. As it is shown in Figure 4.6. and Table 4.4, eight of the proteins were recognized by human sera collected from the CDI patient group (CD0999, CD1119, CD1131, CD1774, CD2177, CD2645, CD0855, CD2672). Control serum samples recognized seven lipoproteins (CD0300, CD0999, CD1119, CD2177, CD2645, CD0855, CD2672). Two lipoproteins (CD1131, CD1774) reacted only with the sera from CDI patients and one lipoprotein (CD0300) was recognized exclusively by control sera. Five lipoproteins (CD0440, CD2365, CD0873, CD1653, CD2029) were not recognised by either the convalescent or control sera, and five lipoproteins (CD0999, CD1119, CD2177, CD2645, CD0855, CD2672) were seroreactive with sera of both groups of patients. CD2645 was highly seroreactive (seroreactivity greater than 15 times of the purified histidine tag) with almost all the tested serum samples. CD2672 and CD2177 was highly reactive with some of the sera.

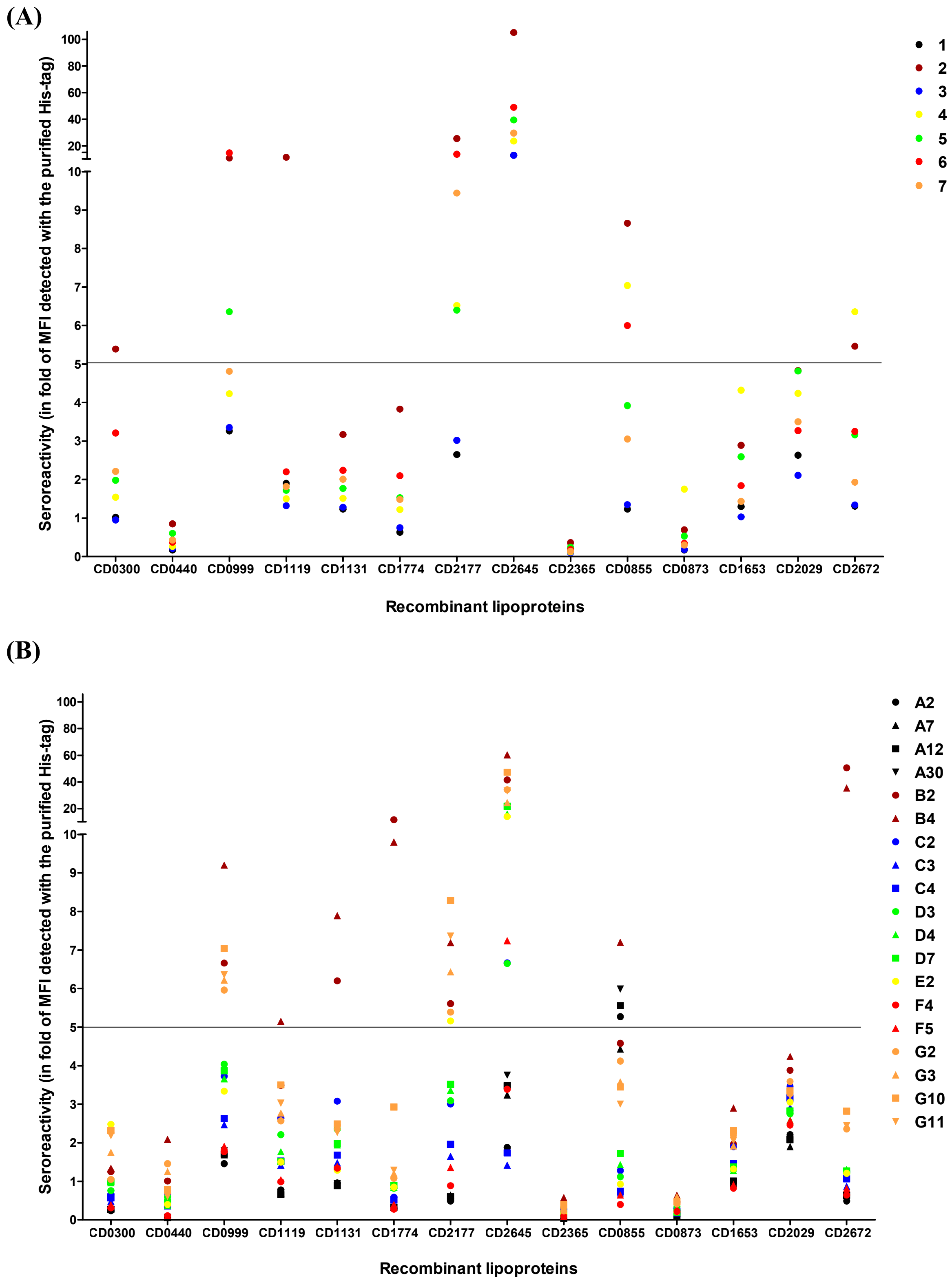


Figure 4.6. IgG seroreactivity of the recombinant lipoproteins of *C. difficile* with human serum samples. Recognition of the recombinant proteins by the serum samples was determined using protein microarray. Histidine tagged recombinant proteins were probed with serum samples collected from a group of patients with no history of CDAD (A) and from a CDI patient (B). The signal (MFI) detected with each sera was normalized to the signal detected with the purified tag (see Figure 4.5). Graphs show seroreactivity of the proteins in fold of the values detected for the purified histidine tag with each serum samples. Proteins were considered seroreactive if the detected MFI signal was five times or more higher than the MFI signal detected for the purified histidine tag. Serum samples are indicated on the right.

Table 4.4 shows the number of patients whose serum sample(s) recognised the recombinant proteins. CD2645 was immunoreactive with sera of most of the patients (13 out of 14 patients). Serum samples of the different patients reacted differently with the different proteins. For example, some proteins were recognised by sera of more patients (e.g., CD2177 by eight, CD0855 and CD0999 by five, CD2672 by three and CD1119 by two patients), others by only one patient (CD1774, CD1131 and CD0300), while five proteins (CD0440, CD2365, CD0873, CD1653, CD2029) were recognised by none of the patient sera. This indicates that the IgG antibody repertoire of the individuals was not consistent. When MFIs measured with each serum were averaged by group and compared, intensities were generally higher in the asymptomatic group than in the CDAD group (Table 4.4).

Table 4.4. Recognition of the recombinant lipoproteins by human sera (summary). Left panel: Figures indicate the number of individuals whose serum sample(s) positively reacted with the recombinant proteins. Right panel: Average seroreactivity of the recombinant proteins (in fold of the MFI detected with the purified His-tag) by patient groups. Values are mean numbers ± standard errors. Average values which indicate positive seroreactivity (five times or more greater than that of the purified histidine tag) are marked with red.

	Recognition by individuals		**Average seroreactivity**	
	Control group	**CDI patient group**	**Control sera**	**Convalescent sera**
CD0300	1	0	2.32 ± 0.58	1.21 ± 0.33
CD0440	0	0	0.41 ± 0.09	0.77 ± 0.28
CD0999	3	2	6.77 ± 1.64	4.43 ± 1.03
CD1119	1	1	3.12 ± 1.38	2.40 ± 0.58
CD1131	0	1	1.88 ± 0.25	2.77 ± 0.89
CD1774	0	1	1.64 ± 0.40	2.51 ± 1.55
CD2177	5	3	9.5 ± 3	4.16 ± 1.08
CD2645	7	6	38.92 ± 12.12	22.99 ± 8.37
CD2365	0	0	0.18 ± 0.03	0.27 ± 0.07
CD0855	3	2	4.46 ± 1.08	3.12 ± 1.00
CD0873	0	0	0.56 ± 0.20	0.35 ± 0.07
CD1653	0	0	2.2 ± 0.43	1.67 ± 0.27
CD2029	0	0	3.62 ± 0.39	3.13 ± 0.25
CD2672	2	1	3.25 ± 0.75	8.37 ± 7.04

Following the dynamics of the antibody reactivity of the serum samples collected at different times during the course of the disease allows monitoring the level of the antibody production to the antigens. IgG profiles of the serum samples collected at various times from six CDI patients were compared in each individual. While antigen profiles of the serum samples collected from the same individuals were only slightly different, reactivity of most of the recognised proteins increased with the sera obtained from four patients (B, D, G and F) at later time points of the disease (Figure 4.6.B). This suggests that more IgG was produced in these patients as the disease progressed further. In the case of the G patient, lower reactivity was measured with the G10 sample relative to the G11 sample (collected on day 10 and 11, respectively) with some of the proteins, which is possibly the result of the metronidazole treatment. This suggests that *C. difficile* started to clear from the gut which is further supported by the lack of the diarrhoea at that time point (Table 4.1).

4.3. Discussion

In this study, IgG antibody recognition of 14 *C. difficile* putative lipoproteins was investigated using convalescent human sera and sera collected from individuals with no history of the disease caused by *C. difficile*. Nine lipoproteins were recognised by human sera, and lipoproteins which are differentially reactive with sera of healthy and CDI patients were identified. Lipoprotein CD0873 has previously been reported to react with a mixture of IgG, IgA and IgM antibodies (Wright *et al.* 2008) but it was not reactive with IgG in the serum samples used in this study. However, CD2672 was found to be immunoreactive in both studies.

Several serum samples from both groups were highly reactive with the CD2645, CD2672 and CD2177 proteins suggesting that high levels of antibodies to these proteins were present in those serum samples. This is indicative of that these lipoproteins may

have a role in eliciting adaptive immune responses in the host during CDI. CD2645 and CD2177 were recognized by sera of several patients (13 and eight, respectively). Variability in the antibody repertoire might reflect the differences in the antigen overlap of the strain(s) that have colonised the individuals at the time of serum collection (symptomatic or asymptomatic colonisation) and/or in the past. As several different *C. difficile* strains infected the CDI patients we obtained sera from (Table 4.1) and presumably the control group patients, recognition of CD2645 and CD2177 by a range of patient sera suggests that these proteins are expressed in a number of *C. difficile* strains. Furthermore, immunoreactivity of CD2645 and CD2177 increased during the course of the disease with serum samples of four and two patients, respectively, which probably mirrors the development of the disease (increasing level of *C. difficile* colonisation, causing more damage to the intestinal epithelium, is likely to increase the production of antibodies to these antigens). Therefore, CD2645 and CD2177 may be useful as new diagnostic markers since the level of antibodies to *C. difficile* in asymptomatic carriers might not change as rapidly as in symptomatic patients. As the existing tools for the diagnosis of CDI are not always reliable and easy to perform (see section 1.3.4), development of a quick and more reliable method would be of a value and the recombinant protein-based diagnostics, which are designed on the basis of seroreactive antigens, would provide such a test.

Serum antibodies to *C. difficile* toxins are found in 60 % of the population (Kelly 1996) as the majority of the individuals encounter *C. difficile* for at least a short period of time during their lifetime. In accordance with this fact, seven lipoproteins were recognised by control sera, indicating production of antibodies against *C. difficile* in the asymptomic group. 3 to 4 % of the adult population are asymptomatic *C. difficile* carriers and this proportion is increasing by age and the time spent in hospital (McFarland *et al.* 1989).

As the serum samples used in this study were obtained from hospitalised elderly, the likelihood of that they are asymptomatic carriers is higher (see page 6).

Several other studies have investigated the immunoreactivity of *C. difficile* proteins and found that the immune system of asymptomatic patients produced antibodies against *C. difficile* proteins and in most of the cases, antibody levels were higher in the healthy individuals than in the CDI patients which may confer protection against the disease (Kelly 1996, Kyne *et al.* 2000, Mulligan *et al.* 1993, Pechine *et al.* 2005, Sanchez-Hurtado *et al.* 2008). Similarly in this study, the intensity of recognition by IgG was generally higher in the asymptomatic group than in the patients with CDI suggesting higher serum antibody levels in the healthy group.

Chapter Five

Role of the Lipoprotein diacylglycerol transferase in the virulence of *C. difficile*

5.1. Introduction

As in many bacterial species, Lgt inactivation in *C. difficile* has an effect on the lipoproteome mainly by causing the release of some lipoproteins from the cytoplasmic membrane. Therefore, disruption of the lipoprotein biosynthetic pathway by Lgt inactivation is a powerful strategy to assess the global contribution of lipoproteins to cellular physiology and virulence. Phenotypes caused by mutation of the *lgt* gene vary between species because lipoproteins have different functions and Lgt inactivation has different effects on the localisation and activity of lipoproteins in the bacterial species.

5.1.1. Lipoprotein processing and virulence

As described in Chapter 3, lipoprotein processing is essential for the viability of Gram-negative bacteria since lipoproteins play key roles in the biogenesis of the Gram-negative bacterial outer membrane. Unprocessed lipoproteins accumulate in the inner membrane and consequently, incorrect outer membrane assembly leads to cell death (Robichon *et al.* 2005, Tokuda 2009). In contrast, Lgt and Lsp are dispensable for the viability of most of the Gram-positive bacteria, even if the bacterium possesses lipoproteins that are essential for viabililty. The reason for this might be that some lipoprotein precursors resemble the mature lipoproteins and are able to fulfil, at least partly, their roles (Venema *et al.* 2003, Widdick *et al.* 2011). This is supported by the results obtained with the PrsA lipoprotein of *B. subtilis*. PrsA is an extracellular chaperone and plays an important role in protein secretion (Jacobs *et al.* 1993). PrsA is essential for the growth of *B. subtilis* (Kontinen & Sarvas 1993), while deletion of the *lgt* gene did not affect viability of the bacteria, although it resulted in impaired protein secretion (Leskela *et al.* 1999). However, there is a report for the essentiality of Lgt for the viability of the Gram-positive *S. coelicolor* (Thompson *et al.* 2010).

Although, Lgt and Lsp are dispensable for the viability of most of the Gram-positive bacteria, lipoprotein processing by Lgt and Lsp is necessary for the virulence of many Gram-positive pathogens (Mei *et al.* 1997, Tidhar *et al.* 2009). For example, a *S. pneumoniae lgt* mutant was highly attenuated in a mouse infection model (Petit *et al.* 2001), while disruption of *lgt* resulted in moderately attenuated virulence of *Streptococcus equi*; only 3 out of 30 mice challenged with the *lgt* mutant exhibited signs of the disease (Hamilton *et al.* 2006). In contrast, inactivation of Lsp did not affect virulence of *Streptococcus suis* (De Greeff *et al.* 2003). However, virulence of an *lspA* mutant of *M. tuberculosis* was markedly attenuated in both cell and mouse infection models (Rampini *et al.* 2008, Sander *et al.* 2004). An *lsp* mutant of *L. monocytogenes* also showed reduced virulence; the LD_{50} of the mutant was 10-fold lower than that of the wild-type (Reglier-Poupet *et al.* 2003). Conversely, an *lgt* mutant of *S. aureus* showed a hypervirulent phenotype in a mouse infection model (Stoll *et al.* 2005). The suggested reason for this is that incorrect lipoprotein processing in the *lgt* mutant of *S. aureus* might allow the bacteria to escape from the immune defence mechanisms of the host, such as recognition by TLRs or production of antibodies.

Overall, there are variations in the effects of Lgt and Lsp inactivation on the virulence of Gram-positive pathogens. Changes in the virulence might be caused by alterations in the virulence factors (e.g., adherence, toxin production, recognition by host immune system), fitness of the pathogens or the combination of both which has to be determined for the individual species. Nevertheless, pleiotropic phenotypes of the *lgt* and *lsp* mutant strains suggest that lipoproteins and lipoprotein processing have species-specific roles in virulence.

5.1.2. Stress factors in the gut

In the human and animal intestinal tract, bacteria have to overcome stressful conditions to survive, such as low oxygen concentration, detergent-like activity of bile salts, competing microflora, and in some cases the presence of antibiotics. Bile salts are synthetised in the liver from cholesterol (primary bile salts; cholic acid (CA) and chenodeoxycholic acid), secreted into the intestine and further metabolised via conjugation to glycine or taurine by the normal microflora (secondary bile salts; deoxycholic acid (DCA) and lithocholic acid). Their major action is to break down fats and oils by emulsifying them into smaller droplets, making them accessible to various enzymes for subsequent digestion. Bile salts also play important roles in the absorption of fat soluble vitamins. The physiological bile concentration in the gut varies between 0.2 and 2 % depending on the amount of the fat released into the duodenum (Whitehead *et al.* 2008). Another function of bile acids is to suppress excessive bacterial colonisation in the small intestine (Ridlon *et al.* 2006). Bile has a potent antimicrobial activity against many microbes by causing damage to the cells, possibly by disrupting the membrane and the cell wall. To resist this, bacteria regulate their gene expression and adapt to bile acid stress by altering the lipid composition of the membranes as prevention from entering bile salts and by increasing the number of efflux pumps to pump out undesirable bile salts that have entered the cell (Gunn 2000, Taranto *et al.* 2003). Earlier studies demonstrated that bile salts play a central role in the germination of *C. difficile* spores. While taurocholate is known to stimulate germination of spores (Sorg & Sonenshein 2008, Wilson 1983), chenodeoxycholate and secondary bile salts act as inhibitors of *C. difficile* spore germination (Sorg & Sonenshein 2009).

Administration of antibiotics, especially broad-spectrum antibiotics, disrupts the balance of the normal gut flora. While most bacteria that compose the intestinal

microflora are sensitive to antibiotics, *C. difficile* is extremely resistant to a wide range of antibiotics, and thereby flourishes under antibiotic treatment. Antibiotics target processes in microbes which do not have an equivalent in humans. Bacterial cell walls are unique prokaryotic structures and targets of several groups of antibiotics, such as β-lactams and glycopeptide antibiotics. β-lactam antibiotics act as competitive inhibitors of the penicillin-binding-protein (PBP), responsible for crosslinking of the peptidoglycan, while glycopeptides prevent incorporation of the NAM/NAG-oligopeptide subunits (N-acetylmuramic acid/N-acetylglucosamine) into the peptidoglycan matrix. Lipoproteins have been demonstrated to be involved in antibiotic resistance by hydrolysing the β-lactam ring of penicillins (e.g., penicillinases in *B. licheniformis*) (Nielsen *et al.* 1981), and in sensing, therby promoting susceptibility of bacterial cells to, antimicrobial peptides (AMPs) as it has been shown in a number of Enterobacteriaceae and in *Salmonella enterica* species (Chang *et al.* 2012, Farris *et al.* 2010).

5.1.3. Aim of this study

This study aimed to investigate the effects of *lgt* inactivation on the physiology and virulence of *C. difficile*. Initially, I studied the growth phenotypes of the Cdi::*lgt* strain, then investigated whether resistance of *C. difficile* to various stress factors was affected by Lgt inactivation. Finally, I studied the behaviour of the Cdi::*lgt* in adhesion.

5.2. Results

5.2.1. *In vitro* growth of *C. difficile* is not affected by mutation of the *lgt* gene

To investigate the effects of *lgt* inactivation on the growth of *C. difficile,* growth of the Cdi::WT, Cdi::*lgt* and complemented Cdi::*lgt* strains were compared in BHI medium. Growth was monitored by measuring the change in OD_{590} and colony forming unit

(CFU) (Figure 5.1). Although, no differences were found in the doubling times of the strains in the exponential phase, the Cdi::*lgt* strain had a longer stationary phase by OD_{590} measurements but not by CFU counts. This shows that the differences in the OD_{590} values are not due to the differences in the number of vegetative cells. The reason of the increased OD_{590} values might be that more lipoproteins are released into the culture medium by Cdi::*lgt* which could potentially affect the optical density of the culture. Complementation of the Cdi::*lgt* strain almost fully restored the OD_{590} measurements in the stationary phase.

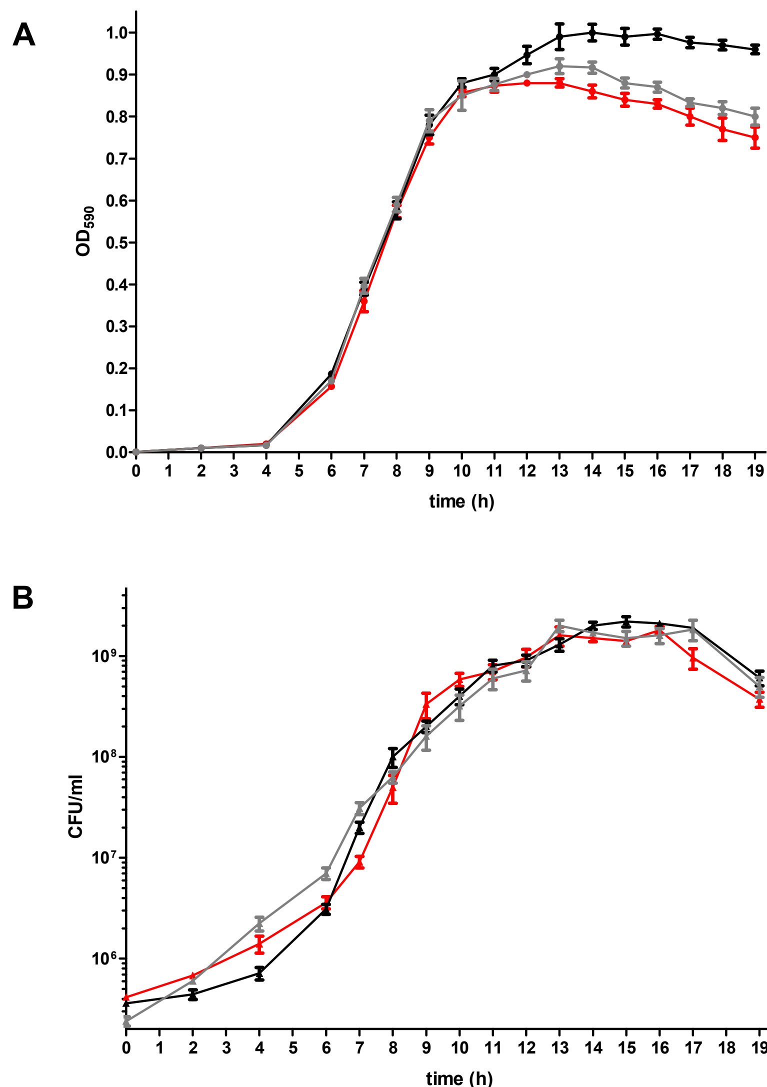


Figure 5.1. Comparison of the growth of the wild-type, *lgt* mutant and complemented *lgt* mutant *C. difficile* in BHI medium. Growth rate of the Cdi::WT ( ), Cdi::*lgt* ( ) and complemented Cdi::*lgt* ( ) strains was assessed by following optical densities of the cultures (A) and determining colony forming units (B) in time. Error bars indicate the standard errors of the means (n=3). Growth curves indicate similar growth of the strains.

5.2.2. Heat-resistant colony formation by *C. difficile* is not affected by *lgt* mutation

In contrast to the vegetative cells, spores of *C. difficile* remain viable after being exposed to high temperatures. Germination is triggered by molecules, such as taurocholate. Cdi::WT, Cdi::*lgt* and complemented Cdi::*lgt* strains grown in liquid cultures for 6 days were tested to compare the number of heat-resistant colonies formed on BHI agar supplemented with 0.1 % taurocholate (Figure 5.2). These colonies represent the number of heat-resistant spores that were germinated. To control the efficacy of the heat treatment in terms of eradicating the vegetative cells, culture of the sporulation deficient *spo0A* mutant of *C. difficile* 630Δ*erm* (Heap *et al.* 2007) was also set up which produced the expected negative result (lack of heat-resistant CFU formation on BHI agar supplemented with taurocholate; data not shown). The number of colonies formed by Cdi::WT, Cdi::*lgt* and complemented Cdi::*lgt* showed no obvious differences between the strains at any time points (except T_0).

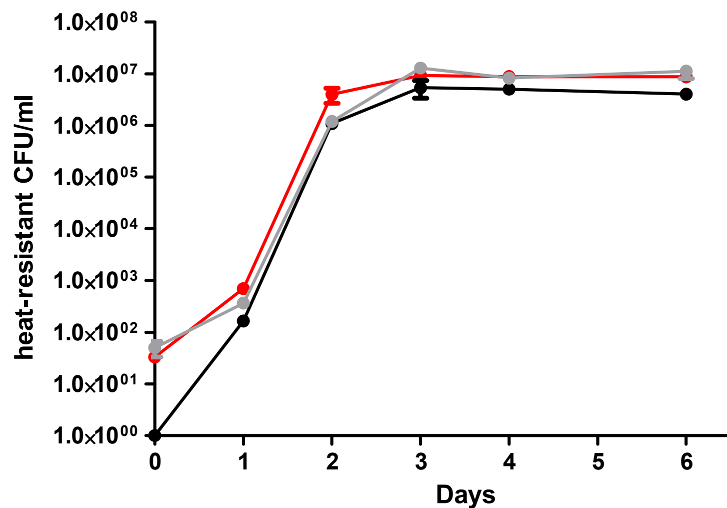


Figure 5.2. Development of heat-resistant CFU by the wild-type, *lgt* mutant and complemented *lgt* mutant *C. difficile*. Cdi::WT (red line), Cdi::*lgt* (black line) and complemented Cdi::*lgt* (grey line) strains were cultivated in BHI medium as described in Chapter 2. An aliquot of the cultures was heated at 69°C for 25 minutes at 24-hour intervals and spores were recovered by plating onto BHI agar supplemented with 0.1 % taurocholate. Error bars are the standard error of the means (n=2). The graph indicates that the numbers of the recovered heat-resistant bacteria were similar in the bacterial cultures.

5.2.3. Effect of *lgt* mutation on the growth of *C. difficile* under intestinal stress conditions

C. difficile is exposed to the detergent-like activity of bile salts in the gastrointestinal tract during infection. Resistance to bile salts depends on the integrity of the bacterial cell wall. Since some lipoproteins are released from the Cdi::*lgt*, which potentially effects cell wall integrity, resistance of the Cdi::WT and *Cdi::lgt* strains to bile salts (CA and DCA) and sodium dodecyl sulfate (SDS) were compared. The activity of bile salts was investigated by determining the minimum inhibitory concentration (MIC) of bile salts (cholic acid : deoxycholic acid = 1:1) against the strains. Although, no differences were observed in the MIC values between the strains (625 µg/ml), lower OD_{590} values of the mutant strain at each bile concentration (Figure 5.3) suggest that the Cdi::*lgt* has a growth defect. However, no differences have been observed in the antimicrobial activity of SDS against the strains at any concentrations (graph is not shown; MIC ≥ 39 µg/ml).

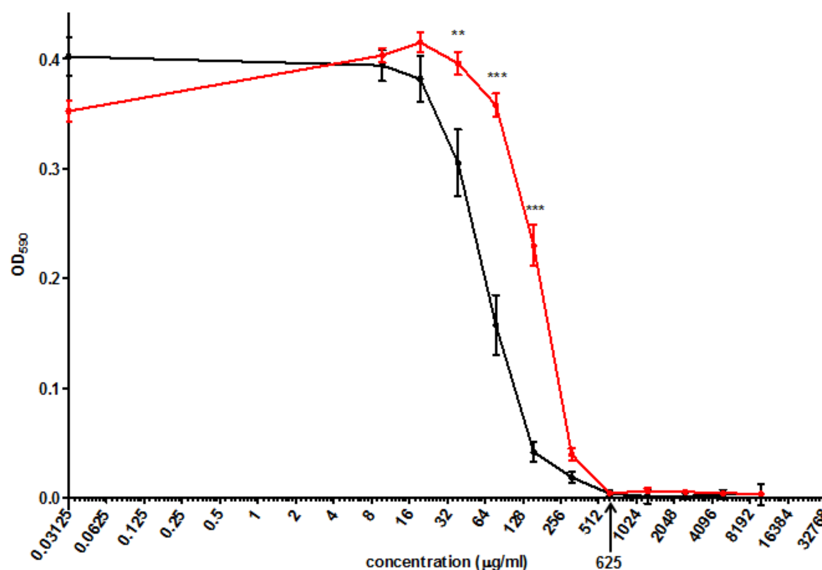


Figure 5.3. Determination of the minimum inhibitory concentration (MIC) of bile salts against the wild-type and the *lgt* mutant *C. difficile*. Cdi::WT (white bars) and Cdi::*lgt* (grey bars) strains were incubated with the mixture of cholic acid and deoxycholic acid (1:1) in the concentration range of 0 and 10mg/ml. Growth of the strains was determined by measuring the optical densities at 570 nm in the wells after 20 hours of incubation. Bars indicate the average of three independent experiments in three technical replicates, and error bars represent the standard errors of the means. P values were obtained using one-way ANOVA with Tukey post test. Asterisks indicate significant differences (** P<0.01; *** P<0.001). Bars indicate that the MIC of bile salts against Cdi::WT and Cdi::lgt were the same, 625 µg/ml.

To further analyze the effect of bile salts on the growth of *C. difficile*, Cdi::WT and Cdi::*lgt* were grown in BHI broth supplemented with various concentrations of bile salts. Growth was monitored by measuring the change in OD_{590} (Figure 5.4). As expected, both strains showed impaired growth in the presence of bile salts in a dose-dependent manner. However, the optical density of the cultures suggest that the Cdi::*lgt* strain had a longer lag phase which correlates with the slower growth of Cdi::*lgt* observed in the MIC assay and demonstrates that the Cdi::*lgt* strain is more susceptible to bile salts than the Cdi::WT. This result indicates that the fitness of the Cdi::*lgt* strain is reduced in the presence of bile salts.

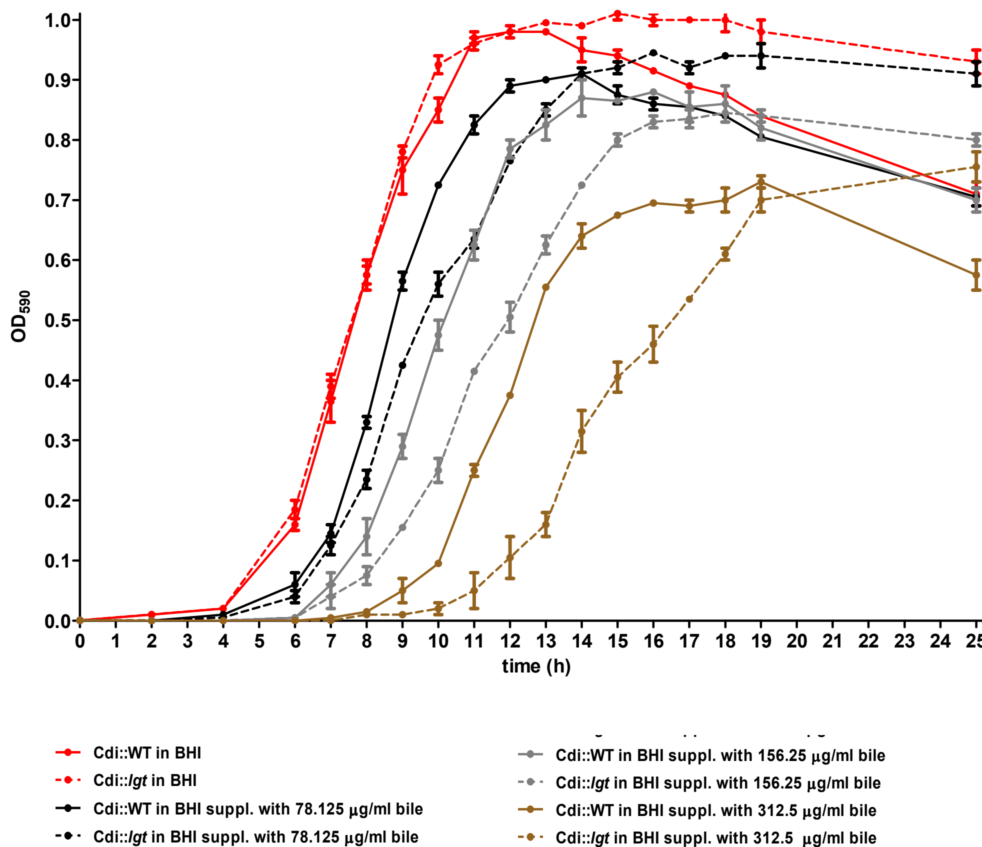


Figure 5.4. Comparison of the growth of the wild-type, *lgt* mutant and complemented *lgt* mutant *C. difficile* in BHI medium supplemented with bile salts. Growth rate of the Cdi::WT (solid line) and the Cdi::*lgt* (dash line) strains in BHI medium supplemented with bile salts (cholic acid : deoxycholic acid = 1:1) in a final concentration of 78.125 µg/ml (black line), 156.25 µg/ml (grey line) and 312.5 µg/ml (brown line) was assessed by following the optical densities of the bacterial cultures in time. Concentrations of bile salts in the medium were chosen on the basis of the MIC value (Figure 5.3). Growth in BHI medium served as a control. Error bars indicate the standard errors of means (n=2). Growth curves indicate that the Cdi::*lgt* strain has a longer lag phase relative to the Cdi::WT when grown in the presence of bile salts.

To study whether antibiotics are able to cross the potentially altered cell wall of Cdi::*lgt*, susceptibility of the Cdi::WT and Cdi::*lgt* strains to antibiotics acting intracellularly was investigated. The minimum inhibitory concentration (MIC) of ciprofloxacin and metronidazole, which are taken up by diffusion, were determined (Table 5.1). As the strains showed no differences in sensitivity to these antibiotics, it is suggested that antibiotics were able to reach their site of action and that the permeability of the cell wall was not altered in the Cdi::*lgt* strain. Changes in the resistance to β-lactam antibiotics can involve reduced affinity of β-lactamases to the antibiotics. This could potentially derive from the lack of the lipid group or the presence of the signal peptide at the amino terminus of β-lactamases. But as investigated in this study, lipoprotein processing by Lgt does not have an effect on the susceptibility of *C. difficile* to the tested β-lactam antibiotics (cefoxitin, dicloxacillin, ampicillin; Table 5.1). Sensitivity of *C. difficile* was also unaffected to the cell wall specific glycopeptide vancomycin (Table 5.1).

Table 5.1. MIC of the antibiotics against the wild-type and the *lgt* mutant *C. difficile.*

Antibiotics	**MIC (µg/ml)**	
	Cdi::WT	Cdi::*lgt*
ciprofloxacin	4	4
metronidazole	0.25	0.25
cefoxitin	128	128
dicloxacillin	16	16
ampicillin	2	2
vancomycin	1	1

5.2.4. Inactivation of the *lgt* gene does not affect the cell morphology of *C. difficile*

To microscopically examine whether morphology of the bacterial cells are affected by Lgt inactivation, the Cdi::WT, Cdi::*lgt* and complemented Cdi::*lgt* were examined by

scanning electron microscopy at the University of Exeter Bioimaging Centre. As it is shown in Figure 5.5, *lgt* mutation did not have any effect on the cell morphology of the strains as no apparent changes have been observed on the micrographs.

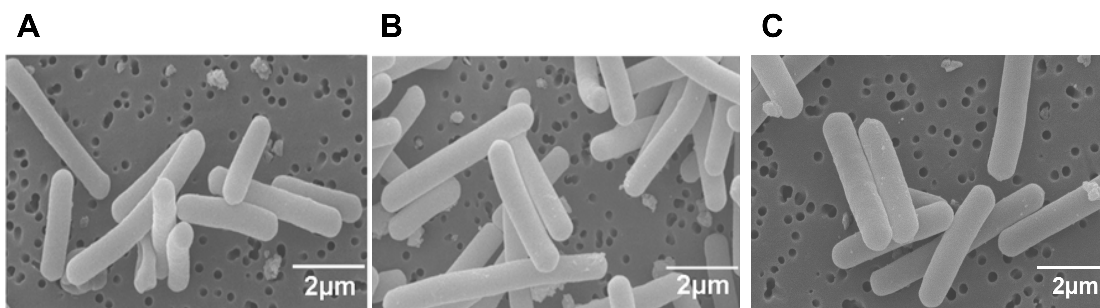


Figure 5.5. Scanning electron microscopy of the wild-type, *lgt* mutant and complemented *lgt* mutant *C. difficile*. The Cdi::WT (A), Cdi::*lgt* (B) and complemented Cdi::*lgt* (C) strains were grown in BHI liquid medium until mid exponential phase, bacterial cells were then harvested and prepared for microscopy. Micrographs show that the cells of the strains look similar.

5.2.5. Lipoprotein processing by Lgt is required for *in vitro* adherence of *C. difficile*

The *C. difficile* 630 genome encodes several putative lipoproteins that may influence the adherence of the bacterial cells, including components of ABC-type transporters and lipoproteins that have similarity to the amino acid sequences of known adhesins from other species. For example, CD1491 (263 amino acids) and CD1653 (265 amino acids) exhibit 40 % (111/271) and 35 % (174/270) amino acid sequence identity to the IlpA adhesin (269 amino acids) of *Vibrio vulnificus* (Lee *et al.* 2010), respectively. The amino acid sequences of CD2672 (appA, 519 amino acids) and CD0855 (oppA, 522 amino acids) are 20 % (124/616) and 21 % (131/621) identical to the BopA adhesin of *Bifidobacterium bifidum* (Guglielmetti *et al.* 2009), respectively, and CD0873 (340 amino acids) has 21 % amino acid identity (82/385) to PsaA of *S. pneumoniae* (Rajam *et al.* 2008). Thus, inactivation of *lgt* might influence the adherence of *C. difficile*, which is an important step in the course of CDI. To investigate the effect of *lgt* mutation

on the adherence ability of *C. difficile*, adherence of the Cdi::WT and Cdi::*lgt* strains to Caco-2 cells was measured. This cell line is commonly used as a human intestinal cell model for investigating interactions *in vitro* between the human gut and bacterial cells (Guglielmetti *et al.* 2009, Parker *et al.* 2010, Tuomola & Salminen 1998), including *C. difficile* (Eveillard *et al.* 1993, Naaber *et al.* 1996). In this study, bacterial cells were incubated with half-differentiated Caco-2 cells (8 days after seeding, 6 days after confluency occurred; Figure 5.6.A) and the number of adherent bacteria was determined by CFU counts. A *fliC* mutant of *C. difficile* 630Δ*erm* which shows increased adherence compared to Cdi::WT (Dingle *et al.* 2011) was used as a control and exhibited the expected increased adherence phenotype (data not shown). After Caco-2 cells were incubated with the Cdi::WT for 2 hours, the number of bacteria adhered to 100 Caco-2 cells was 39.2 ± 2.6 (Figure 5.6.C). In the same experiment, the number of Cdi::*lgt* bacterial cells adhered to Caco-2 cells was significantly lower, i.e., 3.6 ± 1.4 per 100 Caco-2 cells ($P<0.001$). These data strongly suggest that lipoprotein processing by Lgt is required for the *in vitro* adherence of *C. difficile*. However, complementation did not restore adherence of the Cdi::*lgt* strain to wild-type level, the reason of which has to be further investigated (see Discussion on page 145). The viability of Caco-2 cells after 2 hours of incubation with *C. difficile* was investigated by MTT assay. Figure 5.6.B shows the results and indicates that the level of viability depends on which strain the cells are incubated with; Caco-2 cells were less viable when incubated with Cdi::WT relative to when incubated with Cdi::*lgt*, which may be associated with the reduced adherence of the Cdi::*lgt* strain. Viability of Caco-2 cells was not significantly affected under anaerobic condition.

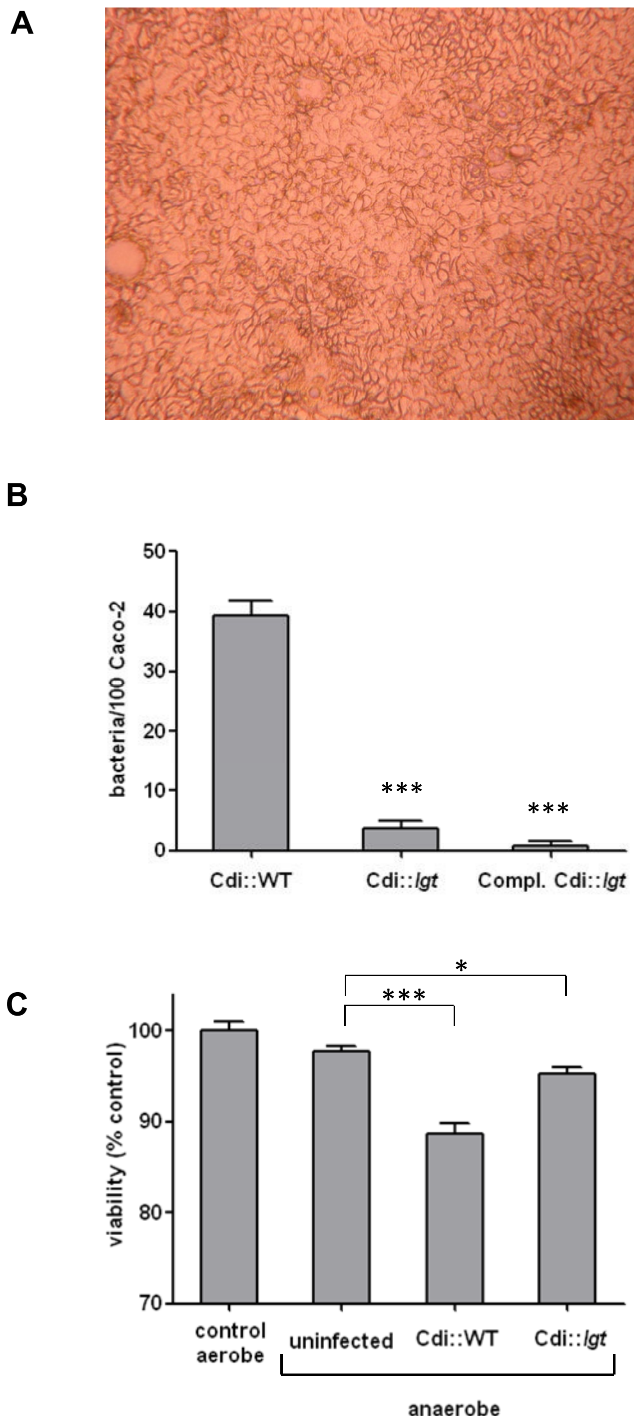


Figure 5.6. Adherence of *C. difficile* to Caco-2 cells is reduced when *lgt* is inactivated. (A) Light microscopic image of Caco-2 cells 6 days after confluency occurred. (B) Adherence of the Cdi::WT, Cdi::*lgt* and complemented Cdi::*lgt* strains to Caco-2 cells maintained for 6 days after confluency occurred (MOI 1:5). Number of the adherent bacteria was evaluated by CFU counts after 2 hours of anaerobe incubation. Adherence assays were performed in triplicate and repeated independently 3 times. (C) Quantification of Caco-2 cell viability by MTT assay under anaerobic conditions without infection and when incubated with the Cdi::WT or the Cdi::*lgt* strains for 2 hours. MTT assay was conducted in triplicate and repeated independently twice. Viability of Caco-2 cells was significantly reduced when incubated with the Cdi::WT. All panels represent mean values with error bars indicating standard errors of means. Statistical comparisons were made using one-way ANOVA with Tukey post test. Significant differences from the control are indicated by asterisks (*** $P<0.001$).

5.2.6. Mouse CDI experiment

In order to assess the effect of *lgt* mutation on the *in vivo* phenotype of *C. difficile*, mice were co-infected with a mixture of wild-type (Cdi::*tetM*) and Cdi::*lgt* spores in a competitive challenge experiment. Faecal shedding of and intestinal colonisation by the strains were then measured at different time points (see below in more detailed). This experiment was performed by Zoe Seager and myself at Imperial College London. The Cdi::*tetM* strain which is sensitive to tetracycline was used as wild-type in the infection to be able to select for *C. difficile* among other gut microbials with erythromycin, and distinguish between the recovered challenge strains (wild-type, which is here the tetracycline sensitive derivative of the *C. difficile* 630Δ*erm* strain (Cdi::*tetM*), and the mutant (Cdi::*lgt*); both strains are resistant to erythromycin because of the inserted ClosTron group II intron) in the faecal and tissue samples.

5.2.6.1. Generation and *in vitro* characterisation of a *tetM* mutant of *C. difficile*

The *tetM* mutant of *C. difficile* (Cdi::*tetM*) was generated using the ClosTron system by Zoe Seager at the Imperial College London. In this strain, the *tetM* gene conferring tetracycline resistance was inactivated by insertion of the group II intron between the 936 and 937 base pairs of the *tetM* gene. Single intron insertion into the genome was confirmed by Southern blot.

The Cdi::*tetM* strain was characterised *in vitro*, in order to confirm that *tetM* inactivation does not affect phenotypes of the wild-type strain and to exclude that the phenotypes of the challenge strains in the *in vivo* CDI experiment are not related to different growth, germination and sporulation rates of the strains. Growth rate of the Cdi::*tetM* strain in BHI broth was investigated by Zoe and was identical to that of the Cdi::WT (data not shown).

Germination rates of the Cdi::*tetM* and Cdi::*lgt* strains were determined as follows: serial dilutions of the challenge suspension containing 0.5 x 10^7 of Cdi::*tetM* and 0.5 x 10^7 of Cdi::*lgt* spores was plated onto BHI agar supplemented with either erythromycin, tetracycline and taurocholate or erythromycin and taurocholate to select for the strains and quantify the recovered spores. The number of colonies grown on BHI agar supplemented with tetracycline was half of those that have grown on the non-tetracycline agar indicating that the Cdi::*tetM* and Cdi::*lgt* strains exhibit similar germination rates.

The number of heat-resistant CFU formed by Cdi::*tetM* and Cdi::*lgt* was determined as described in 5.2.2. Briefly, bacterial cultures were heated to kill vegetative cells but not spores, and then plated onto BHI agar supplemented with taurocholate to recover viable spores. Since germination rates of the spores are similar, the number of heat-resistant CFU grown on BHI agar supplemented with taurocholate reflect the same numbers of germinated spores in each culture and thereby the sporualtion rate of the strains. The level of heat-resistant colony formation was determined at 24 hour intervals over 6 days and as shown in Figure 5.7, no difference in the development of heat-resistant CFU was observed between the Cdi::*tetM* and Cdi::*lgt* strains. This suggests that sporulation rates of the Cdi::*tetM* and Cdi::*lgt* are also similar. However, spores formed by the strains may have different heat-resistance, in which case comparison of the number of heat-resistant colonies does not reflect the differences in sporulation rates. Therefore, to more accurately compare sporulation rates of the strains, the numbers of spores in the bacterial cultures could have been determined by microscopic counting.

The numbers of heat-resistant colonies developed by the Cdi::WT, Cdi::*tetM* and Cdi::*lgt* strains (Figure 5.2. and 5.7) were similar. Assuming that germination rate of Cdi::*tetM* are similar to that of Cdi::WT, the observations described in this section

suggest that germination and sporulation rates of the the Cdi::WT and Cdi::*lgt* strains are also similar.

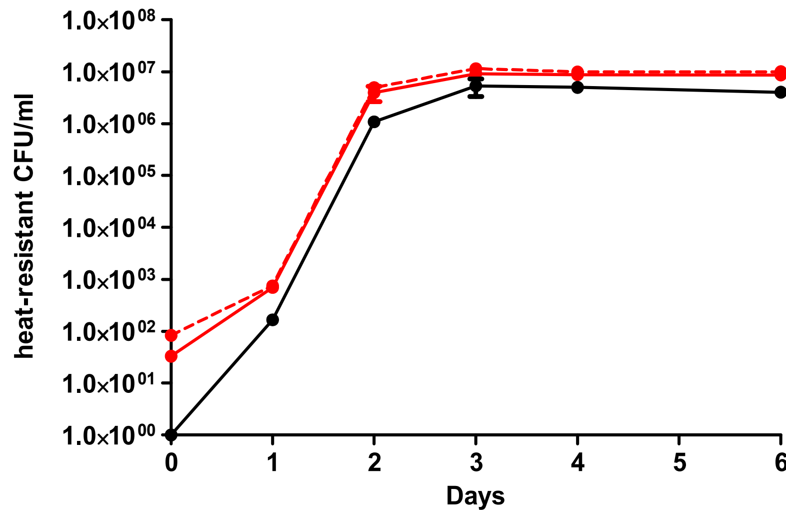


Figure 5.7. Development of heat-resistant CFU by the wild-type, *lgt* mutant and *tetM* mutant *C. difficile*. Cdi::WT ( ), Cdi::*lgt* ( ) and Cdi::*tetM* ( ) strains were cultivated in BHI medium as described in Chapter 2. An aliquot of the cultures was heated at 69°C for 25 min at 24-hour intervals and spores were recovered by plating onto BHI agar supplemented with 0.1 % taurocholate. Error bars are the standard error of the means (n=2). The graph indicates that the numbers of the recovered heat-resistant bacteria were similar in the bacterial cultures. Results with the Cdi::WT and the Cdi::*lgt* strains are taken from Figure 5.2.

Next, Cdi::*tetM* and Cdi::*lgt* were cultured together in liquid medium in order to assess whether Cdi::*tetM* and Cdi::*lgt* compete for growth. Growth of the strains was monitored by determining the CFU/ml of each strain at hourly intervals. Comparison of the curves revealed that the growth of the strains was similar (Figure 5.8) suggesting that the growth rate of the Cdi::*tetM* and the Cdi::*lgt* is not affected in the presence of Cdi::*lgt* and Cdi::*tetM*, respectively. Growth of the Cdi::WT and Cdi::*lgt* when cultured together was also determined by following the optical density of the culture and as shown in Figure 5.8, the growth curve by OD_{590} measurements was indistuingishable from that of the Cdi::*tetM* and Cdi::*lgt* culture.

Taken together, Cdi::*tetM* and Cdi::*lgt* exhibit similar growth, germination and sporulation rates in the laboratory. However, it has to be borne in mind that the *in vitro* observations do not always reflect the *in vivo* events.

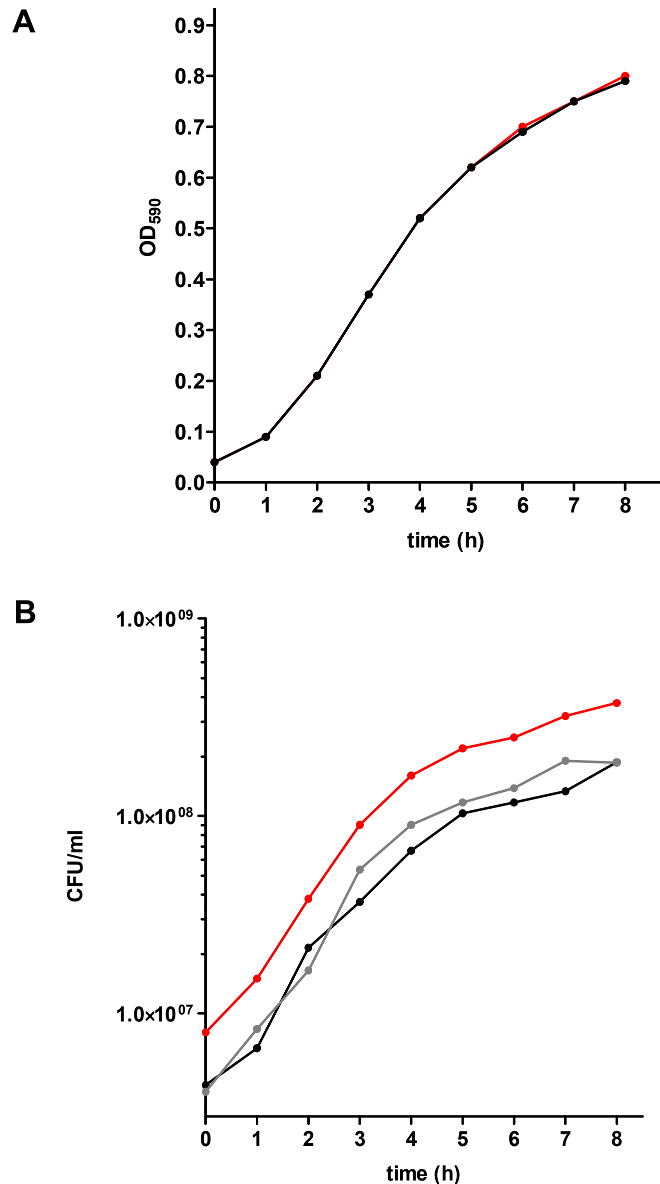


Figure 5.8. Competition growth curve of the wild-type, *lgt* mutant and *tetM* mutant of *C. difficile*. Liquid medium was inoculated with equal number of vegetative cells of each strain. (A) Bacterial growth was monitored by measuring optical density of the cultures at hourly intervals. Symbols: ( ) Cdi::WT and Cdi::*lgt* cultured together in BHI medium; ( ) Cdi::*tetM* and Cdi::*lgt* cultured together in BHI medium. (B) Comparison of the growth of the Cdi::*tetM* ( ) and the Cdi::*lgt* ( ) strains when cultured together in BHI medium. Growth rate was assessed by determining the number of colony forming units at hourly intervals.An aliquot of the cultures was 10-fold serially diluted and plated onto BHI agar supplemented with appropriate antibiotics. CFU/ml for Cdi::*tetM* strain was calculated by subtracting the number of CFU grown on the tetracycline-containing agar (Cdi::*lgt* only) from the number of CFU grown on the non-tetracycline agar (both Cdi::*tetM* and Cdi::*lgt*). ( ) represent the total CFU/ml (Cdi::*tetM* and Cdi::*lgt*). Curves indicate that the growth rate of the strains is similar when cultured together.

5.2.6.2. Effect of *lgt* mutation on colonisation of *C. difficile* in mice

The effect of *lgt* inactivation on the *in vivo* phenotype of *C. difficile* was studied in mice. Figure 5.9. shows the mouse CDI experiment plan. Briefly, mice were treated with antibiotics to disrupt their normal gut flora, making them susceptible for CDI. Mice were then simultaneously challenged with equal numbers of wild-type (Cdi::*tetM*) and Cdi::*lgt* spores. Faecal shedding of and intestinal colonisation by the strains were measured at different time points. At the time points indicated, when bacterial shedding was not observed, shedding was re-induced by further antibiotic treatment.

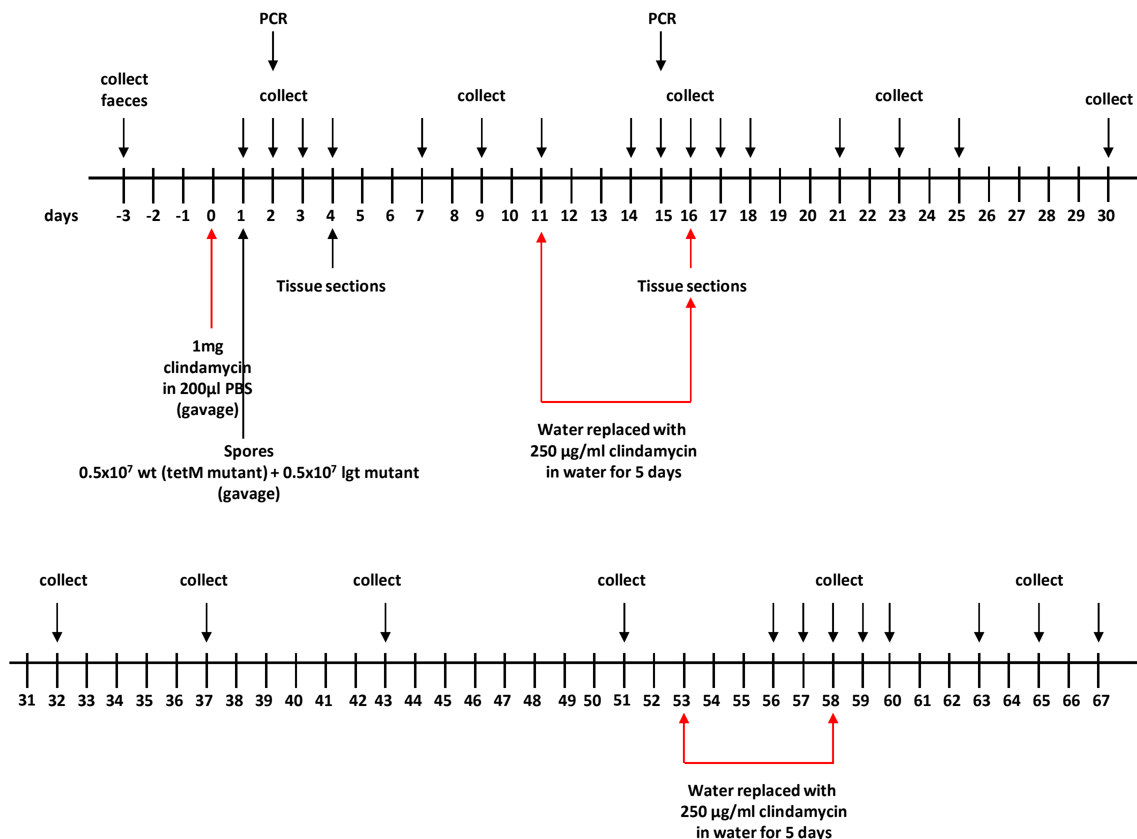


Figure 5.9. Schematic overview of the mouse CDI competition experiment. In order to determine the *in vivo* phenotype of the Cdi::*lgt* strain relative to that of the wild-type (Cdi::*tetM*) strain, 10 mice were co-infected with equal amounts (0.5 x 10^7) of wild type (Cdi::*tetM*) and Cdi::*lgt* spores. Prior to the challenge, mice were treated with 1 mg of clindamycin to disrupt their intestinal microflora and make them susceptible for CDI. Faecal and tissue samples were collected at different time points as indicated by arrows. 11 and 53 days after challenge, mice were re-exposed to clindamycin for 5 days. On day 2 and 15, genomic DNA was extracted from 80-80 single colonies (see Table 5.2) and PCR was performed to confirm that the recovered strains are identical to the challenge strains and grow on the appropriate medium.

To ensure that mice were not infected with *C. difficile* prior to the commencement of the experiment, faecal samples were collected 4 days before the challenge and plated onto Braziers agar. No bacterial outgrowth confirmed the absence of *C. difficile*. On day 3, one mouse from the infected group was culled because it exhibited symptoms of CDI and mice generally do not develop obvious clinical symptoms of the disease in this infection model (Lawley *et al.* 2009a). On day 4, the control group was also culled as they become infected with *C. difficile*, probably during the faecal sample collection.

Figure 5.10 shows the faecal shedding of the Cdi::WT and the Cdi::*lgt* strains over two months after challenge. Cdi::*lgt* shed more vegetative cells than Cdi::WT during the first 6 days (Figure 5.10.A) that might be associated with the reduced adherence of the Cdi::*lgt* observed *in vitro*. Difference in shedding was also observed following the second clindamycin treatment when the Cdi::*lgt* spores were shed with delay (Figure 5.10.B). However, the most striking difference in the shedding rate was observed 2 months post-challenge following the third antibiotic treatment when neither vegetative cells nor spores of the Cdi::*lgt* strain were shed at all (Figure 5.10). This suggests that the intestine of mice was not colonised by the Cdi::*lgt* strain at that time point which is in a good agreement with the reduced adherence phenotype of the Cdi::*lgt*.

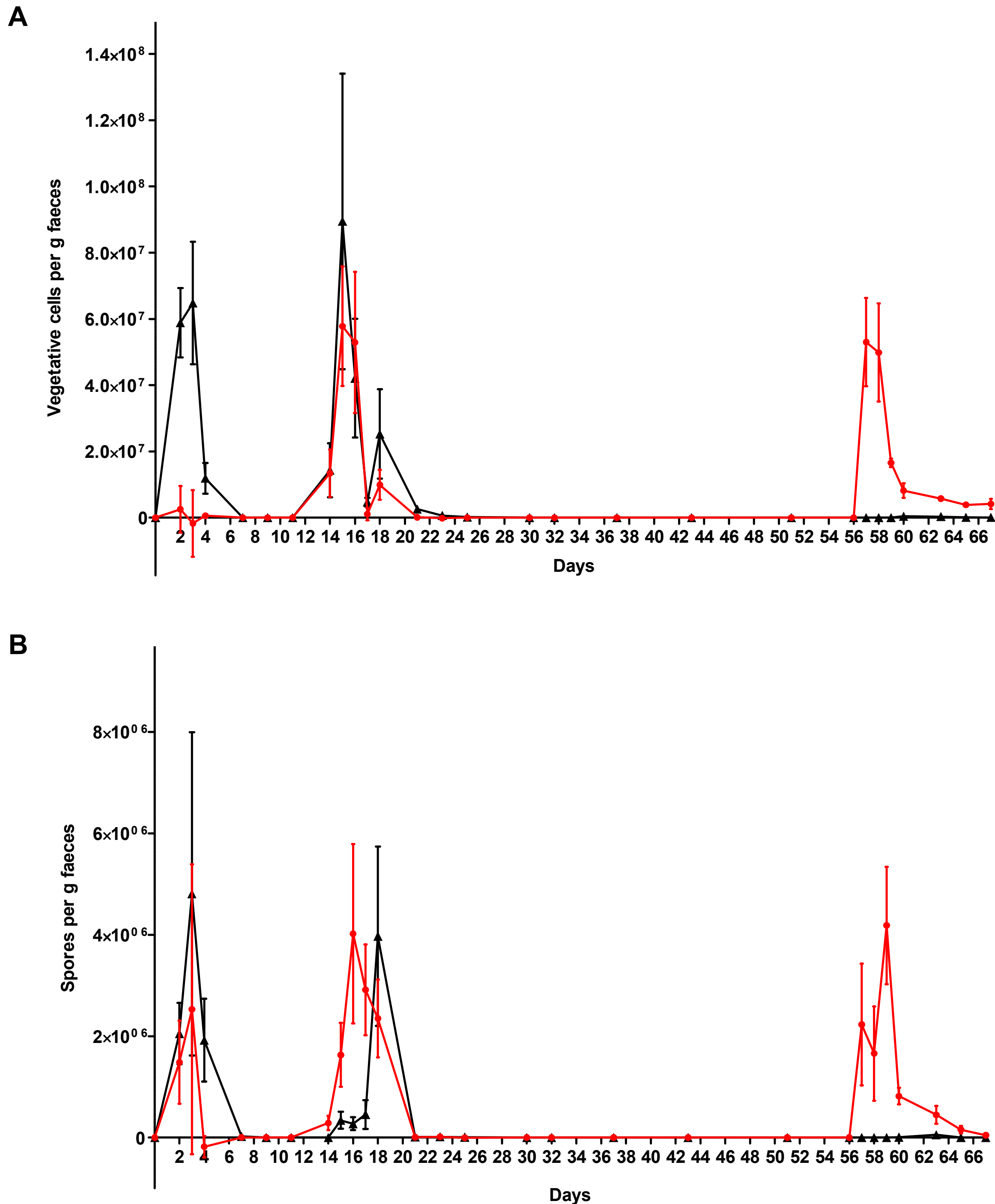


Figure 5.10. Effect of Lgt inactivation on the faecal shedding of *C. difficile* by mice. Faecal shedding of *C. difficile* vegetative cells (A) and spores (B) was monitored for 66 days after mice were co-infected with the mixture of Cdi::*tetM* ( ) and Cdi::*lgt* spores ( ) (1:1). Faeces were collected, resuspended in PBS and plated onto Brazier agar supplemented with the appropriate antibiotics to determine the number of vegetative cells of each strain in the faeces. In parallel, samples were plated onto Brazier agar supplemented with the appropriate antibiotics and with 0.1 % taurocholate to determine the number of spores of each strain in the faeces. Graphs show that the faecal shedding of the Cdi::*lgt* strain was increased in the first peak and was abolished in the third peak.

Tissue sections were taken during the first and second shedding peak on day 4 and 16, respectively. We found that the majority of *C. difficile* was unattached and located in the caecum (Figure 5.11). 4 days after challenge, the numbers of Cdi::*lgt* vegetative cells and spores were much higher in the caecal content than that of the Cdi::*tetM*. This is interesting in the context of that neither the Cdi::*tetM* vegetative cells nor the spores were shed into the faeces while both the Cdi::*lgt* vegetative cells and the spores were detected in the faeces at that time point. These results raise the question why Cdi::*tetM* is present in such small numbers both in the faeces and in the gut, and which part of the intestinal tract is colonised by Cdi::*tetM* at that time. On day 16, vegetative cells of both strains were present in similar amounts but lower numbers of Cdi::*lgt* spores were found in the caecal content of mice than those of the Cdi::*tetM* spores (Figure 5.11). However, this difference was not statistically significant.

Taken together, the findings described in this section suggest that lipoprotein processing by Lgt affects faecal shedding of *C. difficile* in mice, especially in long term infection.

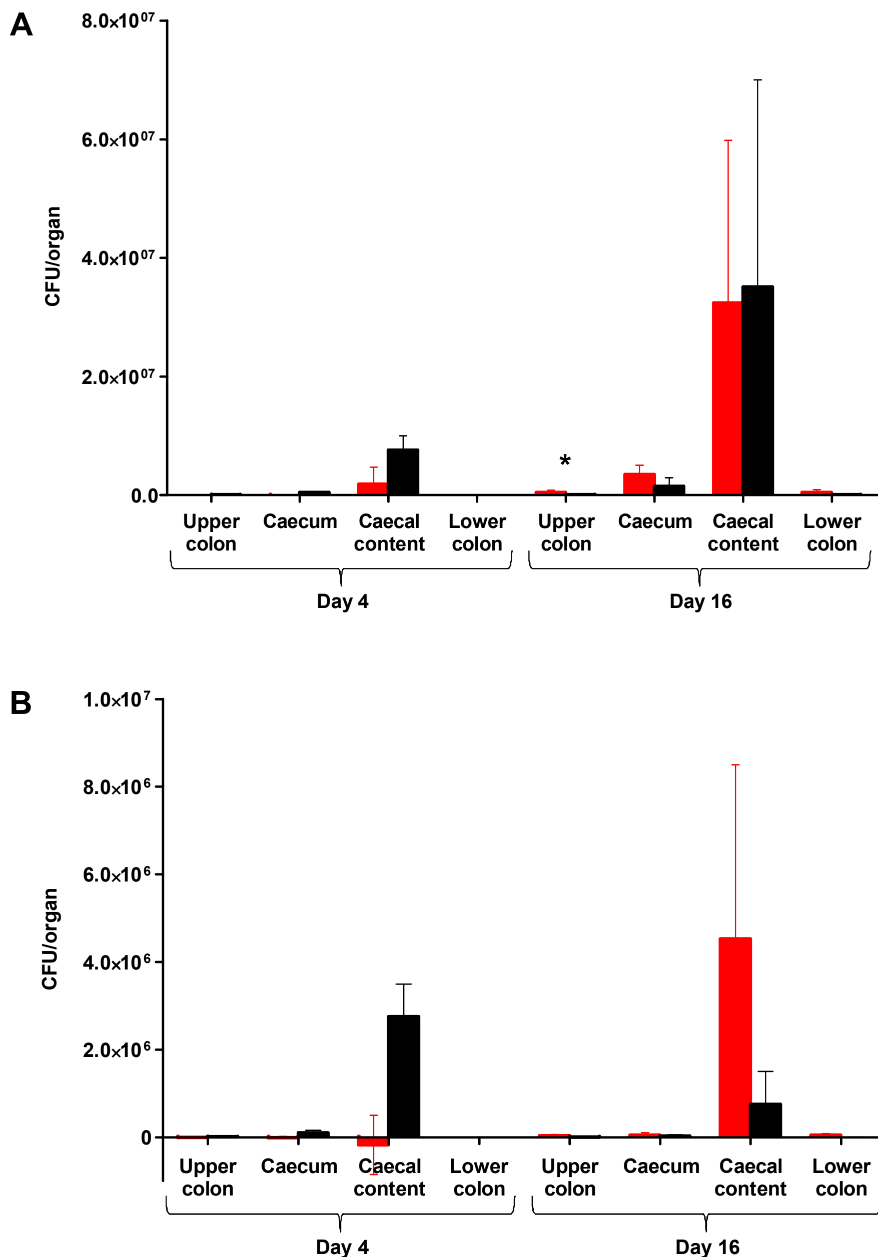


Figure 5.11. Effect of Lgt inactivation on the colonisation of *C. difficile* in mice. Colonisation of *C. difficile* vegetative cells (A) and spores (B) in the mouse large intestinal sections was determined 4 and 16 days after mice were co-infected with the mixture of Cdi::*tetM* (■) and Cdi::*lgt* (■) spores. The caecum and the colon were isolated from two mice at each time, washed, weighed and homogenized. Dilutions were plated for viable counts and the number of CFU per organ was determined for both the vegetative cells and the spores as described in Figure 5.8. Significant difference is indicated by asterisk (* P<0.05; two-tailed t test).

5.2.6.3. Confirmation of the bacterial strains recovered from mice

Genomic DNA was purified on day 2 and day 15 from the bacterial strains recovered from two mice on each day (40 single colonies per mouse per cage per day) grown on agar plates without taurocholate (20 colonies from tetracycline, 20 colonies from non-tetracycline plates per mouse) (Figure 5.9). PCR was performed using primers flanking the intron insertion sites (*lgt* F, *lgt* R and *tetM* F, *tetM* R) to confirm that the recovered strains are identical to the challenge strains and grow on the appropriate medium. As it is shown in Table 5.2, growth of the strains on the selective agar was as expected: Cdi::*tetM* did not grow on agar supplemented with tetracycline and both strains grew on the non-tetracycline agar. Ratio of the strains grown on the non-tetracycline plates also reflects the number of vegetative cells shed in to the faeces on day 2 and day 15; high numbers of Cdi::*lgt* colonies from day 2 and similar numbers of Cdi*::tetM* and Cdi::*lgt* colonies from day 15 (Figure 5.10.A).

Table 5.2. PCR identification of the bacteria isolated from infected mice. PCR was performed on a total of 160 single colonies (isolated from four mice on the 2nd and the 15th day of the experiment) using primers flanking the intron insertion sites (*lgt* F, *lgt* R; *tetM* F, *tetM* R) to confirm that bacteria re-isolated from the infected mice were identical to the challenge strains and grow on the appropriate medium.

		***lgt* F, *lgt* R**			***tetM* F, *tetM* R**		
Day 2		**+**	**-**	**No visible band**	**+**	**-**	**No visible band**
Non-tetracycline plates	Mouse 1	17	2	1	2	18	0
	Mouse 10	20	0	0	0	20	0
	Total	37	2	1	2	38	0
Tetracycline plates	Mouse 1	8	0	12	0	20	0
	Mouse 10	14	0	6	0	19	1
	Total	22	0	18	0	39	1
Day 15							
Non-tetracycline plates	Mouse 3	18	0	2	0	18	2
	Mouse 9	0	20	0	16	0	4
	Total	18	20	2	16	18	6
Tetracycline plates	Mouse 3	20	0	0	0	18	2
	Mouse 9	20	0	0	0	15	5
	Total	40	0	0	0	33	7

5.3. Discussion

So far, several *lgt* mutants of Gram-positive pathogens have been described which exhibited pleiotropic phenotypes. Lgt inactivation has various effects depending on the role of the lipoproteins and how lipoprotein processing (therefore activity and localisation of lipoproteins) is affected by *lgt* mutation in the various bacterial species. Like several *lgt* mutants published in the literature (Baumgartner *et al.* 2007, Bray *et al.* 2009, Chimalapati *et al.* 2012, Hamilton *et al.* 2006), the *lgt* mutant strain of *C. difficile* exhibited no differences in growth in rich medium (growth rate and cell morphology).

However, *lgt* mutants of *S. aureus* and *S. pneumoniae*, which grow normally in complete media, exhibit growth defects in minimal media (Chimalapati *et al.* 2012, Stoll *et al.* 2005). Similarly to Lgt, Lsp is dispensable for the growth of most of the Gram-positive bacteria in rich medium, causing no or only slight growth defects *in vitro* (De Greeff *et al.* 2003, Reglier-Poupet *et al.* 2003, Sander *et al.* 2004, Tjalsma *et al.* 1999, Venema *et al.* 2003). In contrast, mutation of the *lgt* and *lsp* genes seems to have major effect on the growth of some pathogens. For example, inactivation of Lgt and Lsp has serious effect on the growth and development of *Streptomyces scabies* (Widdick *et al.* 2011). *Mycobacterium smegmatis* showed growth retardation when one of its two *lgt* genes was inactivated (Tschumi *et al.* 2012). No obvious phenotype of *S. coelicolor* has been observed when the two *lgt* homologues were deleted separately; but the *lgt* double mutant could not be isolated, perhaps because Lgt activity is essential for viability. Moreover, an *lsp* mutant of *S. coelicolor* exhibited weak growth and formed small flat colonies suggesting that lipoprotein biosynthesis might play an important role in the growth of *S. coelicolor* (Thompson *et al.* 2010).

In this study, changes have been observed in the *in vitro* growth of the Cdi::*lgt* strain in the presence of bile salts (longer lag phase). This may affect the fitness of the bacteria, which might be crucial in the gut. Data published on *S. pneumoniae* also described that the *lgt* mutant was more sensitive to lysis by bile salt (Chimalapati *et al.* 2012). This might be due to that the release of some proteins from the membrane of the *lgt* mutants may result in changes in the bacterial cell wall which could potentially alter the ability of the bacteria to grow or survive while exposed to bile stress. However, scanning electronmicrographs did not show any differences in the morphology of *C. difficile* when *lgt* was inactivated. Cell envelope alterations were observed in a *Bacillus anthracis lgt* mutant, as manifested by a decreased hydrophobicity of the bacterial surface (Okugawa *et al.* 2012).

Although, growth of the *lgt* mutant was affected in the presence of bile salts, germination of the *lgt* mutant spores triggered by the bile salt taurocholate was unaffected. Likewise, the *lgt* mutant exhibited similar sporulation rate as the wild-type. These results suggest that lipoproteins do not play a role in the sporulation or germination of *C. difficile*, or the activity of the lipoproteins involved in these processes is not affected by Lgt inactivation. In contrast, *lgt* mutation reduced the rate of sporulation and germination in *B. subtilis* and *B. anthracis* (Dartois *et al.* 1997, Igarashi *et al.* 2004, Okugawa *et al.* 2012, Robinson *et al.* 1998).

Several lipoproteins of Gram-positive pathogens have been reported to bind to or to be involved in bacterial adherence to cultured epithelial cells (Blevins *et al.* 2008, Cron *et al.* 2009, Guglielmetti *et al.* 2009, Hermans *et al.* 2006, Jin *et al.* 2001, Lee *et al.* 2010, Neelakanta *et al.* 2007, Rajam *et al.* 2008, Spellerberg *et al.* 1999, Verma *et al.* 2009). Reduced adherence of *C. difficile* to Caco-2 cells when lipoprotein processing is disrupted by *lgt* inactivation suggests that lipoproteins of *C. difficile* might also play a role in adhesion. The pattern of Cdi::*lgt* shedding in the *in vivo* infection model further supports the role of lipoproteins in adherence. Reduced adherence of an *lgt* mutant of *S. agalactiae* to endothelial cells has also been described previously (Bray *et al.* 2009). Colonisation is generally multifactorial and involves several adhesins. Therefore, the markedly reduced adherence of the Cdi::*lgt* strain to Caco-2 cells suggests that lipoproteins of *C. difficile* may have a major role in adhesion. It has been demonstrated that several lipoproteins are involved in the colonisation of some bacterial species, such as *S. pneumoniae* (e.g., PsaA, PpmA, SlrA) (Cron *et al.* 2009, Hermans *et al.* 2006, Novak *et al.* 1998) and the Gram-negative *B. burgdorferi* (e.g., DbpA, OspB, BmpA) (Blevins *et al.* 2008, Neelakanta *et al.* 2007, Verma *et al.* 2009) (see Chapter 6 for more details).

A single band in the *lgt* mutant detected by Southern blotting (Figure 3.2) is indicative of that the intron disrupted the *lgt* gene only. Furthermore, transcription from the *lgt* gene in the complemented *lgt* mutant but not in the *lgt* mutant (Figure 3.4) supports that the complemented plasmid construct is correct. However, variations in the phenotype restoration of the *lgt* mutant by complementation have been observed. Although, several phenotypes of the *lgt* mutant of *C. difficile* were fully or partially restored to wild-type level by complementation (e.g., detergent protein profile, protein lipidation, OD_{590} measurements in stationary phase growth; see Chapter 3 and 5 results), adherence phenotype of the *lgt* mutant was not restored at all. This could be the cause of that disruption of the *lgt* gene might affect the transcription of other genes (not the neighbouring genes as their transcription was not affected; see Figure 3.4) on the chromosome. Expression of the proteins coded by the affected genes is not restored by introduction of the complemented plasmid into the mutant strain. This could cause failure of the phenotype restoration of complementated strains, raising the question of whether adherence phenotype exhibited by the *lgt* mutant is solely attributed to the inactivation of the *lgt* gene. On the other hand, level of the *lgt* transcription was much higher in the complemented *lgt* mutant than in the wild-type strain (Figure 3.4) which could also affect restoration of the phenotype. Therefore, results obtained by the complemented *lgt* mutant could both support and disprove the implication of Lgt in the adherence of *C. difficile*. The role of Lgt could be further investigated in future experiments using a mutant constructed by gene deletion (method for gene mutation through allelic exchange has recently been developed in Clostridia) and complementing the mutant by placing the intact *lgt* gene back into the chromosome. An alternative way of mutant generation would be to inactivate Lgt by frame-shift or point mutation of the *lgt* gene to minimalise the changes in the genome. However, currently these DNA

modifications are not possible due to the lack of method and knowledge about the active site of Lgt in *C. difficile*.

Chapter Six

Lipoprotein CD0873 is a putative adhesin of *C. difficile*

6.1. Introduction

As described in the previous chapter, disruption of the lipoprotein biosynthetic pathway facilitates the study of the roles of lipoproteins in the physiology and virulence of bacterial species. However, it does not provide information about whether alteration of a single lipoprotein, or global effects of several altered lipoproteins cause the phenotypic changes. In order to identify candidate lipoproteins contributing to the altered phenotypes exhibited by *lgt* and *lsp* strains, the lipoproteome of the pathogen has to be screened, which can be performed in a numbers of ways. The role of the selected lipoproteins can then be investigated in phenotypic assays.

6.1.1. Colonisation by *C. difficile*

Colonisation is an early and critical step in the pathogenic process of CDI. This is supported by a study carried out in a hamster model of infection: colonisation of the intestinal tract by a nontoxigenic strain prevented intestinal colonisation by another toxigenic strain in a subsequent challenge which protected the animals from developing disease (Sambol *et al.* 2002). Interaction of the bacterial surface proteins with intestinal tissues of the host is necessary for adherence of the pathogen. Therefore, surface proteins are potential colonisation factors and are considered to contribute to the virulence of *C. difficile* (Rupnik *et al.* 2009). Although, a number of colonisation factors have been identified and characterised in other bacterial pathogens (Hoiczyk *et al.* 2000, Moschioni *et al.* 2010, Rogers *et al.* 2011, Romero-Steiner *et al.* 2003, Stalhammar-Carlemalm *et al.* 1999), little is known about the colonisation mechanism of *C. difficile*. To date, only a few molecular determinants of *C. difficile* involved in adhesion have been identified, including the following: surface-layer proteins (Calabi *et al.* 2001, Calabi *et al.* 2002, Cerquetti *et al.* 2000, Eveillard *et al.* 1993, Karjalainen *et al.* 2001, Waligora *et al.* 1999), flagellar proteins (Tasteyre *et al.* 2001a, Tasteyre *et al.* 2001b),

cell wall proteins, such as Cwp66 (Waligora *et al.* 2001) and Cwp84 (Janoir *et al.* 2007), a fibronectin binding protein (Fbp68) (Hennequin *et al.* 2003), the GroEL heat shock protein (Hennequin *et al.* 2001a, Hennequin *et al.* 2001b); and the recently described CbpA collagen binding protein (Tulli *et al.* 2013).

6.1.2. Lipoproteins and ABC-type transporters involved in colonisation of bacterial species

Previous studies have demonstrated the contribution of lipoproteins to the adherence of several bacterial pathogens which highlight the importance of lipoproteins in virulence. The multifunctional PsaA (pneumococcal surface adhesion A) of *S. pneumoniae* is one of the best characterised lipoproteins involved in adherence and currently is extensively studied as a potential vaccine component. PsaA is a substrate-binding protein of an ABC-type transporter specific for Mn^{2+} (Rajam *et al.* 2008). Furthermore, PsaA acts as an adhesin with E-cadherin being its putative receptor on nasopharyngeal epithelial cells (Anderton *et al*. 2007) and plays key role in pneumococcal virulence. Other lipoproteins that play a role in the adherence of *S. pneumoniae* have been described. For example, AmiA, PlpA, OppA, all of these are components of ABC transport systems (Cundell *et al.* 1995), and the lipoproteins PpmA and SlrA (Cron *et al.* 2009, Hermans *et al.* 2006) have been demonstrated to contribute to pneumococcal colonisation. Lipoproteins of ABC-type transporters have been shown to be directly or indirectly involved in the adherence of several other pathogens, such as *Streptococcus parasanguis* (FimA) (Fenno *et al.* 1989, Fenno *et al.* 1995), *S. sanguis* (SsaB) (Ganeshkumar *et al.* 1993, Ganeshkumar *et al.* 1991), *S. gordonii* (ScaA) (Kolenbrander *et al.* 1994) and *Mycoplasma hominis* (OppA) (Hopfe *et al.* 2011).

Three lipoproteins of *B. burgdorferi,* DbpA and BmpA extracellular matrix binding proteins (decorin and laminin-binding, respectively) (Blevins *et al.* 2008, Verma *et al.*

2009) and OmpA, contribute to bacterial adherence in ticks (Neelakanta *et al.* 2007). Furthermore, the role of the Lsp lipoprotein of S*treptococcus pyogenes* (named Lsp for lipoprotein of *Streptococcus pyogenes*) in binding to eukaryotic cells (pulmonary epithelial cells) and extracellular matrix components (fibronectin, laminin) has been demonstrated (Elsner *et al.* 2002). The first adhesin identified in *B. bifidum*, BopA (Guglielmetti *et al.* 2008) is also the member of the lipoprotein family like the well-characterised IlpA adhesin of *V. vulnificus* (Lee *et al.* 2010). To date, the role of lipoproteins in the adherence of *C. difficile* has not been studied.

6.1.3. Aim of this study

It has been described in the previous chapter that lipoprotein processing by Lgt is necessary for the adherence of *C. difficile*, which suggests that lipoproteins may have a role in adhesion. This study aimed to identify lipoprotein(s) with the potential of being involved in the adherence of *C. difficile* and to investigate the role of the candidate lipoproteins in adhesion.

6.2. Results

6.2.1. *In silico* identification of the CD0873 lipoprotein as a putative adhesin of *C. difficile*

In order to identify lipoproteins that might play a role in the adherence of *C. difficile*, I searched for genes in the published transcriptomic data that are upregulated during infection and encode elements of ABC-transport systems that have lipoprotein components. Transcriptional changes in *C. difficile* after infection of Caco-2 cells have been evaluated by Janvilisri *et al.*. (Janvilisri *et al.* 2010) who showed that expression levels of two ATP-binding proteins (CD0874, CD0301) were increased 60 minutes after infection. Both proteins are components of ABC-type transporters (CD0875-CD0873

and CD0302-CD0300) and comprise lipoproteins as solute-binding proteins (CD0873 and CD0300, respectively).

I next sought to determine whether *C. difficile* has an orthologue of the lipoprotein PsaA of *S. pneumoniae*, arguably the best characterised bacterial adhesin and solute-binding lipoprotein of an ABC-type transporter. A BLASTP search was performed with the amino acid sequence of PsaA (SP_1650) as the query sequence to probe solute-binding lipoproteins of *C. difficile* 630. This revealed that CD0873 was the most similar of all *C. difficile* solute-binding lipoproteins to PsaA (Table 6.1).

Table 6.1. Identity of the amino acid sequence of PsaA from *S. pneumoniae* to the amino acid sequences of solute-binding lipoproteins from *C. difficile* 630.

Sequence	Length	Amino acid identity (%)
SP_PsaA	309	
CD0873	340	21
CD2365	363	20
CD0876	345	20
CD1589	320	20
CD2999	379	19
CD0999	331	19
CD0300	355	19
CD3528	355	19
CD1979	337	19
CD2953	349	19
CD2878	312	19
CD0869	270	19
CD3414	436	18
CD1873	425	18
CD1027	350	18
CD3525	355	18
CD0750	271	18
CD3268	280	18
CD2177	267	18
CD1774	267	18
CD2311	270	18
CD1491	263	17
CD2174	265	17
CD0855	522	16
CD2672	519	16
CD3200	342	16
CD1484	330	16
CD2989	319	15

To further investigate the potential of CD0873 for adhesin function, amino acid sequence of the CD0873 protein (340 amino acids containing a 23-amino acid lipoprotein signal peptide cleavage motif) was used as a query sequence in a BLAST search against all protein sequences in RCSB PDB. This resulted in two protein hits: SP_1069 of *S. pneumoniae* and VC_1101 of *V. cholerae*. CD0873 has 33 % (120/353)

and 35 % (122/340) amino acid sequence identity, to these proteins, respectively. SP_1069 and VC_1101 are annotated as having a metal binding motif (GO: 0046872) as does PsaA of *S. pneumoniae*. In addition, tryptophan and phenylalanine as ligands were described for SP_1069 and VC_1101, respectively (www.rcsborg/pdb).

The hypothetical structure of CD0873 was predicted using SWISS-MODEL server (www.swissmodel.expasy.org). This search generated two structure models which was based on two protein templates, the SP_1069 (E value: 0.00e-1), and the BAB2_0673 lipoprotein (E value: 2.90e-7) of *Brucella abortus* (Figure 6.1). However, the potential error for the model is high in the latter case. Similarly to PsaA and VC_1101, BAB2_0673 also has a metal cation ligand (manganese, zinc and magnesium ion, respectively).

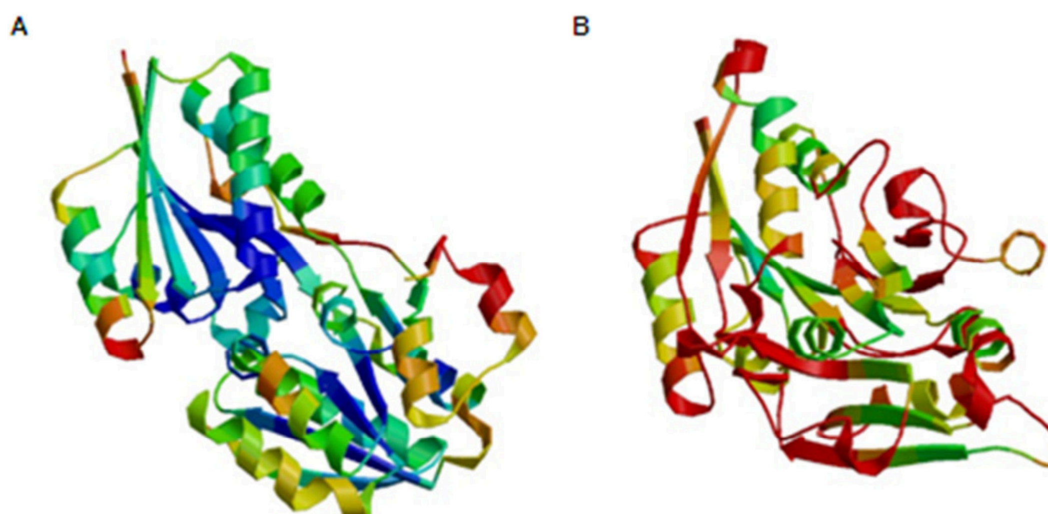


Figure 6.1. Hypothetical models for the structure of the CD0873 protein from *C. difficile* 630. Structure of the CD0873 protein was predicted using SWISS-MODEL server. Structure models are based on the structure of the (A) SP_1069 protein of *S. pneumoniae* and that of the (B) BAB2_0673 protein of *Brucella abortus*. Colours indicate the estimated inaccuracy of the model using a colour gradient from blue (more reliable regions) to red (potentially unreliable regions). Global score of the whole model is 0.734 for model A (~SP_1069) and 0.32 for model B (~BAB2_0673), which reflect the reliability of the predicted model ranging from 0 (less reliable) to 1 (more reliable).

6.2.2. Interaction of the recombinant CD0873 protein with metal ions and amino acids

On the basis of the sequence and/or structure similarities of the CD0873 protein to PsaA, SP_1069, VC_1101 and BAB2_0673, I aimed to experimentally investigate whether metal cations and amino acids interact with CD0873. The recombinant CD0873 protein was purified, the His-tag was cleaved by TEV protease and removed as described in Chapter 2 (Figure 6.2) and the protein was subjected to protein thermal shift assays using differential scanning fluorometry (DSF). Interaction of a protein with other molecules can affect protein stability, for example by changing the protein structure and its conformational stability, which can cause changes in the melting temperature (T_m) of the protein. Protein thermal shift assays are used to investigate protein-ligand interactions through monitoring thermostability of proteins in the presence of various compounds. Higher T_m value indicates greater stability of a protein. Ligands usually increase protein stability. However, there are reports for protein destabilisation in the presence of the ligands (Cimmperman *et al.* 2008).

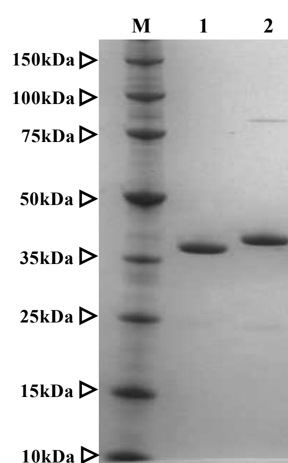


Figure 6.2. SDS-PAGE of the recombinant CD0873 protein. The histidine-tagged recombinant CD0873 (Lane 2) was produced as described in Chapter 4 and the histidine tag was removed by treating the purified protein with TEV protease (Lane 1). Proteins were subjected to SDS-PAGE and stained with SimplyBlue™ SafeStain. Lane M, protein ladder. The difference in the size of the proteins indicates that cleavage of the histidine tag from the recombinant CD0873 protein was successful.

Initially, the T_m of CD0873 was determined in various buffers recommended for initial screening by Niesen *et al.*. (2007) (Niesen *et al.* 2007) (Appendix III). As indicated in Figure 6.3, the protein seemed to be stable in almost all the tested buffers apart from the ones creating extreme conditions (e.g., high pH) when the protein was destabilised. Based on this result, I decided to use the reference buffer (100 mM HEPES in 150 mM NaCl, pH 7.5) in further experiments.

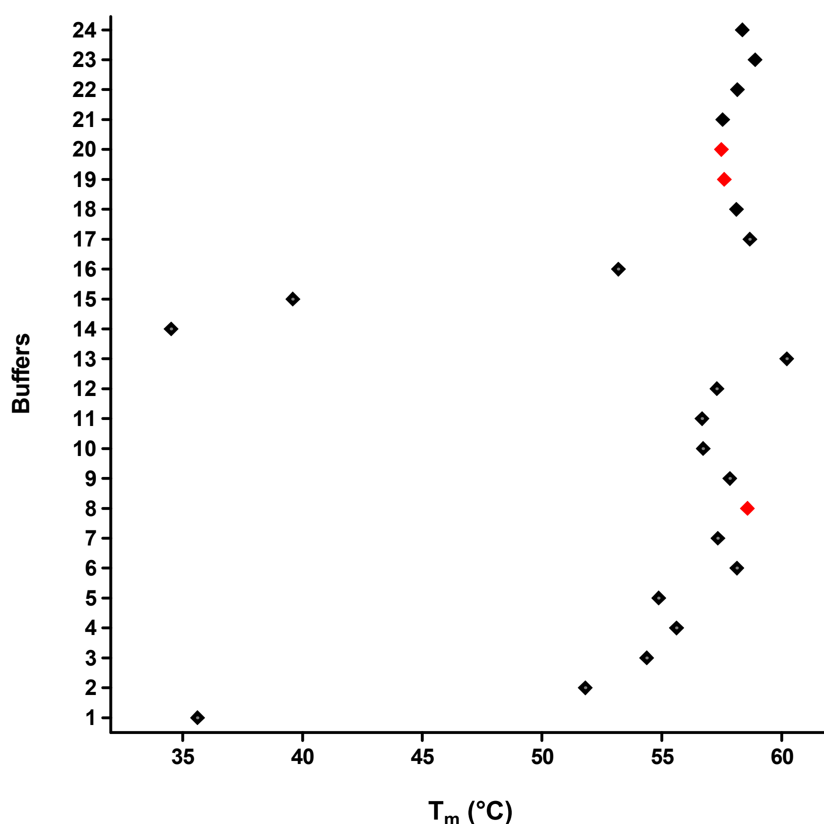


Figure 6.3. Determination of the stability of the recombinant CD0873 protein in various buffers. Melting temperature (T_m) of the recombinant CD0873 protein in 22 buffers (numbers correspond to buffers listed in Appendix III) was determined by thermal shift assay using differential scanning fluorometry. T_m indicated by red was measured in the reference buffer (100 mM HEPES in 150 mM NaCl, pH 7.5).

As metal ions are ligands of the PsaA, SP_1069, VC_1101 and BAB2_0673 proteins, which show amino acid sequence and/or structure similarities to the CD00873 protein, it was hypothetised that metal ions, particularly metal cations may interact with CD0873.

Several metal cations were screened by DSF to determine the T_m of CD0873 protein. Figure 6.4. shows that no significant changes in the melting temperature (T_m) of the protein were observed in the presence of Ca^{2+}, Mg^{2+} and Mn^{2+} ions. However, when Co^{2+} was added to the protein, the measured T_m value was slightly lower, while addition of Fe^{2+} or Zn^{2+} caused more significant reduction in the T_m, relative to the protein only control.

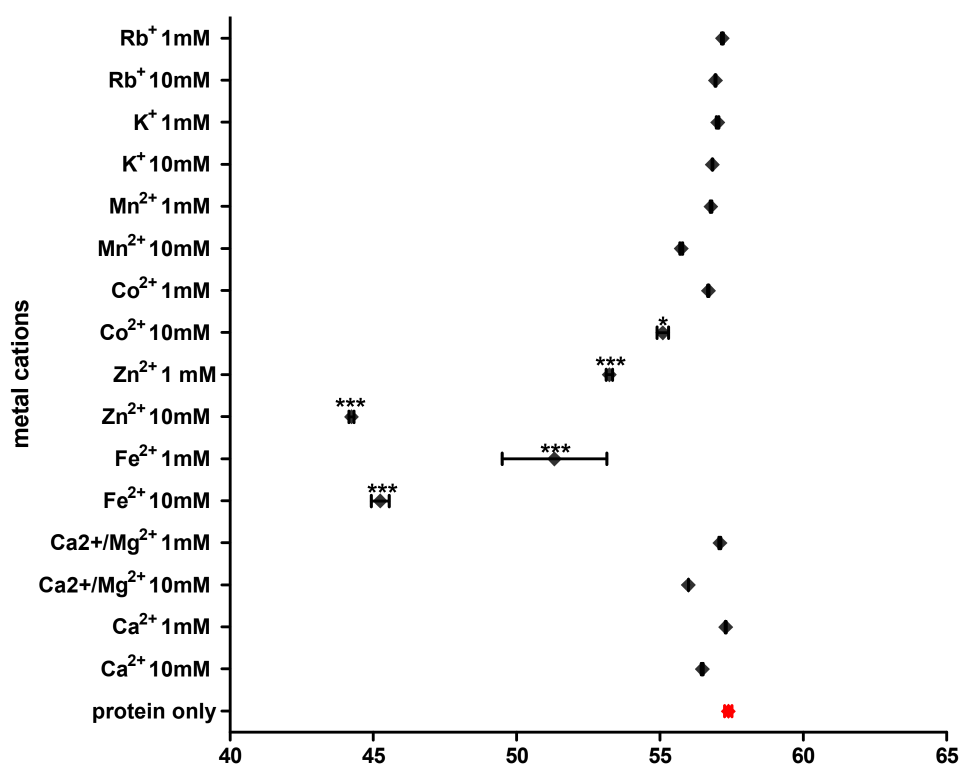


Figure 6.4. Determination of the stability of the recombinant CD0873 protein in the presence of metal cations. Melting temperature (T_m) of the recombinant CD0873 protein in the presence of $CaCl_2$, $CaCl_2$ + $MgCl_2$, $FeCl_2$, $ZnCl_2$, $CoCl_2$ and $MgCl_2$ were determined by thermal shift assay using differential scanning fluorometry. KCl and RbCl served as negative controls. P values were calculated using one-way ANOVA (n=2). Significant differences relative to the protein only control are indicated by asterisks (* $P<0.05$; *** $P<0.001$). The graph shows that T_m of the recombinant CD0873 decreased in the presence of Fe^{2+} and Zn^{2+} and Co^{2+}.

The effect of iron and zinc ions on the melting temperature of CD0873 protein was further investigated by addition of these ions in various concentrations to the protein.

Figure 6.5.A shows that the T_m values decreased gradually in a dose-dependent manner when Fe^{2+}, Fe^{3+} or Zn^{2+} were added to the protein. This reduction in the T_m values with increasing metal concentrations suggests destabilization of the protein. To visualize these effects, the CD0873 protein was incubated with Fe^{2+}, Fe^{3+} and Zn^{2+} and subjected to native PAGE (Figure 6.5.B). Migration of the protein indicates that CD0873 forms a protein dimer under native conditions (~75 kDa) since the predicted molecular weight of CD0873 is ~37 kDa. The mobility of CD0873 was not affected in the presence of iron and zinc ions indicating that the protein remained in dimer form. However, decreasing intensity of the protein bands with increasing concentrations of ferrous and ferric ions suggests that these ions destabilise the protein in a dose dependent manner. As indicated in Figure 6.5.B, CD0873 was more susceptible to Fe^{3+}, as complete destabilisation of CD0873 was observed at 20 mM of Fe^{3+}, while a portion of the protein remained intact when 50 mM of Fe^{2+} was added. Despite the dose-dependent reduction in T_m, CD0873 does not seem to undergo major structural changes in the presence of Zn^{2+} since no decomposition or `band-shift` was observed after native PAGE.

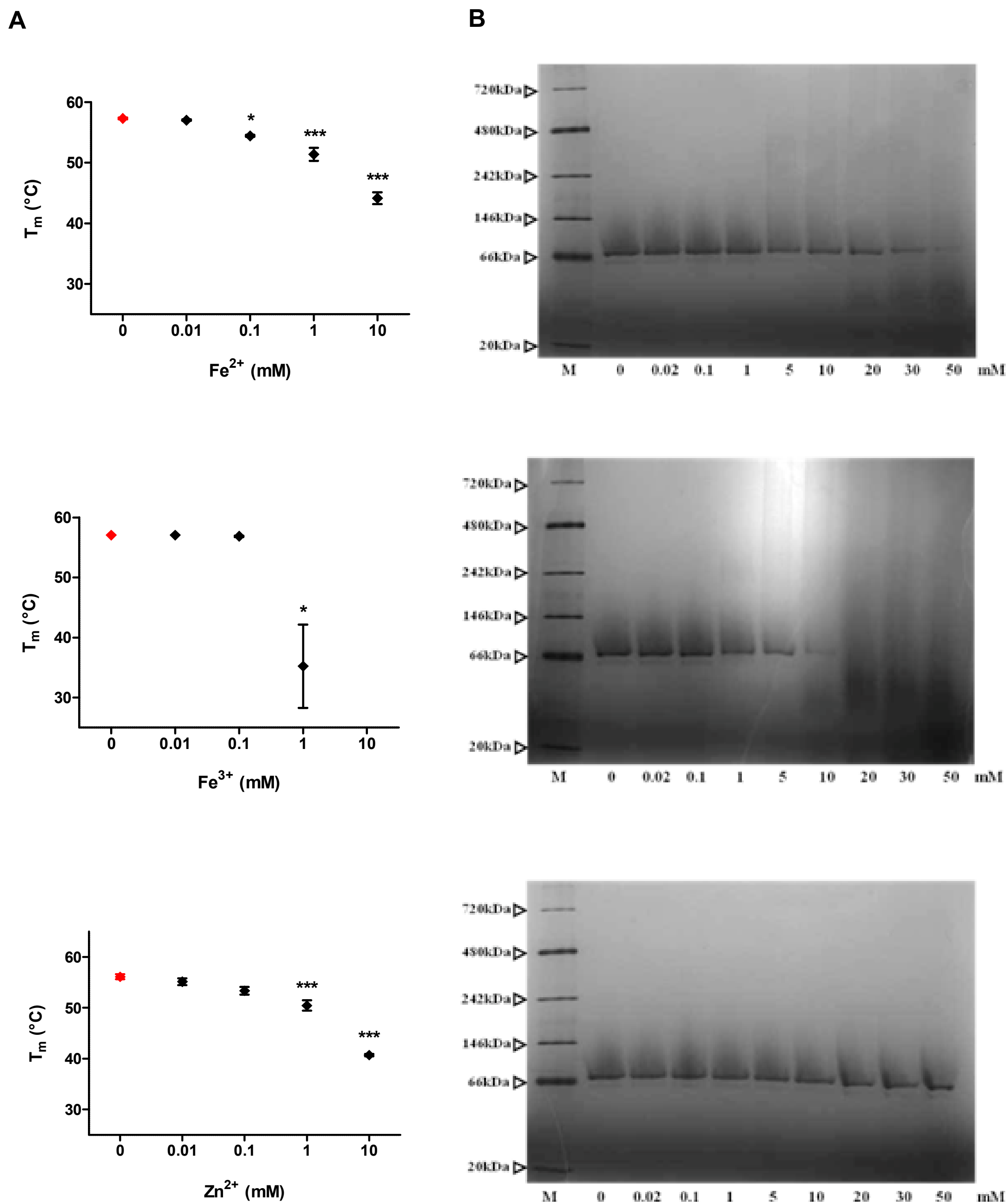


Figure 6.5. The recombinant CD0873 protein is destabilized in the presence of ferrous and ferric ions. Stability of the recombinant CD0873 protein in the presence of Fe^{2+}, Fe^{3+} and Zn^{2+} was evaluated by (A) thermal shift assay. Melting temperature (T_m) of the CD0873 protein (0.1 mg/ml, ~2.7 mM) in the presence of 0.01, 0.1, 1 or 10 mM of Fe^{2+}, Fe^{3+} or Zn^{2+} was determined using differential scanning fluorometry. Data are means with standard error of means of two independent experiments carried out in duplicates. Asterisks indicate change in the T_m which was significantly different relative to control (*$P<0.05$; *** $P<0.001$; one-way ANOVA). (B) and by native PAGE. The CD0873 protein (5 μM) was incubated with Fe^{2+} (top), Fe^{3+} (middle) or Zn^{2+} (bottom) in the 0.02 - 50 mM concentration range and subjected to native PAGE and Coomassie staining. Molecular weight standard is indicated on the left. Position of the protein bands corresponds to the size of the protein dimer.

I next addressed the question of whether amino acids affect the stability of CD0873. To investigate this, 19 amino acids were screened by DSF. As shown in Figure 6.6, stability of the CD0873 protein increased in the presence of phenylalanine and histidine ($\Delta T_{m(Phe)}$= 1.672 ± 0.1876, $\Delta T_{m(His)}$= 0.3247 ± 0.003315). Considering the sequence similarity of the CD0873 protein to the VC_1101 protein, these results suggest interaction of the CD0873 protein with phenylalanine.

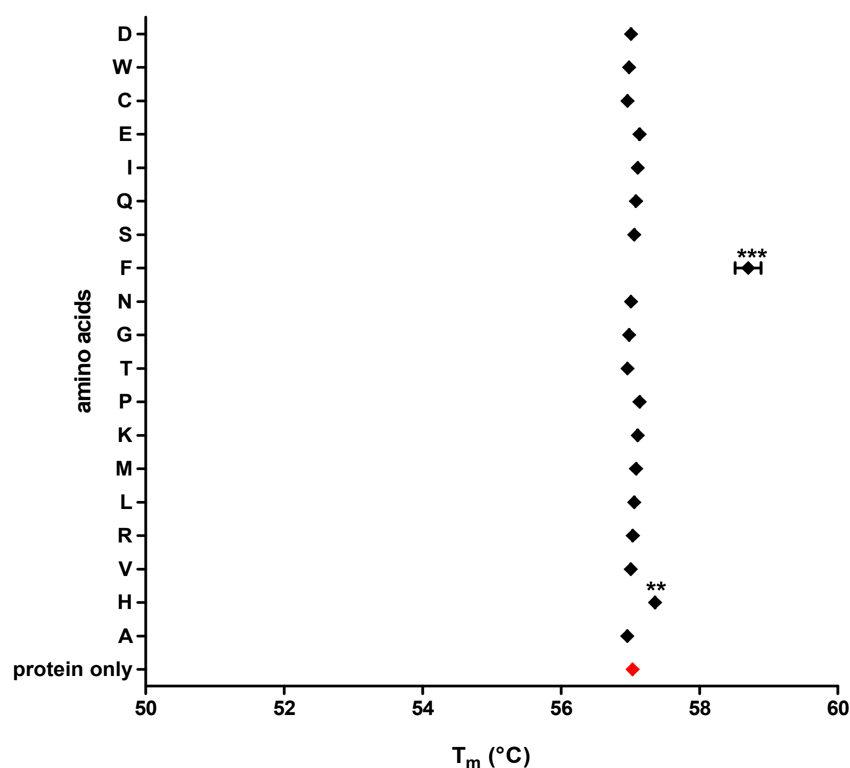


Figure 6.6. Determination of the stability of the recombinant CD0873 protein in the presence of amino acids. Melting temperature (T_m) of the recombinant CD0873 protein in the presence of 19 amino acids (indicated by single-letter amino acid codes on the left) was determined by thermal shift assay using differential scanning fluorometry. Significant differences relative to the protein only control are indicated by asterisks (** P<0.01; *** P<0.001; one-way ANOVA). The graph indicates that the T_m of the recombinant CD0873 protein increased in the presence of phenylalnine.

Taken together, DSF and native PAGE results suggest interaction of the CD0873 protein with phenylalanine, ferrous, ferric and zinc ions.

Crystallography of the CD0873 protein was also attempted but, in spite of several attempts, the protein failed to crystallise and I was unable to solve the crystal structure of the protein. One reason for the failure of the CD0873 crystallisation could be the extreme solubility of the protein. The highest achieved concentration of the recombinant protein was more than 300 mg/ml and the protein was still in solution. Another reason could be the extreme resistance of the protein. This was investigated by protease treatment of the recombinant protein using chymotrypsin, trypsin and proteinase K, which is shown in Figure 6.7. Resistance of CD0873 was also reflected in the thermal shift assays, where the T_m of the protein was not highly affected under most of the tested conditions (Figure 6.3, 6.4, 6.6). T_m of the protein decreased under certain conditions but this does not promote the formation of stable protein crystals.

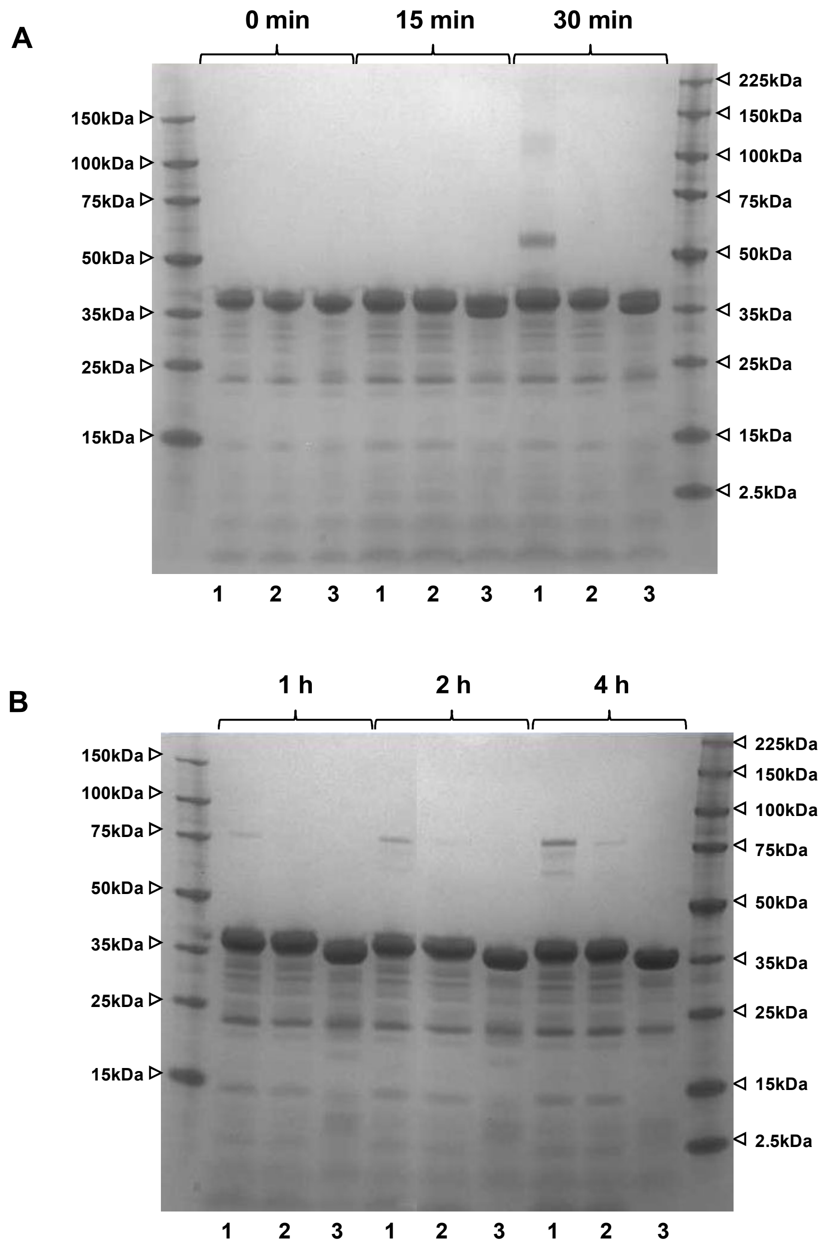


Figure 6.7. Effect of protease treatment on the decomposition of the recombinant CD0873 protein. 100 µl of 1 mg/ml CD0873 recombinant protein was mixed with 100 µl of 1 µg/ml of protease - chymotrypsin (Lane 1), trypsin (Lane 2) and proteinase K (Lane 3) - and incubated at 4℃ for periods of times (0, 15, 30 minutes, 1, 2 and 4 hours) when 20 µl an aliquot was removed from the samples and mixed with 20 µl of 2x Laemmli buffer. After boiling for 5 minutes, 17 µl of the mixture was subjected to SDS-PAGE and stained with SimplyBlue™ SafeStain. This image shows that the protease treatments did not have major effect on the structure of the CD0873 protein.

6.2.3. Generation and complementation of a CD0873 mutant of *C. difficile*

A CD0873 mutant of *C. difficile* (Cdi::CD0873) was generated by inactivating the CD0873 gene using the the pMTL007C-E2::Cdi-0873-317|318a retargeted plasmid in the ClosTron insertional mutagenesis system (Heap *et al.* 2010, Heap *et al.* 2007). In this construct, the group II intron is designed to be inserted into the CD0873 gene in antisense orientation after the 317th base pair. (Figure 6.8.B). To select the integrant clones, erythromycin-resistance colonies were picked and PCR analysis was performed to amplify the intron insertion site using gene-specific screening primer pairs (CD0873 F and CD0873 R) (Figure 6.8.C). Correct size of the PCR products with the CD0873 F and CD0873 R primers confirmed that the integration occurred: in the Cdi::CD0873 strain, a 2.1 kb DNA fragment was amplified, while the 253 bp amplicon showed the intact CD0873 gene in the Cdi::WT. DNA sequencing of the PCR product obtained from the mutant confirmed that the integration occurred into the desired site.

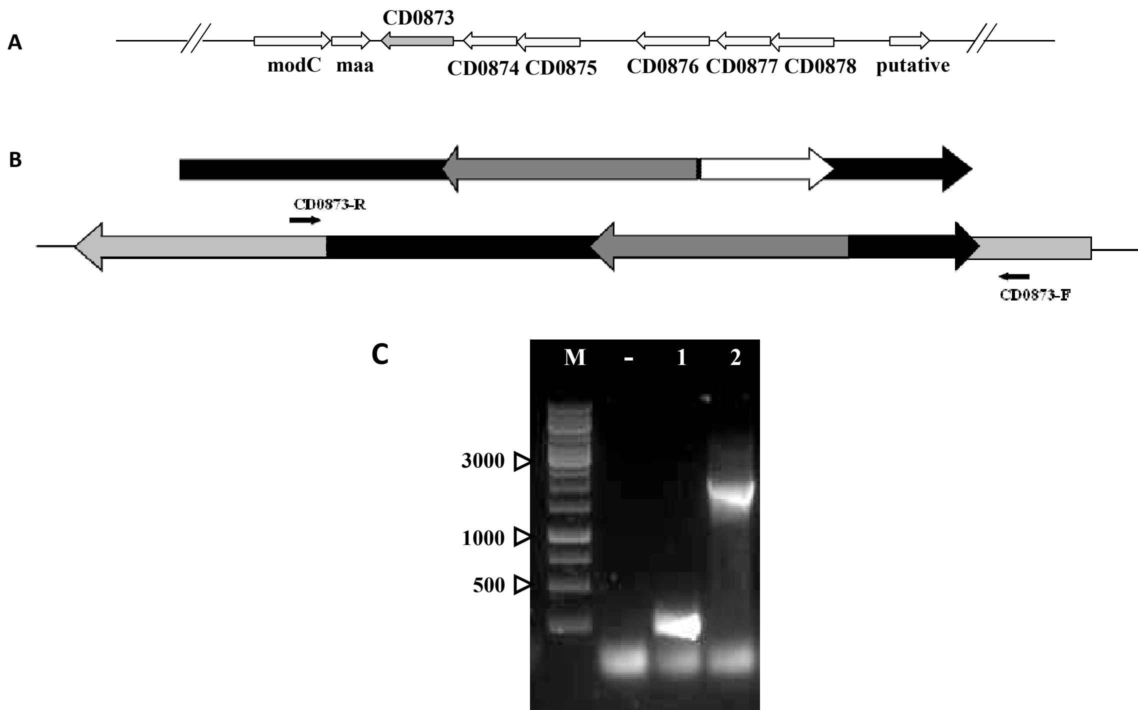


Figure 6.8. Construction and PCR confirmation of the CD0873 mutant *C. difficile*. (A) Schematic representation of the CD0873 region on the chromosome of *C. difficile*. (B) Schematic representation of the ClosTron system. The group II intron (black arrow) contains an erythromycin-resistance gene, *erm*RAM (dark grey arrow). Transcription from *erm*RAM is interrupted by a group I intron (white arrow) which splices out after integration of group II intron into the target gene occured, here into the CD0873 gene (light grey arrow). Primers amplifying the CD0873 gene around the integration site are indicated on the diagram. (C) Confirmation of the CD0873 mutant by PCR using the CD0873 F and CD0873 R primers. Lane 1, Cdi::WT (253 bp); Lane 2, Cdi::CD0873 (2102 bp); -, water (negative) control. Expected sizes of the products are indicated in the brackets. The molecular weight marker is indicated on the left in bp.

A complemented Cdi::CD0873 strain was generated by introducing the pRPF144::Cdi-0873 plasmid (Figure 6.9) into the Cdi::CD0873 strain. The pRPF144 plasmid was chosen for generation of the complementation construct because the pMTL84151 failed to restore some phenotypes of Cdi::*lgt* (see Chapter 3 and 5). Vector pRPF144 was designed and constructed at the Imperial College London and is based on the pMTL960 backbone (Fagan & Fairweather 2011). It contains the constitutive P_{cwp2} promoter (cloned with *Kpn*I and *Sac*I restriction enzymes) and the *gusA* gene along with its own ribosomal binding site (RBS) (cloned with *Sac*I and *Bam*HI). For complementation of the Cdi::CD0873, the CD0873 gene and 22 bp of the 5` upstream region containing the

ribosomal binding site (RBS, Shine-Dalgarno sequence) was amplified using the Cdi::WT genomic DNA as template, and CD0873 *Sac*I F and CD0873 *Bam*HI R oligonucleotides as primers. Primers were designed to allow subsequent cleavage of the fragment with *Sac*I at the 5` end and with *Bam*HI at the 3` end. The cleaved PCR product (from pGEM holding vector following sequencing) was ligated to the pRPF144 plasmid previously cut with *Sac*I and *Bam*HI. The ligation reaction was then transformed into *E. coli* CA434. Subsequently, pRPF144::Cdi-0873 was transferred from *E. coli* CA434 into Cdi::CD0873 by conjugation and positive complements (thiamphenicol resistant colonies) were screened by PCR.

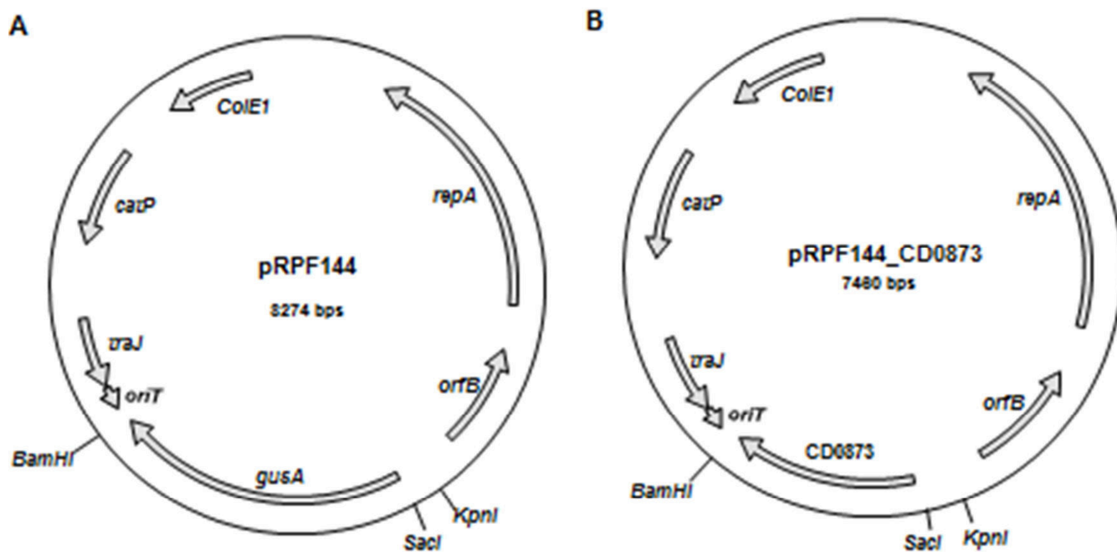


Figure 6.9. Map of the pRPF144 plasmid. (A) Plasmid pRPF144. The pRPF144 plasmid is based on the pMTL960 backbone and encorporates the constitutive P_{cwp2} promoter cloned with *Kpn*I and *Sac*I restriction enzymes, and the *gusA* gene and its ribosomal binding site (RBS) cloned with *Sac*I and *Bam*HI. (B) The pRPF144 plasmid encompassing the CD0873 structural gene and the gene`s own Shine-Dalgarno sequence. The g*usA* gene and its RBS is replaced by a 1,055 bp fragment encompassing the CD0873 gene and a 22 bp 5` region containing the Shine-Dalgarno sequence. TraJ, conjugal transfer protein; CatP, chloramphenicol acetyltransferase (conferring chloramphenicol resistance in *E. coli* and thiamphenicol -methyl-sulfonyl analogue of chloramphenicol- in *C. difficile*); ColE1, Gram-negative replicon; repA and orfB, Gram-positive replicon; GusA, β-D-glucuronidase from *E. coli* K12.

The Cdi::CD0873 and complemented Cdi::CD0873 strains were characterized by reverse transcriptase PCR (RT-PCR) to ensure that the CD0873 gene is no longer expressed and to check whether the expression of the genes upstream and downstream of the CD0873 gene are affected. RNA was extracted from *C. difficile* at early logarithmic phase. RT-PCR analysis of the strains showed continued transcription of the genes upstream and downstream of the CD0873 gene, lack of CD0873 transcription in the mutant and restored transcription from the CD0873 gene in the complemented strain (Figure 6.10).

Figure 6.10. Transcriptional analysis of the CD0873 gene, and the genes upstream and downstream of the CD0873 gene in the CD0873 mutant and the complemented CD0873 mutant of *C. difficile*. Transcriptional analysis was performed by RT-PCR using primer pairs listed in Table 2.4. Lanes: +, Cdi::WT DNA (positive) control; -, water (negative) control; 1, 3, 5, DNAse treated RNA; 2, 4, 6, cDNA; 1, 2, Cdi::WT; 3, 4, Cdi::CD0873; 5, 6, complemented Cdi::CD0873. Sizes of the RT-PCR products: *maa*-RT F, R: 509 bp; CD0873-RT F, R: 884 bp; CD0874-RT F, R: 255 bp.

To confirm that the CD0873 lipoprotein is absent in the Cdi::CD0873 strain, immunoblot analysis using antisera raised against the CD0873 protein was performed on whole bacterial cell extracts prepared by following the protocol described previously (Fagan & Fairweather 2011). Figure 6.11. shows that disruption of the CD0873 gene results in the absence of CD0873 lipoprotein from the *C. difficile* cells. In addition, presence of the CD0873 protein in the cell lysate of the complementated Cdi::CD0873 strain indicates that complementation successfully restored the absence of CD0873.

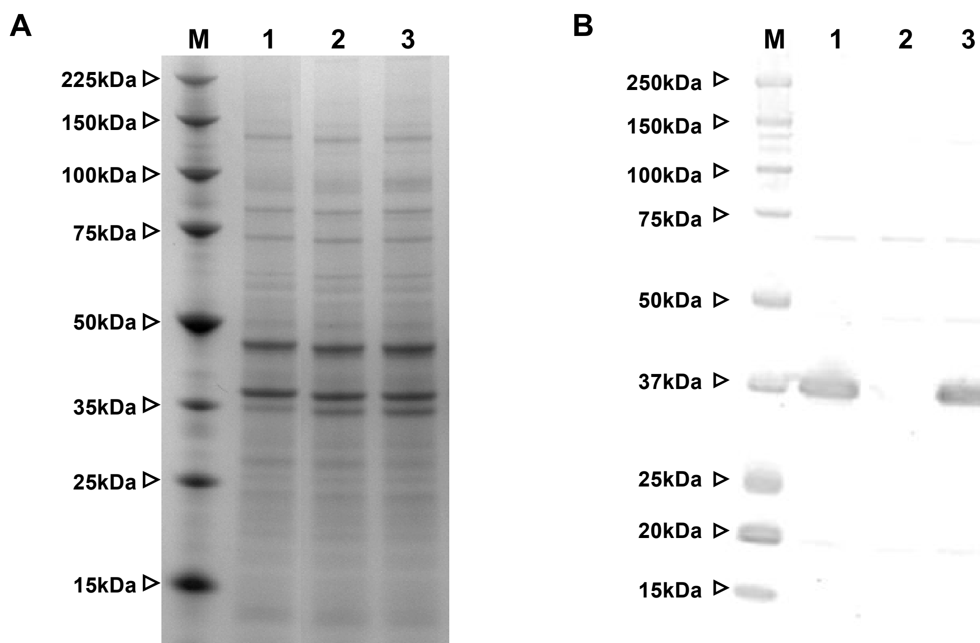


Figure 6.11. The CD0873 lipoprotein is not expressed in the CD0873 mutant of *C. difficile*. Total proteins were extracted from the Cdi::WT (Lane 1), Cdi::CD0873 (Lane 2) and complemented Cdi::CD0873 strains, separated by SDS-PAGE followed by staining with SimplyBlue™ SafeStain (A), and then probed with rabbit polyclonal anti-CD0873 antibodies (1:2,500) (B). Protein molecular weight marker is shown on lanes M. This image shows that the anti-CD0873 antibodies do not react with the whole cell lysates of Cdi::CD0873 but do react with the whole cell lysates of the complemented Cdi::CD0873 strain.

Taken together, PCR, Western blot and RT-PCR analyses indicated that the CD0873 gene was successfully disrupted and inactivated in the Cdi::CD0873 strain. Furthermore, it was also confirmed that the levels of transcription from the CD0873 gene and the production of the CD0873 protein are similar in the complemented Cdi::CD0873 to those in the Cdi::WT strain.

6.2.4. Effect of CD0873 mutation on the growth of *C. difficile*

Growth of the Cdi::CD0873 strain was determined in BHI liquid medium by measuring the change in OD_{590} and colony forming unit (CFU) over the time. Figure 6.12. shows the growth curves of the Cdi::WT and Cdi::CD0873 and indicates that the growth rate of *C. difficile* is not affected by CD0873 mutation in BHI medium.

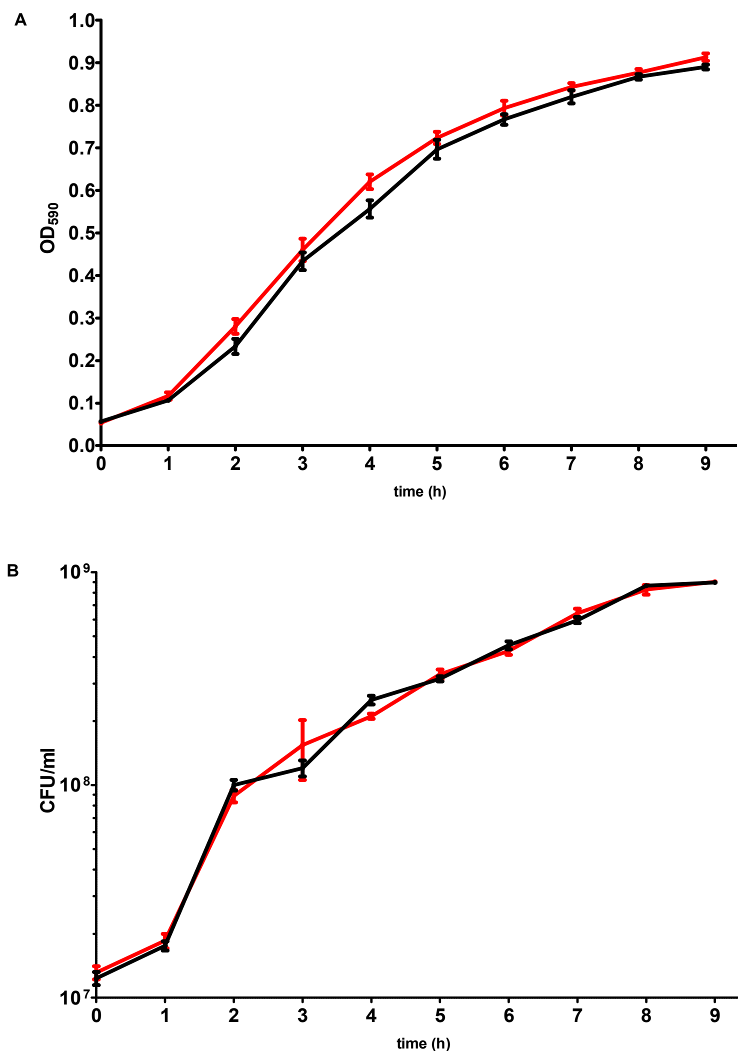


Figure 6.12. Comparison of the growth of the wild-type and CD0873 mutant *C. difficile* in BHI medium. Growth rate of the Cdi::WT (red line) and Cdi::CD0873 (black line) strains was assessed by following the optical densities of the cultures (A) and by determining the number of colony forming units (B) in time. Error bars indicate the standard errors of the means (n=2). Growth curves indicate similar growth of the strains.

To further investigate the interaction of the CD0873 protein with metal ions and to determine whether Fe^{2+}, Fe^{3+} or Zn^{2+} are substrates of CD0873, growth rates of the Cdi::WT and Cdi::CD0873 were compared in chemically defined media (CDM). $FeSO_4$ was omitted in CDM - Fe^{2+}, CDM - Fe^{2+} + Fe^{3+} and CDM - Fe^{2+} + Zn^{2+} and replaced with $FeCl_3$ in CDM - Fe^{2+} + Fe^{3+} and with $ZnSO_4$ in CDM - Fe^{2+} + Zn^{2+}, respectively. CDM was further supplemented with $ZnSO_4$ in CDM + Zn^{2+}. As indicated in Figures 6.13. and 6.14, mutation of the CD0873 gene caused a slightly decreased growth of *C.*

difficile in CDM but this difference was not significant (Table 6.2). Similarly, Cdi::CD0873 showed slower growth rate under Fe^{2+}, Fe^{3+} and Zn^{2+} deplete conditions than the Cdi::WT (Figure 6.13 and 6.14.A) but this reduction does not appear to be significant either, except at the 6h time point, when the growth of the Cdi::CD0873 in CDM was compared to the growth of the Cdi::CD0873 in CDM- Fe^{2+} (Table 6.2). Zn^{2+} repletion did not affect the growth of either the Cdi::WT or Cdi::CD0873 strains (Figure 6.14.B).

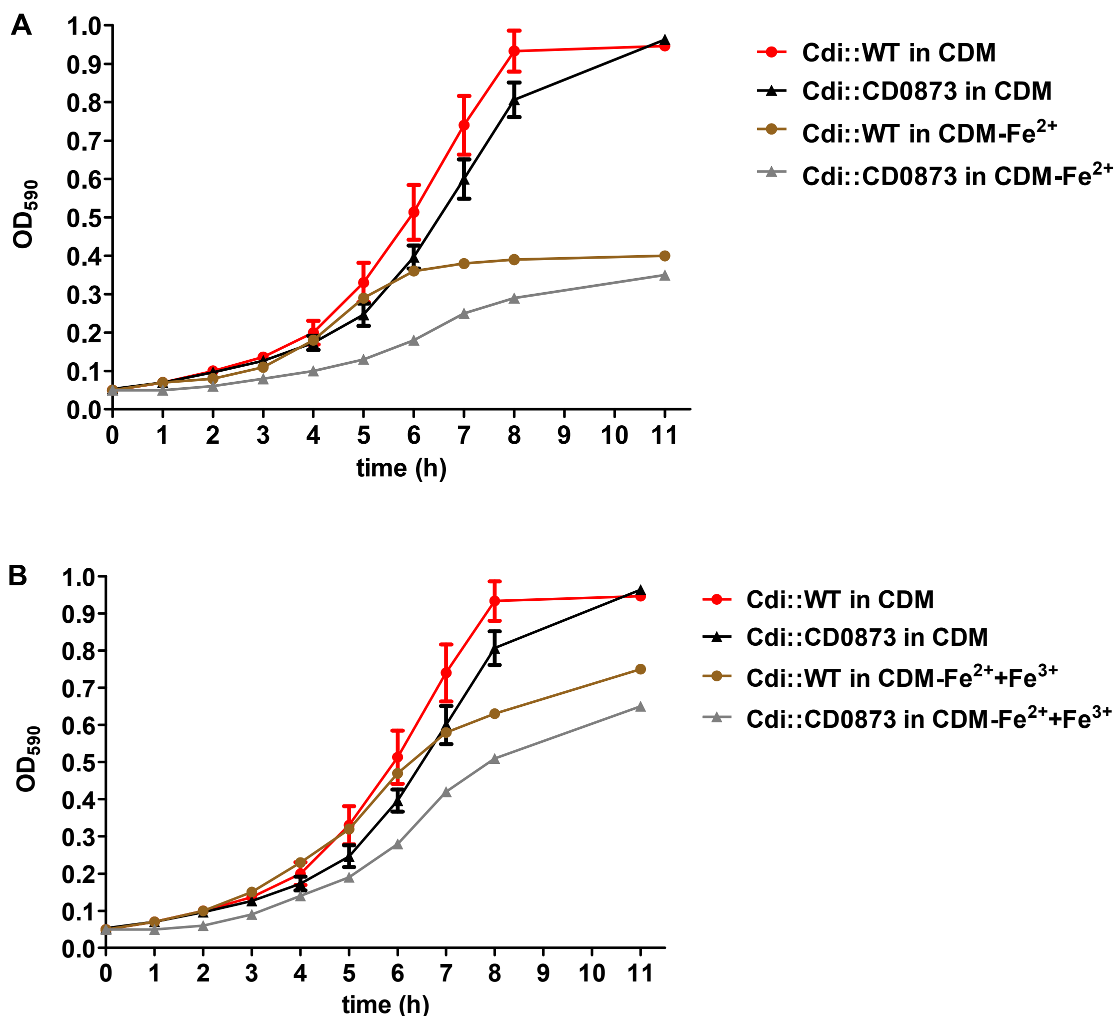


Figure 6.13. Effect of the presence of iron on the growth of the wild-type and the CD0873 mutant *C. difficile* in CDM. The Cdi::WT and Cdi::CD0873 strains were cultivated in CDM, CDM - Fe^{2+} or CDM - Fe^{2+} + Fe^{3+} medium. Growth rate of the strains was assessed by following the optical densities of the cultures over 11 hours. Composition of the CDM is shown in Table 2.1. $FeSO_4$ (15 µM) was omitted in CDM - Fe^{2+} and CDM - Fe^{2+} + Fe^{3+}, and replaced with $FeCl_3$ (15 µM) in CDM - Fe^{2+} + Fe^{3+}. Error bars indicate standard errors of the means (n=3). Statistical analyses of the curves are summarized in Table 6.2.

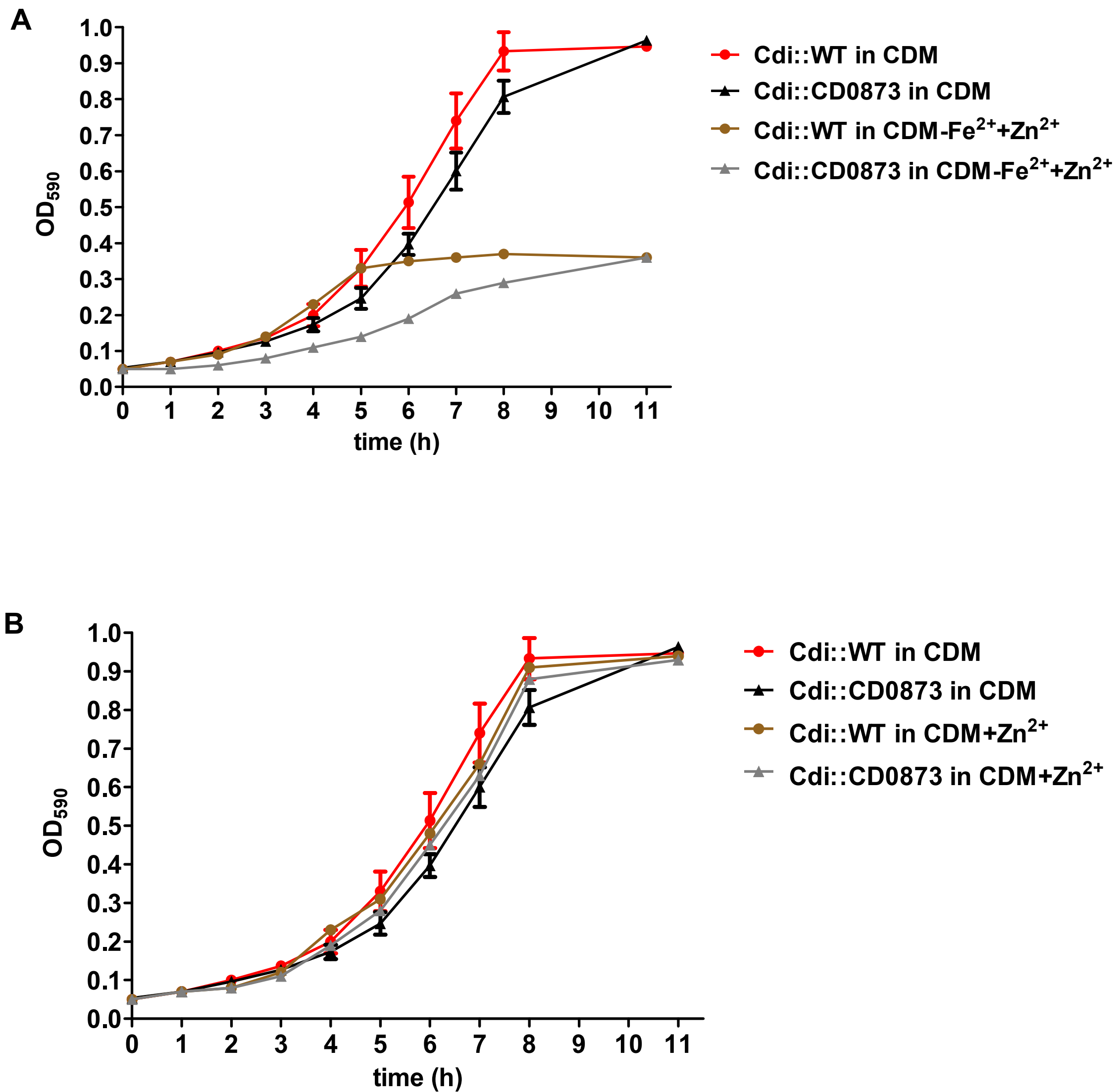


Figure 6.14. Effect of the presence of zinc on the growth of the wild-type and the CD0873 mutant *C. difficile* in CDM. The Cdi::WT and the Cdi::CD0873 strains were cultivated in CDM, CDM - Fe^{2+} + Zn^{2+} or CDM + Zn^{2+} medium. Growth rate of the strains was assessed by following the optical densities of the cultures over 11 hours. Composition of the CDM is shown in Table 2.1. $FeSO_4$ (15 µM) was omitted and replaced with $ZnSO_4$ (15 µM) in CDM - Fe^{2+} + Zn^{2+}. CDM was further supplemented with $ZnSO_4$ in CDM + Zn^{2+}. Error bars indicate standard errors of the means (n=3). Statistical analyses of the curves are summarized in Table 6.2.

Based on the findings of the thermal shift assay (Figure 6.6), phenylalanine was hypothetised to be the substrate of the CD0873 protein. To further investigate the interaction of the CD0873 protein with phenylalanine, growth rates of the Cdi::WT and Cdi::CD0873 strains were compared under phenylalanine-deplete condition in

chemically defined media (CDM). Although, the T_m of the recombinant CD0873 was not affected in the presence of tyrosine, growth of the Cdi::CD0873 under tyrosine-deplete condition was also investigated because tyrosine is highly similar to phenylalanine. Tyrosine was omitted in CDM - Tyr, phenylalanine was omitted in CDM - Phe, while CDM - Tyr - Phe was lacking both the tyrosine and the phenylalanine. CDM lacking tryptophan served as a negative growth control since tryptophan is essential for the growth of *C. difficile* (Karasawa *et al.* 1995). As expected, the growth of *C. difficile* was inhibited under tryptophan deficient conditions (data not shown). Interestingly, the growth rate of *C. difficile* decreased when tyrosine was lacking from the culture medium but the growth was not affected when both the phenylalanine and the tyrosine were omitted (Figure 6.15). Depletion of the tyrosine and the combination of the lack of the tyrosine and the phenylalanine had the same effect on the growth of the Cdi::CD0873 and the Cdi::WT (i.e., reduced growth in CDM-Tyr and unaffected growth rate in CDM-Tyr-Phe relative to growth in CDM). While growth of the Cdi::WT was not affected under phenylalanine deplete conditions, growth rate of the Cdi::CD0873 strain was slightly increased in CDM-Phe. Based on the effect of phenylalanine on the stability of the recombinant CD0873 protein (Figure 6.6), the CD0873 protein was presumed to be involved in the uptake of phenylalanine and growth of the Cdi::CD0873 strain was expected to be decreased under phenylalanine deficient condition. Nevertheless, the increase in the growth rate of Cdi::CD0873 was not significantly different from that of the Cdi::WT.

Taken together, growth of the Cdi::CD0873 strain was not significantly affected under iron and zinc ion, tyrosine and phenylalanine deplete conditions which suggest that CD0873 does not have a key role, if it has any, in the uptake of these molecules.

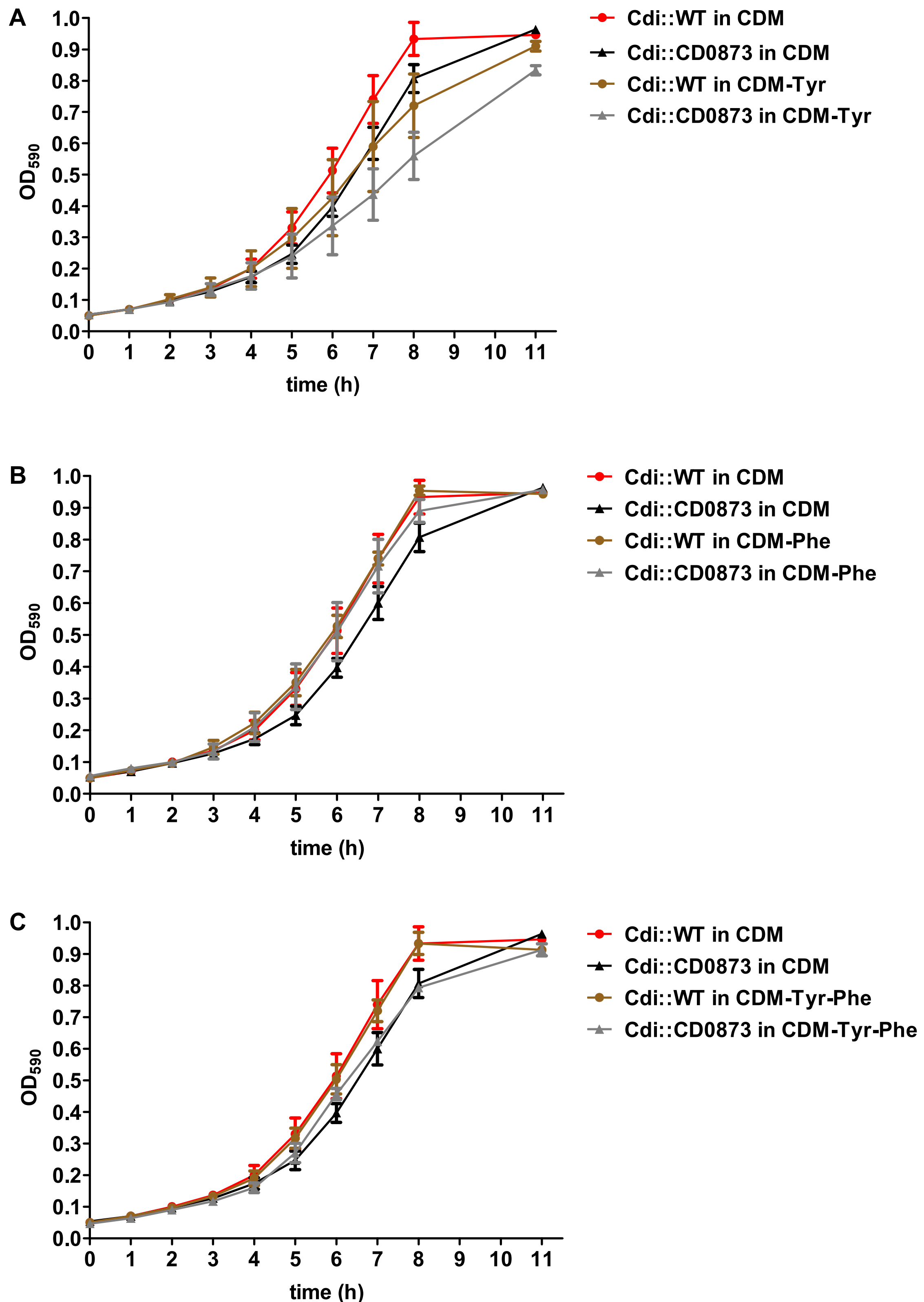


Figure 6.15. Effect of the presence of tyrosine and phenylalanine on the growth of the wild-type and the CD0873 mutant *C. difficile* in CDM. The Cdi::WT and the Cdi::CD0873 strains were cultivated in CDM, CDM – Tyr, CDM - Phe or CDM - Tyr - Phe medium. Growth rate of the strains was assessed by following the optical densities of the cultures over 11 hours. Composition of the CDM is shown in Table 2.1. Tyrosine (100 mg/l) was omitted in CDM - Tyr, phenylalanine (300 mg/l) was omitted in CDM - Phe, while CDM - Tyr - Phe was lacking both the tyrosine and the phenylalanine. Error bars indicate standard errors of the means (n=3). Statistical analyses of the curves are summarized in Table 6.2.

Table 6.2. Statistical analysis of the results of the CDM growth experiments (summary).The left panel shows the statistical analysis of the growth rate of the Cdi::WT versus that of the Cdi::CD0873 strain in various media. The right panel indicates the statistical analysis of the growth of the strains individually (left, Cdi::WT; right, Cdi::CD0873) in CDM versus in the various modified CDM. Significant differences between the growth rates of the strains are marked by red colour. Differences were determined using 2-way ANOVA with Bonferroni post tests (* P<0.05, ** P<0.01, *** P<0.001, ns = no significant difference at any time points).

Cdi::WT vs Cdi::CD0873 in	
CDM	ns
CDM-Fe^{2+}	ns
CDM-Fe^{2+}+Fe^{3+}	ns
CDM-Fe^{2+}+Zn^{2+}	ns
CDM+Zn^{2+}	ns
CDM-Tyr	ns
CDM-Phe	ns
CDM-Tyr-Phe	8h **

Cdi::WT in CDM vs Cdi::WT in	
CDM-Fe^{2+}	7h *** 8h *** 11h ***
CDM-Fe^{2+}+Fe^{3+}	8h **
CDM-Fe^{2+}+Zn^{2+}	7h *** 8h *** 11h ***
CDM+Zn^{2+}	ns
CDM-Tyr	ns
CDM-Phe	ns
CDM-Tyr-Phe	ns

Cdi::CD0873 in CDM vs Cdi::CD0873 in	
CDM-Fe^{2+}	6h * 7h *** 8h *** 11h ***
CDM-Fe^{2+}+Fe^{3+}	8h **
CDM-Fe^{2+}+Zn^{2+}	7h *** 8h *** 11h ***
CDM+Zn^{2+}	ns
CDM-Tyr	8h *
CDM-Phe	ns
CDM-Tyr-Phe	ns

6.2.5. The CD0873 lipoprotein is exposed to the bacterial cell surface

Lipoproteins are typically localised on the outer side of the cytoplasmic membrane in Gram-positive bacteria. In Chapter 3, the CD0873 lipoprotein was shown to be present in the cell membrane. This study aimed to determine whether the CD0873 lipoprotein is accessible on the cell surface. Using immuno-fluorescence microscopy (Rosanna Leuzzi, Novartis Vaccines and Diagnostics, Siena, Italy), it was demonstrated that the CD0873 antisera is able to bind to whole Cdi::WT cells (Figure 6.16), suggesting that CD0873 is exposed to the surface of the bacterial cells. In addition, lack of immunofluorescence in the Cdi::CD0873 strain confirmed that CD0873 is no longer present on the cell surface, while restoration of the signal showed that CD0873 is re-expressed and accessible on the surface in the complemented Cdi::CD0873 strain (Figure 6.16). However, distribution of the CD0873 protein in the complemented Cdi::CD0873 is slightly different to that of the Cdi::WT. While the protein is uniformly distributed on the surface of most of the Cdi::WT cells and arranged into small islets on only few of the Cdi::WT cells, CD0873 is typically arranged to the end of the rod-shaped cells in the complemented Cdi::CD0873. Taken together, these results indicate that CD0873 is attached to the cytoplasmic membrane and exposed to the bacterial cell surface giving the possibility of the protein to interact with external factors.

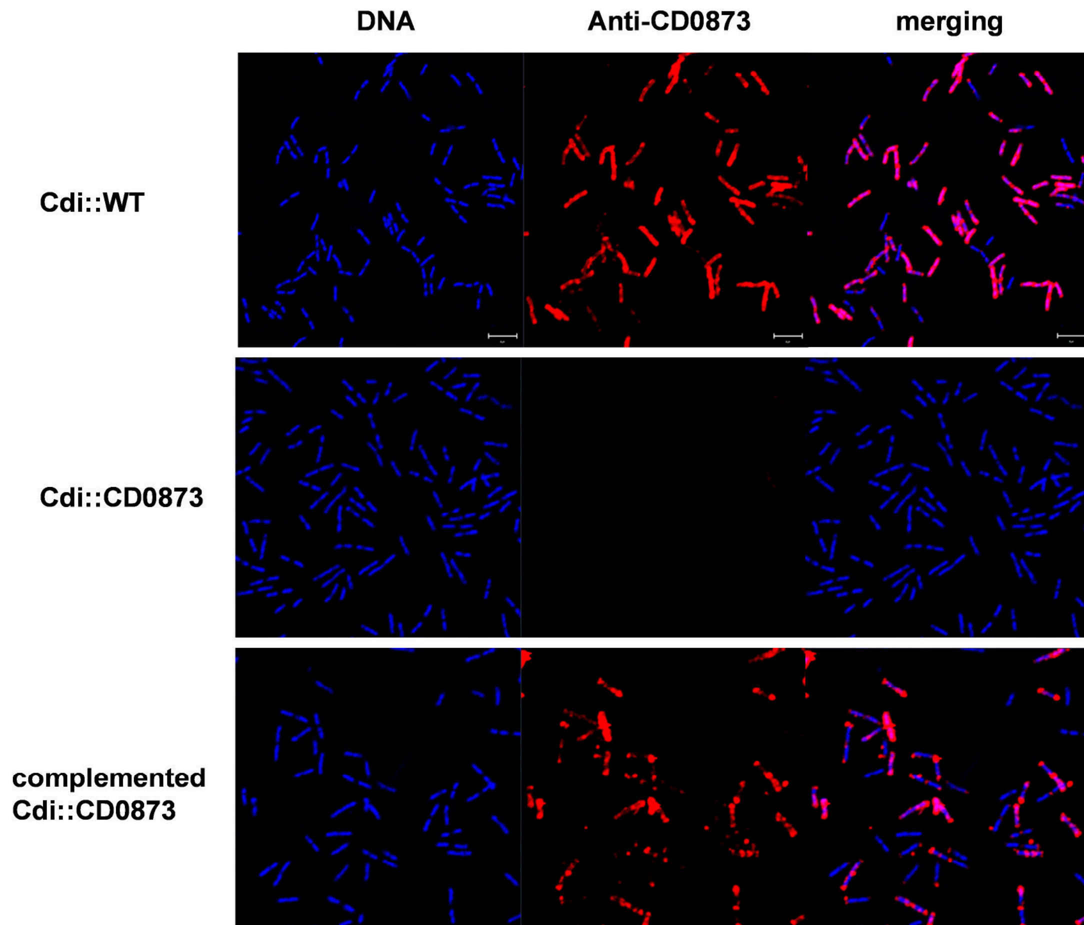


Figure 6.16. The CD0873 lipoprotein is exposed to the surface of *C. difficile*. Immunofluorescence microscopy of the Cdi::WT, Cdi::CD0873 and complemented Cdi::CD0873 strains using anti-CD0873 antibodies and Fluo568 conjugated anti-rabbit secondary antibodies (red). DNA was stained with DAPI (blue). This image shows that the CD0873 antibodies are able to bind to whole Cdi::WT cells but not to Cdi::CD0873 cells. Binding of the CD0873 antibodies to the surface of *C. difficile* can be restored by complementation.

6.2.6. Cytoaherence of *C. difficile* is inhibited by anti-CD0873 antibodies

Based on the observation of that CD0873 is exposed to the bacterial cell surface, I examined the role of the CD0873 protein in the interactions of *C. difficile* with Caco-2 cells. I aimed to investigate whether masking of CD0873 on the surface of *C. difficile* with antisera against CD0873 effects bacterial adherence to Caco-2 cells. Initially, viability of the Caco-2 cells in the presence of the rabbit immune sera under anaerobic conditions was determined. As observed by MTT assay, after incubation with the immune sera at 10^{-1} dilution, viability of the Caco-2 cells increased (Figure 6.17).

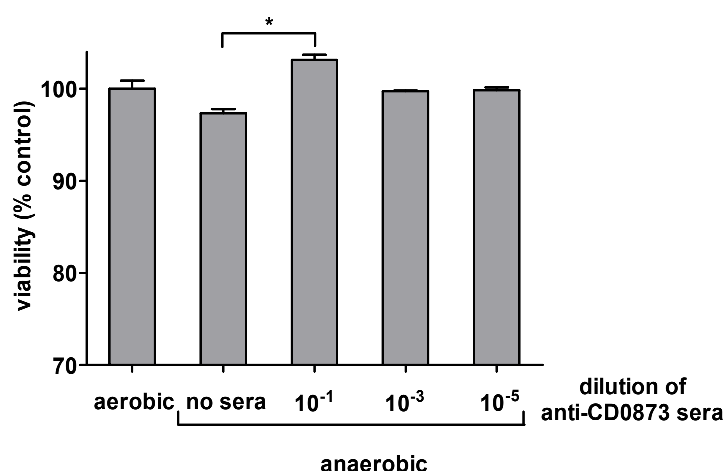


Figure 6.17. Determination of the viability of the Caco-2 cells in the presence of anti-CD0873 sera. Quantification of Caco-2 cell viability was performed by MTT assay under anaerobic conditions after 2 hours of incubation. MTT assay was conducted in triplicate. Significant difference is indicated by asterisk (*P<0.05; two-tailed t test).

Prior to the adherence assay, the Cdi::WT was incubated with anti-CD0873 sera serially diluted (10^{-1}, 10^{-3}, 10^{-5}) in EMEM for 30 minutes. When the Cdi::WT was pretreated with the CD0873-specific antisera, the number of bacteria bound to the cells was lower at each antibody dilution relative to the no sera control and the number of adhered bacteria decreased in a dose dependent manner when evaluated by CFU counts (Figure 6.18.A). Further investigations using an on-cell western assay revealed similar results

(Figure 6.18.B): when pretreated with the CD0873 immune sera, level of the bacterial adherence was significantly reduced relative to both the no sera and the pre-immune sera controls. These results suggest that anti-CD0873 antibodies inhibit the adherence of *C. difficile* to Caco-2 cells.

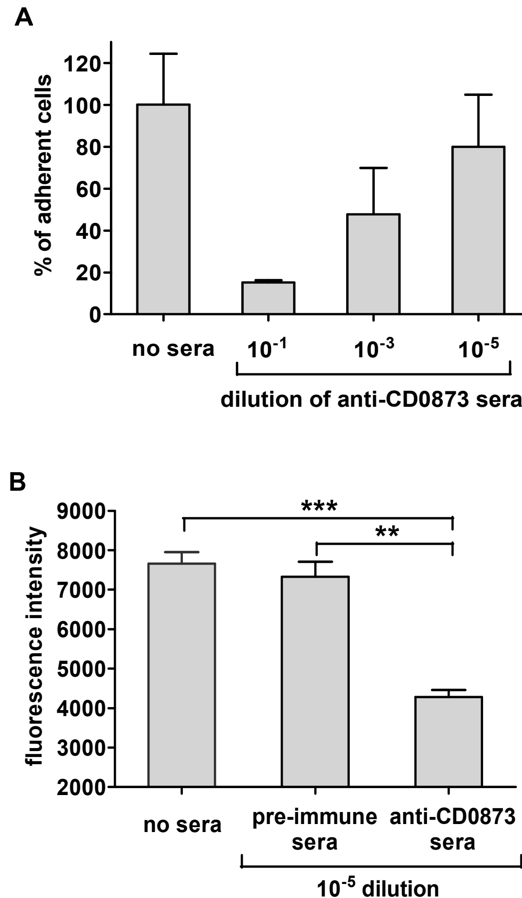


Figure 6.18. Adherence of *C. difficle* to Caco-2 cells is inhibited by CD0873 antibodies. (A) Adherence of the Cdi::WT to Caco-2 monolayers after the bacterial cells were preincubated with CD0873 antisera. Cell-binding was measured by CFU counts as described in the section 2.7.5.1. Results are presented as the proportion of the bacteria bound to the Caco-2 cells without pretreatment of the bacteria with CD0873 antisera. The assay was performed in triplicate in two independent experiments. (B) Adherence of the Cdi::WT to Caco-2 monolayers after preincubation of the bacteria with either CD0873 antisera or preimmune sera (n=3). Cell-binding was determined by on-cell western assay by measuring the fluorescence intensity as described in section 2.6.8. Intensities indicated on the graph are normalised to the intensities measured with the Caco-2 cells only control. 1, Caco-2 + Cdi::WT; 2, Caco-2 + Cdi::WT + CD0873 antisera (10^{-5} dilution); 3, Caco-2 only. Statistically significant differences are indicated by asterisks (n=3) (** $P < 0.01$; *** $P < 0.001$; one-way ANOVA).

6.2.7. Effect of CD0873 mutation on the adherence of *C. difficile* to Caco-2 cell line

The role of CD0873 in adhesion was further studied by investigating the ability of the Cdi::CD0873 strain to adhere to Caco-2 cells. After Caco-2 cells were incubated with the Cdi::WT strain for 2 hours, the number of bacteria adhered to 100 Caco-2 cells was 43.03 ± 3.63 (Figure 6.19.A). In the same experiment, in the case of the Cdi::CD0873 strain, the number of bacterial cells adhered to the Caco-2 cells was significantly lower, i.e., 0.51 ± 0.07 per 100 Caco-2 cells. However, complementation of the Cdi::CD0873 strain did not restore adherence phenotype of the mutant to that of the wild-type level. Adherence of the Cdi::CD0873 was further studied by immuno-fluorescence microscopy using anti-*C. difficile* serum (Magdalena Kasendra, Novartis Vaccines and Diagnostics, Siena, Italy). Adherence of *C. difficile* was reduced when Caco-2 cells were challenged with the Cdi::CD0873 strain compared to when Caco-2 cells were incubated with the Cdi::WT strain (Figure 6.19.B). Adherence of the Cdi::CD0873 strain could be restored to that of the Cdi::WT level after introduction of the pRPF144::Cdi-0873 complementation plasmid. The reason for the contradictory results observed using the complemented Cdi::CD0873 strain, in the same assay but obtained by two different methods, is not known. These results are in good agreement with the finding that the adherence of *C. difficile* is inhibited by CD0873 antibodies and suggest that the CD0873 lipoprotein is involved in adhesion.

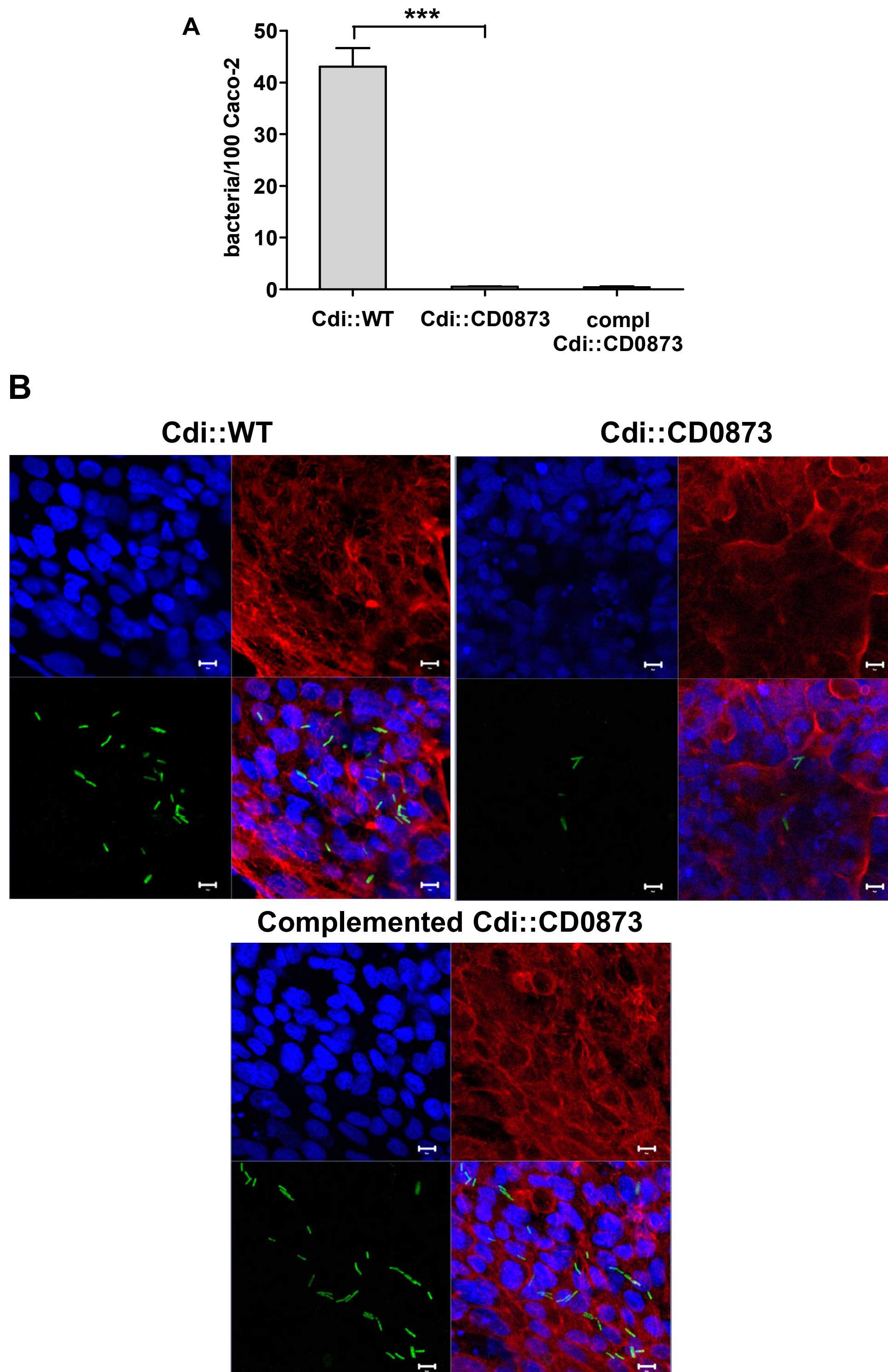


Figure 6.19. Mutation of the CD0873 gene results in reduced adherence of *C. difficile* to Caco-2 cells. (A) Adherence of the Cdi::WT, Cdi::CD0873 and complemented Cdi::CD0873 strains to Caco-2 monolayers measured by CFU counts. Values are mean numbers of the adherent bacteria per 100 Caco-2 cells with standard error of means of three separate experiments performed in triplicates. Statistical analysis between values for the Cdi::WT versus those for the Cdi::CD0873 and the complemented Cdi::CD0873 strains was performed by two-tailed T test (*** $P<0.001$). (B) Adherence of *C. difficile* assessed by confocal immunofluorescent microscopy. Bacteria were labeled by anti-*C. difficile* primary antibodies and secondary fluorescent antibodies (Alexa488, green). DNA and cellular actin were stained with DAPI (blue) and Phalloidin-Alexa568 (red), respectively.

6.2.8. The CD0873 lipoprotein binds to Caco-2 cells

6.2.8.1. Binding of the recombinant CD0873 protein to Caco-2 cells

In order to investigate whether CD0873 has a direct or indirect role in adhesion, interaction of the non-His tagged recombinant CD0873 protein with Caco-2 cells was studied. Recombinant form of the ε prototoxin (inactive, His-tagged) from *C. perfringens* (Bokori-Brown *et al.* 2013) was used as a negative (washing) control. After 2 hours of aerobic incubation and subsequent washing steps, Caco-2 cells were lysed and subjected to Western blot analysis using anti-CD0873 antibodies to determine whether the protein bound to the Caco-2 cells. Figure 6.20. clearly shows that the recombinant CD0873 protein was able to bind to Caco-2 cells. A lack of signal when Caco-2 cells were incubated with media only, and with the negative protein control, indicates specific binding of the CD0873 protein. Staining of the proteins separated by SDS-PAGE shows that similar levels of Caco-2 cell lysates were loaded suggesting that the absence of the immunoreactive bands in the negative controls (ε prototoxin or no protein) is not due to a difference in the amounts of the cell lysates analysed by Western blotting. This result provides evidence for that CD0873 is directly involved in adhesion and suggests that CD0873 is an adhesin of *C. difficile*.

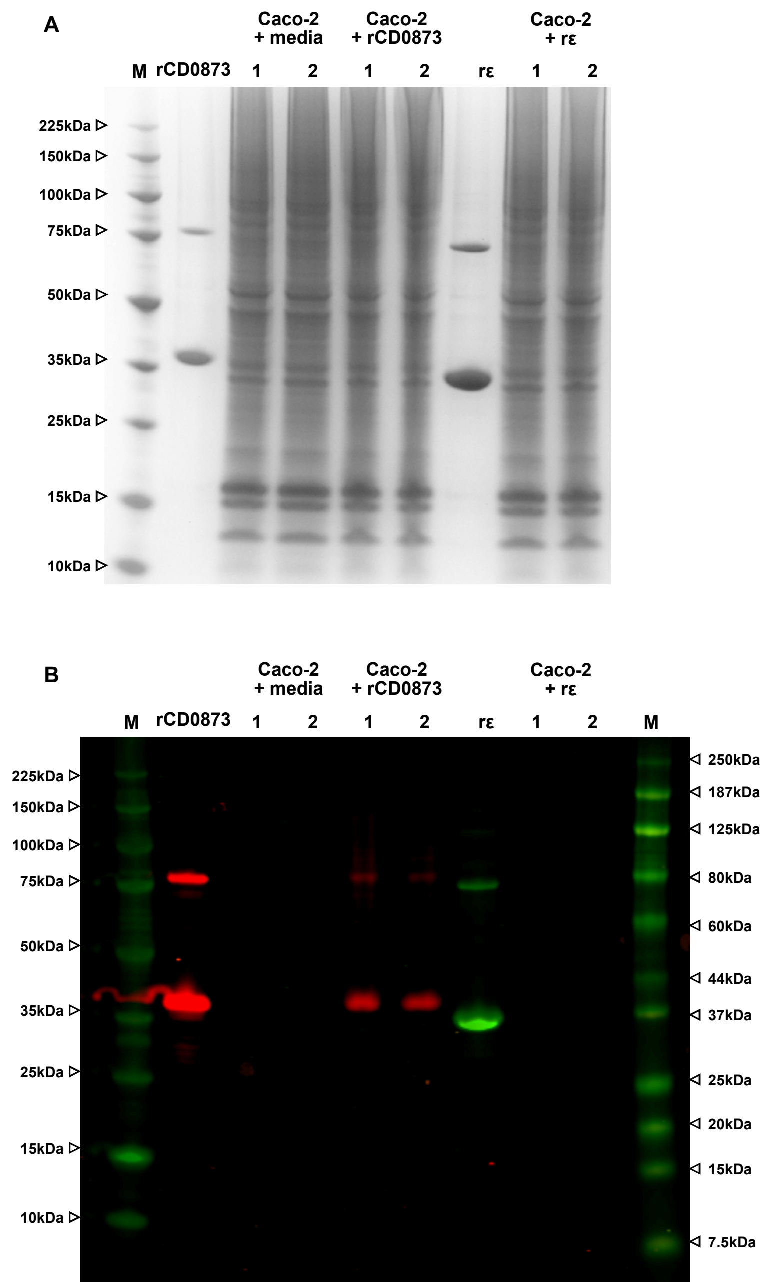


Figure 6.20. The recombinant CD0873 protein binds to Caco-2 cells. Binding of the recombinant CD0873 protein (rCD0873) to Caco-2 cells was determined by CD0873 immunoblot. Caco-2 cells (7 x 10^5) were incubated with 100 µg of rCD0873 for 2 hours (no protein and recombinant ε prototoxin of *C. perfringens* – rε – served as negative controls), then cells were washed, lysed and analysed by Western blotting using rabbit anti-CD0873 antibodies (1:2500) to see if Caco-2 cells were bound by the recombinant CD0873 protein. (A) Loading control of the binding assay. Proteins of Caco-2 cell lysates that were analyzed for protein binding were separated by SDS-PAGE and stained with SimplyBlue™ SafeStain. (B) CD0873 immunoblot of Caco-2 cells lysates. Lanes: rCD0873, rCD0873 alone; Caco-2 + media, Caco-2 cells alone; Caco-2 + rCD0873, Caco-2 cells with rCD0873; rε, rε alone; Caco-2 + rε, Caco-2 cells with rε; M, Protein ladder. Recombinant ε was detected by mouse anti-His antibodies. Technical replicates are indicated by numbers.

6.2.8.2. Binding of the native CD0873 lipoprotein to Caco-2 cells

Interaction of the native CD0873 lipoprotein with Caco-2 cells was demonstrated by incubating *C. difficile* bacterial lysates with Caco-2 cells followed by Western blot analysis as described above. The assay was performed by adding whole cell lysates of the Cdi::WT, Cdi::*lgt*, complemented Cdi::*lgt*, Cdi::*lspA* and Cdi::*lspB* to Caco-2 cells. The signals detected from the CD0873 immunoblot analysis is shown on Figure 6.21. Ponceau staining of the proteins transferred onto the nitrocellulose membrane shows similar levels of Caco-2 cell lysates suggesting that comparable amount of proteins were analysed by Western blotting. Immunoblot indicates that the CD0873 lipoprotein present in the total protein extract of the Cdi::WT, Cdi::*lspA* and Cdi::*lspB* strains is able to bind to the Caco-2 cells. In contrast, CD0873 expressed in the Cdi::*lgt* strain is not able to bind to the Caco-2 cells, while complementation of Cdi::*lgt* restored the binding ability of the protein. This finding is interesting in the context that CD0873 might possess the N-terminal signal peptide in the Cdi::*lgt* (Figure 3.10). Moreover, some of the CD0873 protein is not proteolytically processed in Cdi::*lspA* either (Figure 3.10) and only the smaller, cleaved form of the protein bound to the Caco-2 cells. These results suggest that CD0873 is unable to bind to Caco-2 cells when the signal peptide is not cleaved off from the protein proposing a negative role of the signal peptide in the binding of the CD0873 protein to Caco-2 cells.

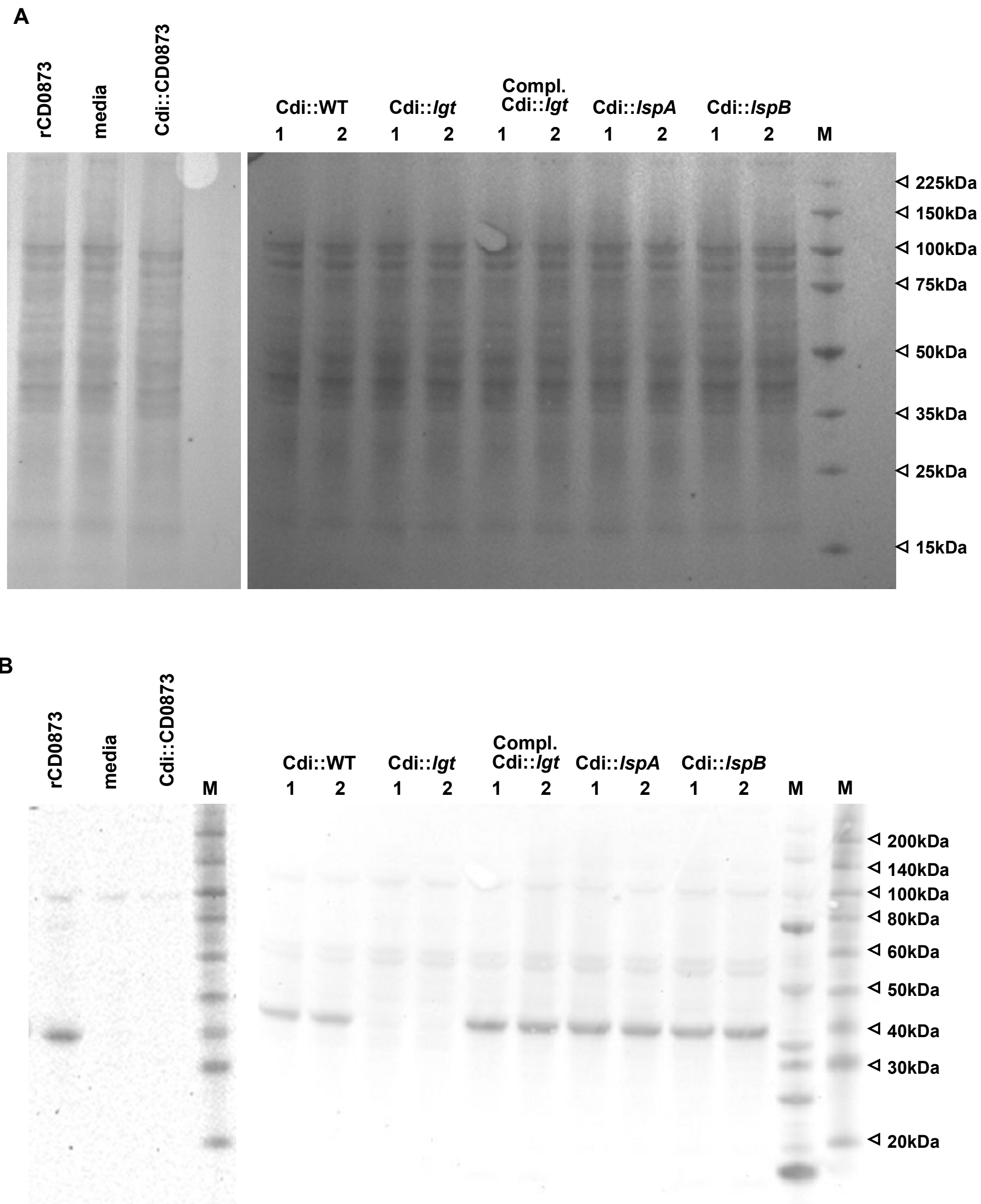


Figure 6.21. The N-terminal signal peptide has a role in the binding of the CD0873 lipoprotein to Caco-2 cells. Binding of the native CD0873 lipoprotein to Caco-2 cells was determined by CD0873 immunoblot. Caco-2 cells (7 x 10^5) were incubated with 1 mg of *C. difficile* cell lysates for 2 hours. Caco-2 cells incubated with the recombinant CD0873 protein (rCD0873) was used as a positive experimental control, Caco-2 cells incubated with the recombinant ε protoxin of *C. perfringens* (not indicated on the image), no protein (media) or whole cell lysate of Cdi::CD0873 were used as negative controls of the experiment. Following the incubation, cells were washed, lysed and analysed by Western blotting using rabbit anti-CD0873 antibodies (1:2500) to see if Caco-2 cells were bound by the native form of the CD0873 protein. (A) Transfer control of the binding assay. Proteins of Caco-2 cell lysates transferred onto nitrocellulose membrane were stained with Ponceau dye prior to the immunoblot to see if similar amount of proteins were to be analyzed for protein binding. The biotinylated protein ladder is not visible in the empty lanes. (B) CD0873 immunoblot of Caco-2 cells lysates. M, Protein ladder. Technical replicates are indicated by numbers. This image shows that only the smaller (presumably uncleaved) form of the CD0873 protein (see Chapter 3) is able to bind to Caco-2 cells.

6.2.9. The CD0873 lipoprotein is expressed in *C. difficile* clinical isolates

I next addressed the question of whether the CD0873 protein is present in other *C. difficile* strains. Blast search analysis revealed that the CD0875-CD0873 ABC transport system is highly conserved and present in several *C. difficile* strains, such as in the NAP07 and the hypervirulent R20291 strains. The CD0873 regions of these strains are shown in Figure 6.22. Interestingly, these transporters have orthologues in several strains which are generally encoded downstream of the CD0875-CD0873 orthologues, for example, the CD0878-CD0876 transporter of the *C. difficile* 630 strain (Figure 6.22). However, CD0876 is not recognised by CD0873 antibodies (Figure 6.11) suggesting that the epitope of the protein is highly altered.

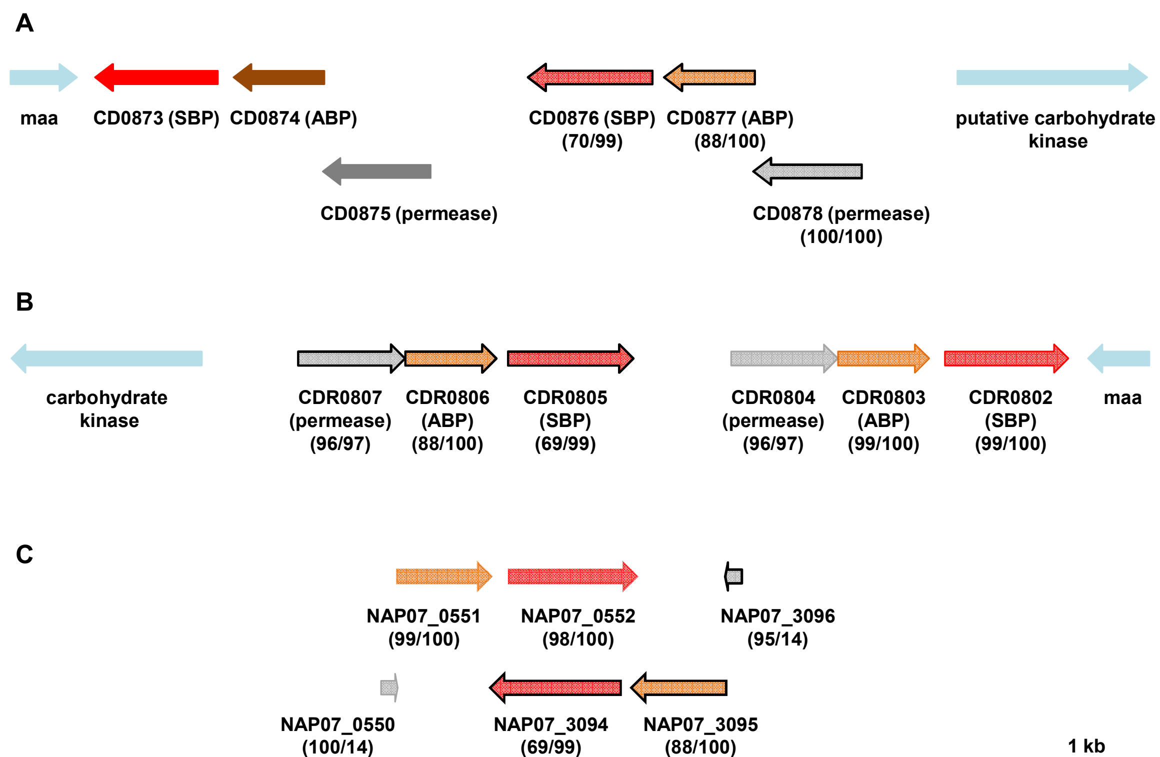


Figure 6.22. Orthologues of the CD0873-CD0875 ABC-type transporter from *C. difficile* 630 in other *C. difficile* strains. The CD0873 region in the (A) *C. difficile* 630 (ribotype 012), (B) R20291 (ribotype 027) and (C) NAP07 (ribotype 078) strains. Figures below the ORFs indicate % identity of the amino acid sequences / query cover (%), relative to the amino acid sequences of the CD0873, CD0874 and CD0875 proteins, respectively (orthologues are indicated by the same but fainter colours). Black and coloured outlines of the arrows refer to the same ABC transport system in each strain. Upstrem and downstream genes are marked with blue arrows. SBP, solute-binding protein; ABP, ATP-binding protein; maa, maltose O-acetyltransferase.

In order to experimentally confirm the expression of CD0873 in clinical strains, whole cell lysates of *C. difficile* clinical isolates belonging to ribotypes 027, 023 and 078 (FA07003754, FA07007522 and FA07004080, respectively, obtained from Royal Devon and Exeter Hospital) were prepared and immunoblotted using sera raised against CD0873. Figure 6.23. shows that the CD0873 antibodies reacted with all the protein extracts indicating that the CD0873 protein is expressed in all the blotted strains.

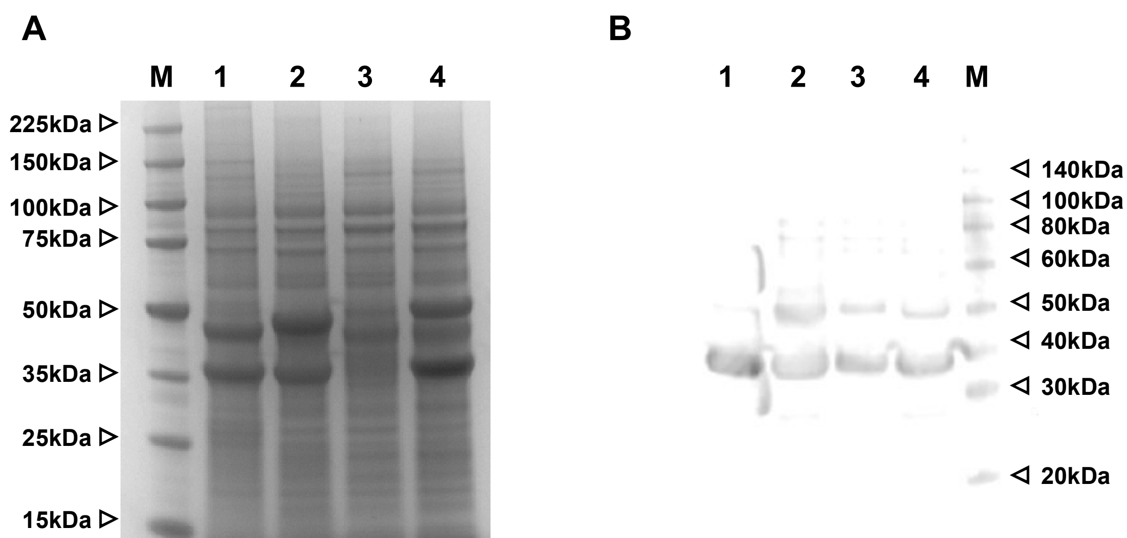


Figure 6.23. The CD0873 homologues are expressed in *C. difficile* clinical isolates. Total proteins were extracted from the *C. difficile* strains, separated by SDS-PAGE and stained with SimplyBlue™ SafeStain (A). *C. difficile* whole cell lysates were probed with rabbit polyclonal anti-CD0873 antibodies (1:2,500) (B). Lane 1, *C. difficile* 630Δerm (012); Lane 2, *C. difficile* FA07003754 (027); Lane 3, *C. difficile* FA07007522 (023). Lane 3, *C. difficile* FA07004080 (078). Figures in brackets refer to the ribotype of the strains. Lane M, molecular weight standard. Western blot analysis indicates that the anti-CD0873 antibodies reacted with whole cell lysates of the probed strains.

To study whether CD0873 homologues of the clinical isolates interact with Caco-2 cells, bacterial lysates of the *C. difficile* strains belonging to ribotypes 027, 023 and 078 were incubated with Caco-2 cells for 2 hours. Cell extract of the *C. difficile* 630Δ*erm* strain (ribotype 012) served as a positive control for this experiment. Protein interactions were examined by blotting the Caco-2 cell lysates with CD0873 antibodies.

Figure 6.24. indicates that all the tested CD0873 homologues were able to bind to Caco-2 cells raising the possibility of that CD0873 is an important adherence determinant of these *C. difficile* strains.

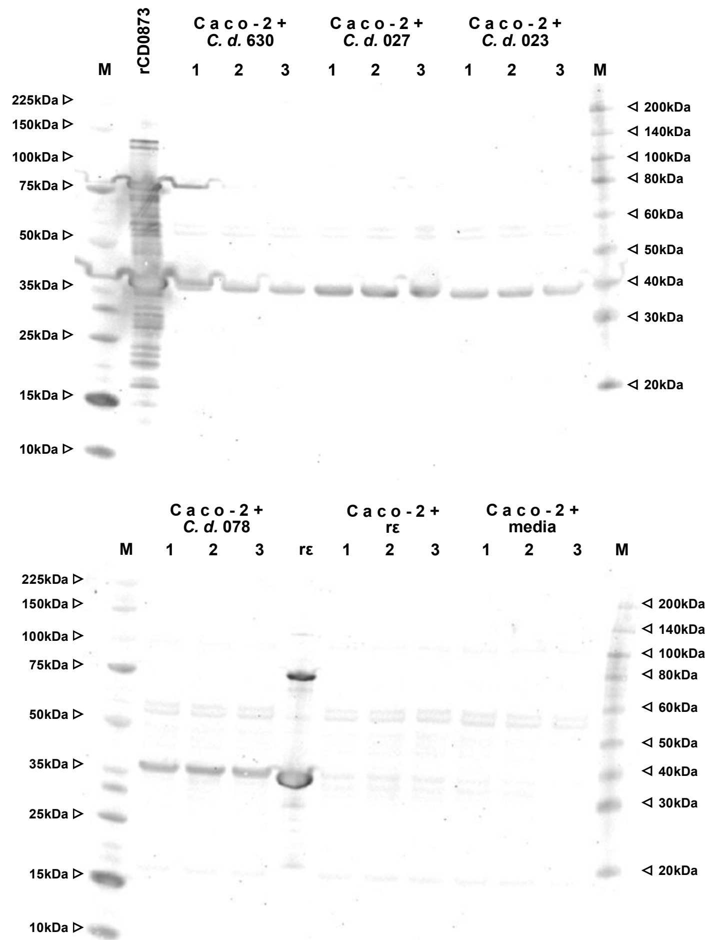


Figure 6.24. Binding of the CD0873 homologues from *C. difficile* clinical isolates to Caco-2 cells. Caco-2 cells (7 x 10^5) were incubated with 1 mg of *C. difficile* cell lysates for 2 hours in parallel with no protein and rε negative controls. Caco-2 cells were then washed, lysed and analyzed by Western blotting using anti-CD0873 antibodies to see if Caco-2 cells were bound by the CD0873 homologues. Lanes on top image: rCD0873, rCD0873 alone; Caco-2 + *C. d.* 630, Caco-2 cells with *C. difficile* 630Δerm (012); Caco-2 + *C. d.* 027, Caco-2 cells with *C. difficile* FA07003754 (027); Caco-2 + *C.d.* 023, Caco-2 cells with *C. difficile* FA07007522 (023); Lanes on bottom image: Caco-2 + *C. d.* 078, Caco-2 cells with *C. difficile* FA07004080 (078); rε, rε alone; Caco-2 + rε, Caco-2 cells with rε; Caco-2 + media, Caco-2 cells alone; M, Protein ladder. Technical replicates are indicated by numbers. This image shows that CD0873 homologues extracted from *C. difficile* clinical isolates were able to bind to Caco-2 cells.

6.3. Discussion

While toxins are mainly responsible for the pathology of pseudomembranous colitis and nosocomial diarrhoea, colonisation is also a crucial process that allows *C. difficile* to infect its host. Therefore, non-toxin virulence factors probably play an important role in the virulence of *C. difficile*. Previous studies suggested that adherence of *C. difficile* to the gastrointestinal tract is multifactorial involving several bacterial proteins and possibly other macromolecules playing direct or indirect roles in the bacterial attachment. So far, only a few proteins have been identified with a probable or proven role in the adherence of *C. difficile*, either to extracellular matrices or gastrointestinal cells. However, no work has yet focused on the characterisation of *C. difficile* lipoproteins as potential adhesins. Impaired adherence of the Cdi::*lgt* strain is highly suggestive of that lipoproteins might contribute to the adherence of *C. difficile*. In this study, the CD0873 lipoprotein (substrate-binding lipoprotein of an ABC transport system) potentially involved in adhesion was identified and characterised.

Adhesins are accessible on the cell surface allowing direct interactions with external factors (e.g., extracellular matrix, intestinal epithelial cells) (Carvalho *et al.* 2012). Earlier studies have reported that while anchored to the membrane by the amino terminal acyl chain, lipoproteins can span the peptidoglycan layer and be displayed on the extracellular surface (Mascioni *et al*. 2010, Rajam *et al.* 2008). Several findings of this work, such as surface exposure of CD0873, antibodies against CD0873 reducing the adherence of *C. difficile* and evidence for the binding of recombinant CD0873 protein to Caco-2 cells suggest that CD0873 functions as an adhesin.

There are some indications that CD0873 is not only involved in adhesion but fulfils other functions in *C. difficile*. Some lipoproteins have previously been shown to have multiple functions in a numbers of bacterial species, such as *Streptococcus*, *Listeria*, *Lactobacillus*, *Lactococcus* and *Leuconostoc* species (Rajam *et al*. 2008). One of the

best-characterised is the PsaA of *S. pneumoniae*. PsaA is the main pneumococcal antigen which binds to target cells (Gor *et al.* 2002, Miyaji *et al.* 2001, Romero-Steiner *et al.* 2006, Anderton *et al*. 2007) and several phenotypes of *S. pneumoniae*, including adherence, virulence, growth and response to oxidative stress are affected by inactivation of the *psaA* gene (Johnston *et al.* 2004). Although, doubts have been raised concerning whether PsaA is exposed to the bacterial surface and functions as adhesin (Johnston *et al.* 2004), a review by Raman *et al*. summarises numbers of previous and more recent findings and suggests direct involvement of PsaA in the adherence of *S. pneumoniae* (Raman *et al*. 2008). Annotated as a solute-binding protein of an ABC-type transporter, CD0873 may play a role in nutrient uptake. Inactivation of the CD0873 gene caused slightly impaired growth in chemically defined media. Although, attempts to identify the substrate of CD0873 have not been successful, it has been shown in this study that the recombinant CD0873 protein interacts with iron, zinc and phenylalanine. However, the mechanism and physiological importance of these interactions are currently unknown. Nevertheless, results of the growth experiments lead to a hypothesis for the regulation of tyrosine and phenylalanine synthesis in *C. difficile*. Synthetic pathways of tyrosine and phenylalanine are linked together (Shikimate pathway; Appendix IV). The difference in the growth of the Cdi::WT when cultured in CDM lacking of either one or both amino acids raise the possibility that there may be a positive feedback loop for this biosynthetic pathway when phenylalanine is not available, but not when tyrosine is lacking from the medium. Specifically, tyrosine may not be synthesised in the presence of phenylalanine which could cause the slight growth defect in the CDM-Tyr medium. In contrast, phenylalanine may be synthesised in the presence of tyrosine, thereby, phenylalanine could be produced when it is lacking from the medium, independently of the presence of tyrosine. In this case, both amino acids

might be available which could potentially be the reason for that *C. difficile* exhibits normal growth in CDM-Phe and CDM-Tyr-Phe medium.

The results described in this chapter suggest that the CD0873 lipoprotein plays a role in the adherence of *C. difficile.* Although, the Cdi::CD0873 strain showed almost a complete loss of adherence to Caco-2 cells, antibodies to the CD0873 protein only partially blocked adhesion. This could be due to that the CD0873 protein may be able to partially fulfil its function when masked by antibodies, while the phenotype of the Cdi::CD0873 strain (when the protein is not translated) reflects the effect of the absence of the protein. However, colonisation is considered to be multifactorial and inactivation of a single gene is not expected to cause such a dramatic reduction in adherence. This could be caused by two reasons. One of the suggested reasons is that the CD0873 protein is not only a putative adhesin but may also disrupt one or more cellular processes which are necessary for adhesion. Alteration in nutrient transport could be such a process. It has been demonstrated in previous other studies that inactivation of ABC transport system genes can indirectly affect adherence of pathogens caused by the resulting changes in the intracellular levels of certain nutrients that could alter expression or posttranslational modification (and consequently activity) of adhesins (Clarke *et al.* 2004, McNab & Jenkinson 1998, Tamura *et al.* 2002). However, this area remains to be further investigated in the case of CD0873. Another reason could be that complementation of the CD0873 mutation did not restore the adherence defect exhibited by the Cdi::CD0873 strain when the bacterial attachment was assessed by CFU counts. This may be attributed to that the CD0873 protein is distributed differently on the surface of the complemented Cdi::CD0873 than that of the Cdi::WT cells or mutation of the CD0873 gene might affect transcription of other gene(s) within the chromosome. But, in contrast to results obtained by CFU counts, complete phenotype restoration was

observed by the complemented Cdi::CD0873 strain in the immunofluorescence microscopy experiments. Therefore, further investigations would be necessary to determine whether the loss of a solute-binding protein or polar effect of the mutation on the genome or the combination of these caused the highly attenuated adherence phenotype of the Cdi::CD0873 strain to Caco-2 cells. Future experiments would include generation of new mutants using other methodologies as described in Chapter 5 (page 145) to exclude the possibility of the effect of CD0873 mutation on the transcription of other genes.

Nevertheless, inhibition of the adherence of *C. difficile* by CD0873 antibodies strongly supports the role of CD0873 in adhesion. Furthermore, direct binding of the recombinant and the native CD0873 protein to Caco-2 cells has also been shown by protein-binding assays. The CD0873 protein extracted from the Cdi::*lgt* strain (uncleaved, possessing the N-terminal signal peptide) did not show any binding to Caco-2 cells and only the smaller form of the CD0873 protein from the Cdi::*lspA* strain (cleaved, no signal peptide) bound to the host cells suggesting inhibitory effect of the signal peptide on the binding of the protein. Binding of the CD0873 homologues from *C. difficile* clinical isolates has also been confirmed which suggests that this protein may have species-wide importance in adhesion. Moreover, the fact that the CD0873 protein is present and likely to be localised on the surface of spores (Lawley *et al.* 2009b) raises the possibility that the CD0873 protein may also play a role in the establishment of the infection at spore stage through contributing to the adherence of *C. difficile* spores. However, due to the lack of time, this hypothesis has not been investigated in this study.

Chapter Seven

General conclusion

7.1. Key findings of this work

It is established that the major virulence factors of *C. difficile* are the two endotoxins produced by the bacteria. However, *C. difficile* is likely to possess a number of other factors that play a role in virulence and are important, for example, in intestinal colonisation and in modulation of the immune responses. This thesis is the initial step towards characterisation of *C. difficile* lipoproteins and their contribution to the pathogenesis of CDI. Numerous previous studies have demonstrated the importance of lipoproteins in virulence of many Gram-positive bacteria. However, there is no previous report investigating the role of lipoproteins and lipoprotein processing in the virulence of *C. difficile*.

The first aim of this work was to identify and functionally characterise the lipoproteins of the *C. difficile* 630 strain. The work presented in Chapter 3 identified 86 putative lipoproteins using bioinformatic tools and 21 of those proteins were detected in the membrane extracts of *C. difficile*. Three lipoprotein biosynthetic enzymes (Lgt, LspA and LspB) were identified and inactivated in order to better understand lipoprotein processing in *C. difficile*. Clear reduction in protein lipidation in the *lgt* mutant was confirmed. This led to the release of several lipoproteins from the cell surface into the extracellular milieu suggesting that lipidation is not required for the cleavage of the N-terminal signal peptide from at least some of the lipoproteins. But further investigations are necessary to prove this hypothesis. Inactivation of the *lspA* gene increased the molecular mass of the majority of lipoproteins suggesting that LspA is a lipoprotein signal peptidase and responsible for cleaving the signal peptide from the majority of lipoproteins. However, role of the LspB enzyme in lipoprotein biosynthesis requires further research.

Furthermore, this project has studied IgG reactivity of 14 recombinant lipoproteins. Nine lipoproteins were recognised by human IgGs, three of those (CD2645, CD2672 and CD2177) showing high seroreactivity.

As lipoproteins fulfil a range of cellular functions in other bacteria, it was suggested that disruption of the lipoprotein biogenesis would affect the physiology and virulence of *C. difficile*. Several phenotypes of the *lgt* mutant were assayed and expected to be altered. Surprisingly, the *lgt* mutant exhibited no difference in growth in rich medium, sporulation or germination rates, or susceptibility to antibiotics. However, the *lgt* mutant was more sensitive to the antimicrobial of activity of bile salts. Inactivation of the *lgt* gene resulted in markedly reduced adherence to Caco-2 cells suggesting that lipoprotein processing by Lgt plays a significant role in the adherence of *C. difficile*. Furthermore, it was demonstrated that mutation of the *lgt* gene affects long-term colonisation in mice.

Using *in silico* approaches, the CD0873 solute-binding lipoprotein of an ABC-type transporter was identified as a candidate lipoprotein involved in adhesion. It has been shown in further research that the recombinant CD0873 protein is destabilised in the presence of iron and stabilised in the presence of phenylalanine. Nevertheless, growth of the CD0873 mutant did not appear to be significantly affected in the absence of certain metal ions (Fe^{2+}, Fe^{3+}, Zn^{2+}) or amino acids (phenylalanine, tyrosine) suggesting that the CD0875-CD0873 transporter does not play a role in the uptake of these molecules. Surface-exposure of the CD0873 lipoprotein was determined by immunofluorescence microscopy and involvement of CD0873 in adhesion was confirmed by an array of methods. It has been demonstrated that the *in vitro* adherence of *C. difficile* was inhibited with antibodies binding to the CD0873 protein and that inactivation of the CD0873 gene reduce the ability of *C. difficle* to adhere to intestinal epithelial cells in

culture. In addition, direct binding of the recombinant CD0873 protein and the native lipoprotein from different strains to Caco-2 cells was demonstrated. Inhibitory role of the N-terminal signal peptide in binding has also been suggested. This is the first study reporting experimental evidence for the role of a lipoprotein in *C. difficile*.

7.2. Final discussion and future perspectives

Although, indications for the role of lipoproteins in the pathogenesis of CDI have been revealed in this study, the project left some questions unanswered. Since lipoprotein biosynthetic enzymes are restricted to bacteria and are potential drug targets, understanding of the lipoprotein biosynthesis and the effects of disrupted lipoprotein biosynthetic pathway are of great importance.

Lipidation of *C. difficile* proteins was not completely abolished in the *lgt* mutant. In the future, it will be important to clarify which enzyme is responsible for protein lipidation in the *lgt* mutant. In some pathogens, attachment of a lipid group can be catalysed by alternative enzymes (Hayashi *et al.* 1985, Kurokawa *et al.* 2009, Tschumi *et al.* 2009). In the \`click\` chemistry assay, biotin is bound to the fluorophore molecule which allows affinity purification of the lipidated proteins from the fractions. Following purification, investigation of the structure and localisation of the acyl chain attached to the lipoproteins present in the *lgt* mutant of *C. difficile* would reveal more insight into the lipidation patterns. Furthermore, it would also be worth elucidating whether the signal peptide is present at the amino-terminus of the lipoproteins found in the culture supernatant of the *lgt* mutant to further confirm that the signal peptide of some lipoproteins are cleaved independently from lipidation, as it is the case in some Gram-positives (Baumgartner *et al.* 2007, De Greeff *et al.* 2003, Denham *et al.* 2009, Henneke *et al.* 2008, Tjalsma *et al.* 1999, Tschumi *et al.* 2012). With more time, investigation

could have been carried out to assess whether inhibition of the Lsp enzymes with globomycin, a specific inhibitor of bacterial lipoprotein signal peptidases, resulted in signal peptide cleavage, addressing the possibility of an Lsp-independent signal peptide cleavage from lipoproteins of *C. difficile*, as it is suggested in *B. subtilis* (Tjalsma *et al.* 1999). Nevertheless, analysis of the lipoproteome in combination with globomycin treatment and inactivation of the known and putative lipoprotein biosynthetic enzymes of *C. difficile* would give even more complex view of lipoprotein processing.

Regarding the immunogenic studies, results could have been enhanced by investigating recognition of the recombinant lipoproteins by other serum immunoglobulins, such as IgM and IgA, and by determining the levels of serum antibodies to the reactive lipoproteins. Quantification of serum antibodies to pathogens potentially allows determining when the individual was exposed to the bacteria, the progression of the infection and the therapeutic response. In case of *C. difficile*, it may also help differentiate between infection and low level of carrier colonisation. Comparison of the levels of antibodies to the wild-type and to the *lgt* mutant (which has a reduced lipoproteome) produced during monoxenic, *in vivo* infection could be another way of determining whether lipoproteins have a role in the acquired immune responses against *C. difficile*.

Lipoproteins activate the innate immune responses through TLR signalling. In accordance with this fact, proinflammatory mediators produced by various host cells was significantly less when the cells were challenged with an *lgt* mutant of *S. aureus* due to the reduced lipoprotein content of the bacterial cells (Stoll *et al.* 2005). In future research, it would be worth exploring whether inactivation of Lgt in *C. difficile* has similar effect on cytokine production.

In the aspect of *C. difficile* adherence, many possibilities exist for future work. It was described in this thesis that disruption of the lipoprotein biogenesis by Lgt inactivation results in reduced adherence of *C. difficile*. Lipoproteins and lipoprotein biosynthetic enzymes are restricted to bacteria and therefore provide potential drug targets against pathogens. As a highly specific inhibitor of the bacterial lipoprotein signal peptidases is available (globomycin), it would also be valuable to study whether inhibition of the signal peptide cleavage by globomycin, effects activity of lipoproteins and their contribution to adherence or other cellular processes. However, a potential therapeutic agent against CDI has to be species-specific for protection of the natural intestinal microflora. Also, further work would be necessary to clarify the effect of *lgt* inactivation on the *in vivo* phenotype of *C. difficile* to enhance the results of the mouse experiment. In particular, investigating the colonisation by the wild-type and the mutant two months after challenge could reveal why the *lgt* mutant was not shed into the faeces (e.g., there was no colonisation by the *lgt* mutant or the *lgt* mutant did colonise the gut but was not shed). However, it can be presumed that the mice were not colonised by the *lgt* mutant at the end of the experiment as faecal shedding of the *lgt* mutant was observed after the previous antibiotic treatments (day 0, day 11-16) when the *lgt* mutant colonised the gut and it is very unlikely not to be the case at later time points. *In vivo* phenotype of the complemented *lgt* mutant would also be necessary to study but currently there is no method available in this infection model to maintain the complementation plasmid in the bacterial cells therefore this investigation is not possible to be performed. However, construction of a deletion mutant and its complemented derivative as discussed in Chapter 5 would resolve this problem.

Lipoproteins of Gram-positive bacteria are often partially active without their lipid anchor (Leskela *et al.*. 1999). Interestingly, the *lgt* mutant exhibited reduction in the adherence at a level similar to that of the CD0873 mutant which may be indicative of

the complete loss of the function of the CD0873 protein (as a putative adhesin and solute-binding protein) in the *lgt* mutant. This hypothesis is supported by that, despite being expressed (see Figure 3.10), the CD0873 protein is not exposed to the surface of the *lgt* mutant cells (preliminary results, unpublished data), thus, probably not able to interact with host cells which supports the observed adherence phenotype of the *lgt* mutant. Markedly reduced adherence of the mutant - which could be due to that mutation of the CD0873 gene may disrupt one or more cellular processes necessary for expression or activity of other adhesins (discussed in Chapter 6) - addresses a further question. Since involvement of other proteins in the adherence of *C. difficile* has already been described, it is important to bare mind that loss and/or inactivity of the CD0873 lipoprotein in the *lgt* and CD0873 mutants might indirectly mask the activity of the known and potentially other (lipo)proteins involved in adhesion. Therefore, this work could be enhanced by determining the substrate of the CD0873 protein as an initial step for investigating whether, apart from being a putative adhesin, the CD0873 lipoprotein is indirectly involved in adhesion as a component of a nutrient uptake system.

Role of CD0873 inactivation in adherence of *C. difficile* could be further investigated in several other experiments. For example, it would be worth assaying the adherence phenotype of the CD0873 mutant by using other human cell lines (such as mucus-producing cell) or tissue sections. Investigating in more detailed the roles of CD0873 homologues in adhesion would also be valuable. Moreover, *C. difficile* overexpressing the CD0873 protein or other bacterial species expressing CD0873 on the bacterial surface could also be assayed. However, the most important follow-up study would be to investigate the *in vivo* phenotype of the CD0873 mutant.

Results of the protein binding assays suggest direct involvement of the CD0873 protein in adhesion. However, this may be arguable, as it was not determined whether the protein is associated to the surface of or taken up by the Caco-2 cells. On the other

hand, the recombinant ε prototoxin used as an experimental control has similar molecular weight to that of the CD0873 protein and was not internalised. In addition, the larger, presumably the uncleaved form of the native CD0873 protein present in the *lgt* mutant and in the *lspA* mutant strains was not taken up by the Caco-2 cells either. Nevertheless, experiments regarding the binding of the CD0873 protein would be necessary to perform in order to entirely exclude its internalisation. Regarding the mechanism of the presumed interaction of the CD0873 protein with Caco-2 cells, the proposed inhibitory role of the signal peptide in binding could be further explored by studying binding of the recombinant CD0873 protein expressed with the signal peptide. Identification of the CD0873 receptor on the surface of Caco-2 cells would be another important milestone of this research. This may also allow solving the structure of the CD0873 protein in complex with the receptor as proteins often crystallise better when bound to other molecules. Knowledge of the structure of the CD0873 protein, especially in bound form, would then greatly improve the possibilities of designing antimicrobial agents that inhibit interaction of the CD0873 protein, thereby *C. difficile*, with host cells.

7.3. Epilogue

C. difficile infection (CDI) has been emerging in recent years across the globe while the prevalent antibiotic treatment with vancomycin and metronidazole is becoming insufficient. Thereby, development of effective vaccination and therapeutic strategies is now of great importance. Knowledge of the pathogenesis is essential in order to move toward new agents and improve medical interventions. Proteins (and other molecules) which are necessary for the virulence of pathogens, and are unique to bacteria or specific for bacterial species, have great importance in the development of these agents. Lipoproteins and their biosynthetic enzymes meet these requirements. Initial steps in the

investigation of the role of lipoproteins in the virulence of *C. difficile* have been made in this work. Further studies of all areas presented in this thesis will enhance our understanding of the pathogenic process of CDI. *C. difficile* research is now rapidly emerging with a range of gene manipulating tools developed in the last few years providing the possibility of new research fields to be discovered and extending our knowledge in many aspects of *C. difficile* virulence.

“Science never solves a problem without creating ten more.”

George Bernard Shaw

Appendices

Appendix I

List of the putative lipoproteins of *C. difficile* 630 and their predicted roles (Chapter 3). Putative lipoproteins were identified by bioinformatics. Annotations of the amino acid sequences were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov). Colour coding refers to the predicted roles of the proteins: Molecule transport, Signal molecules, Redox processes, Protein folding, Other roles, Unknown roles.

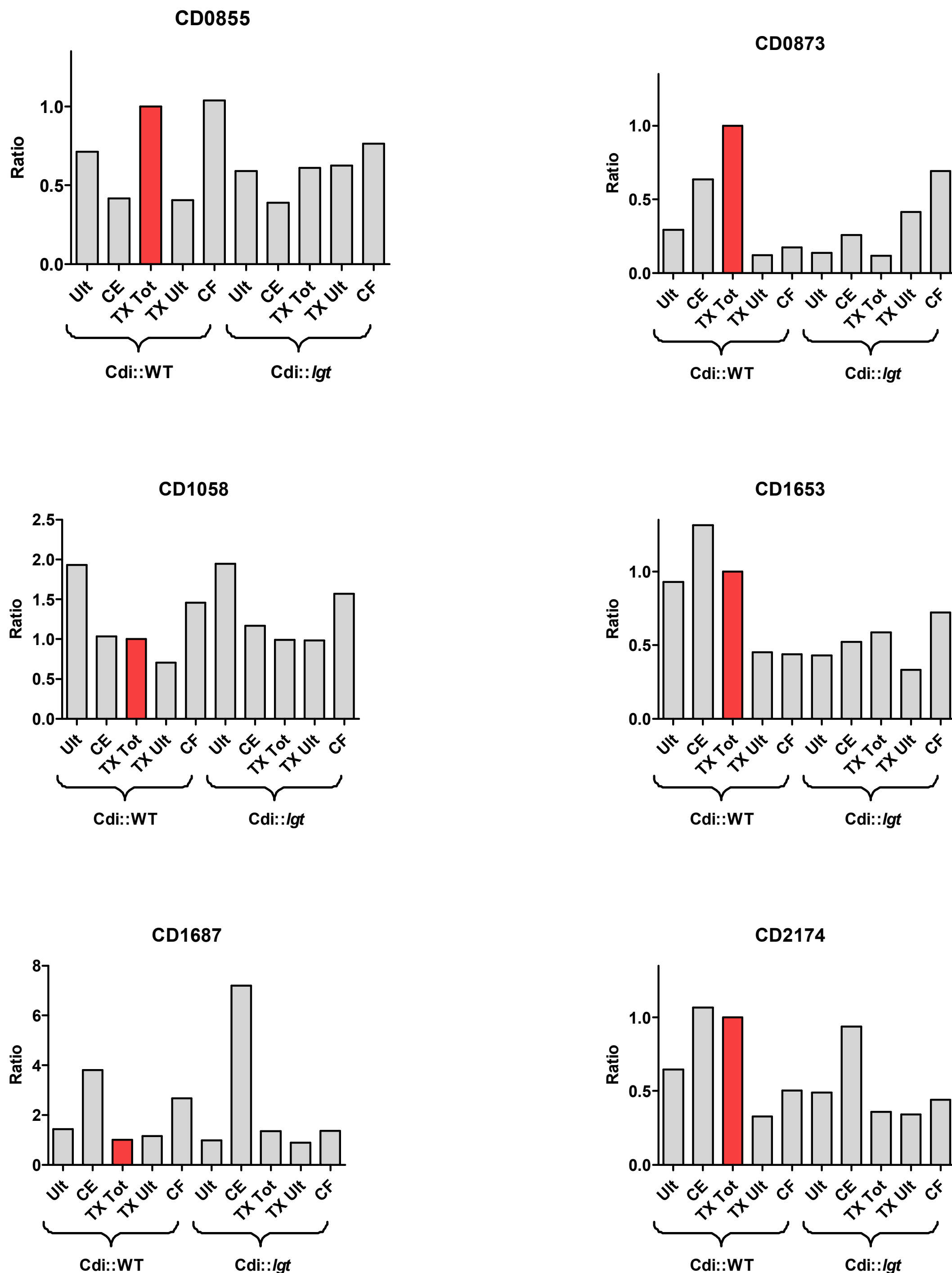
Protein code	Gene	Cysteine Position	Length (amino acids)
CD0173	hypothetical protein	20	355
CD0199	putative membrane-associated nucleotidase	20	463
CD0300	D-ribose ABC transporter	23	320
CD0420	putative cell surface protein	24	513
CD0430	hypothetical protein	24	230
CD0440	cell surface protein	21	386
CD0535	chemotaxis protein	29	161
CD0545	putative lipoprotein	23	203
CD0569	putative outer membrane lipoprotein	23	334
CD0689	putative nucleotide phosphodiesterase	21	641
CD0690	putative nucleotide phosphodiesterase	21	634
CD0747	putative lipoprotein	22	486
CD0750	amino acid ABC transporter, substrate-binding protein	23	271
CD0855	oligopeptide ABC transporter, substrate-binding lipoprotein	21	522
CD0869	molybdenum ABC transporter, substrate-binding protein	21	270
CD0873	ABC transporter, substrate-binding lipoprotein	24	340
CD0876	ABC transporter, substrate-binding lipoprotein	24	345
CD0957	putative phage lipoprotein	22	186
CD0999	ABC transporter, substrate-binding lipoprotein	21	331
CD1027	spermidine/putrescine ABC transporter, substrate-binding lipoprotein	24	350
CD1058	3-hydroxybutyryl-CoA dehydrogenase	22	281
CD1080	putative lipoprotein	20	244
CD1119	putative lipoprotein	26	229
CD1131	putative solute-binding lipoprotein	20	571
CD1156	hypothetical protein	21	360
CD1198	stage III sporulation protein AG	32	184
CD1232	putative lipoprotein	23	273
CD1233	cell surface protein	20	475
CD1348	putative lipoprotein	20	178
CD1387	putative allophanate hydrolase subunit	30	328
CD1476	putative signalling protein	21	965
CD1484	putative aliphatic sulfonates ABC transporter, substrate-binding lipoprotein	25	330
CD1486	putative lipoprotein	22	199
CD1491	putative D-methionine ABC transporter, substrate-binding lipoprotein	22	263
CD1507	putative thioredoxin	21	194
CD1509	putative lipoprotein	21	189

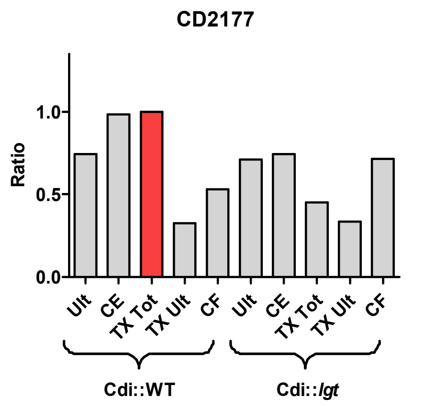
CD1546	putative hemolysin-like membrane protein	22	217
CD1557	putative peptidyl-prolyl isomerase	20	318
CD1589	putative ribose ABC transporter, substrate-binding lipoprotein	23	320
CD1622	putative lipoprotein	21	205
CD1653	putative lipoprotein	22	265
CD1687	putative lipoprotein	28	281
CD1766	putative lipoprotein	20	154
CD1774	amino acid ABC transporter, substrate-binding protein	24	267
CD1781	putative lipoprotein	20	462
CD1873	ABC transporter, permease protein	29	425
CD1940	hypothetical protein	25	151
CD1979	ABC transporter, substrate-binding protein	23	337
CD1992	putative lipoprotein	22	281
CD2029	putative lipoprotein	20	205
CD2052	putative lipoprotein	25	225
CD2077	dihydroorotate dehydrogenase, catalytic subunit	27	361
CD2174	amino-acid ABC transporter, substrate-binding protein	23	265
CD2177	amino-acid ABC transporter, substrate-binding protein	21	267
CD2227	radical SAM superfamily lipoprotein	21	285
CD2311	ABC transporter, substrate-binding protein	21	270
CD2365	putative sulfonate ABC transporter, solute-binding lipoprotein	24	363
CD2406	putative lipoprotein	19	407
CD2478	metallo beta-lactamase superfamily lipoprotein	19	278
CD2482	putative lipoprotein	19	123
CD2538	putative lipoprotein	20	294
CD2550	ABC transporter sugar-family extracellular solute-binding protein	29	430
CD2645	putative extracellular solute-binding protein	24	424
CD2663	putative signalling protein	27	759
CD2672	oligopeptide ABC transporter, substrate-binding protein	22	519
CD2689	putative lipoprotein	21	408
CD2701	putative lipoprotein	21	395
CD2713	cell surface protein	30	653
CD2719	polysaccharide deacetylase	22	312
CD2763	putative lipoprotein	20	156
CD2858	putative transporter	23	429
CD2878	putative ferrichrome ABC transporter, substrate-binding protein	23	312
CD2887	putative signalling protein	28	384
CD2907	phage lipoprotein	22	186
CD2953	ABC transporter, substrate-binding protein	20	349
CD2989	ABC transporter, substrate-binding protein	23	319
CD2999	putative iron ABC transporter, substrate-binding protein	30	379
CD3200	putative ABC transporter, permease protein	34	342
CD3268	putative phosphate ABC transporter, substrate-binding protein	23	280

CD3414	ABC transporter, solute-binding protein	25	436
CD3464	putative lipoprotein	22	196
CD3476	putative ATP synthase protein	31	75
CD3500	putative foldase lipoprotein (late stage protein export lipoprotein)	20	331
CD3525	putative iron ABC transporter, solute-binding protein	23	355
CD3528	putative iron ABC transporter, solute-binding lipoprotein	23	355
CD3669	hypothetical protein	22	191

Appendix II

Quantification of the lipoproteins in the protein extracts of the wild-type and the *lgt* mutant *C. difficile* (Chapter 3). Abundance of the lipoproteins was determined by Progenesis LC-MS software. On the graphs, abundance of each lipoprotein is plotted in ratio relative to the abundance of the lipoprotein measured in the TX Tot fraction of the Cdi::WT (shown by red bars). Ult - ultracentrifuged (crude membrane), CE - carbonate extracted, TX Tot - Triton X-114 extracted fraction of total cell lysate, TX Ult - Triton X-114 extracted fraction of ultracentrifuged (crude membrane) fraction, CF - culture filtrate. Bottom panel shows the abundance of lipoproteins relative to the abundance of CD0873 measured in the TX Tot fraction of the Cdi::WT.




CD2177
Ratio
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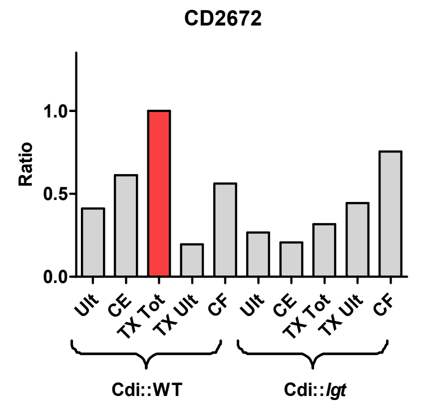
0.0
Ult
CE
TX Tot
TX Ult
CF
Ult
CE
TX Tot
TX Ult
CF
Cdi::WT
Cdi::*lgt*

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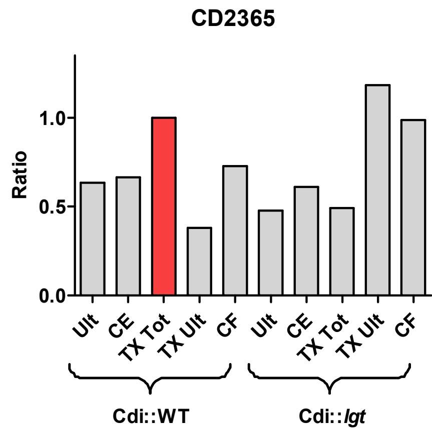
0.0
Ult
CE
TX Tot
TX Ult
CF
Ult
CE
TX Tot
TX Ult
CF
Cdi::WT
Cdi::*lgt*

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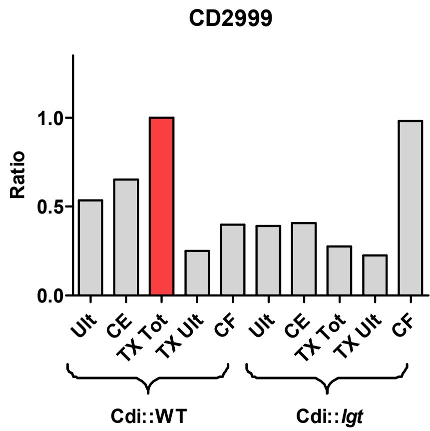
TX Tot
TX Ult
CF
Ult
CE
TX Tot
TX Ult
CF
Cdi::WT
Cdi::*lgt*

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Ult
CE
TX Tot
TX Ult
CF
Ult
CE
TX Tot
TX Ult
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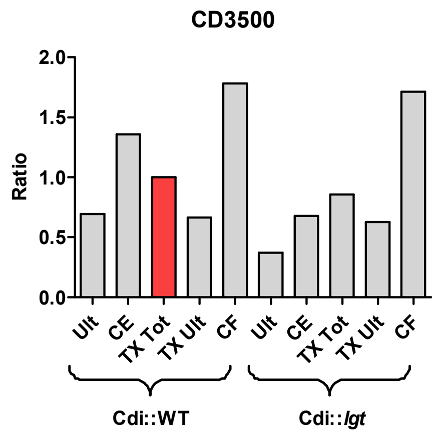
Cdi::*lgt*

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Ult
CE
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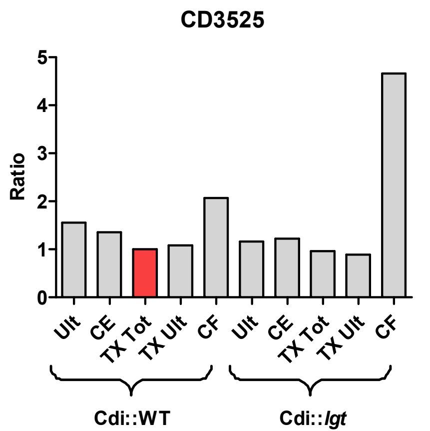
CF
Cdi::WT
Cdi::*lgt*

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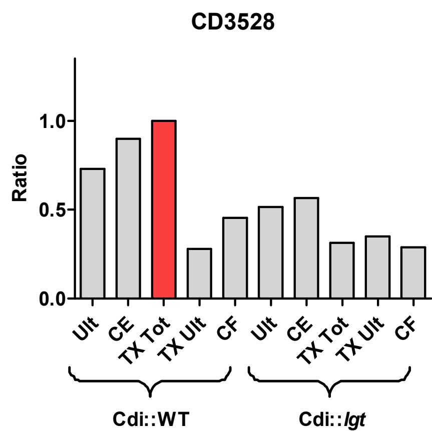
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TX Tot
TX Ult
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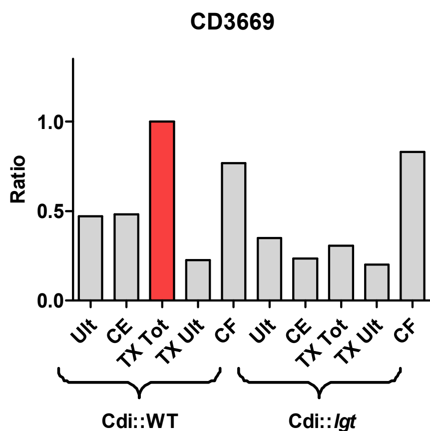
Cdi::*lgt*

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0.0
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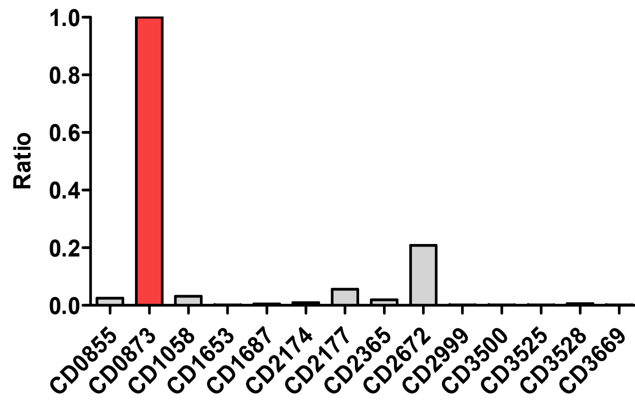
CE
TX Tot
TX Ult
CF
Ult
CE
TX Tot
TX Ult
CF
Cdi::WT
Cdi::*lgt*


1.0
0.8
0.6
0.4
0.2
0.0
Ratio
CD0855
CD0873
CD1058
CD1653
CD1687
CD2174
CD2177
CD2365
CD2672
CD2999
CD3500
CD3525
CD3528
CD3669

Appendix III

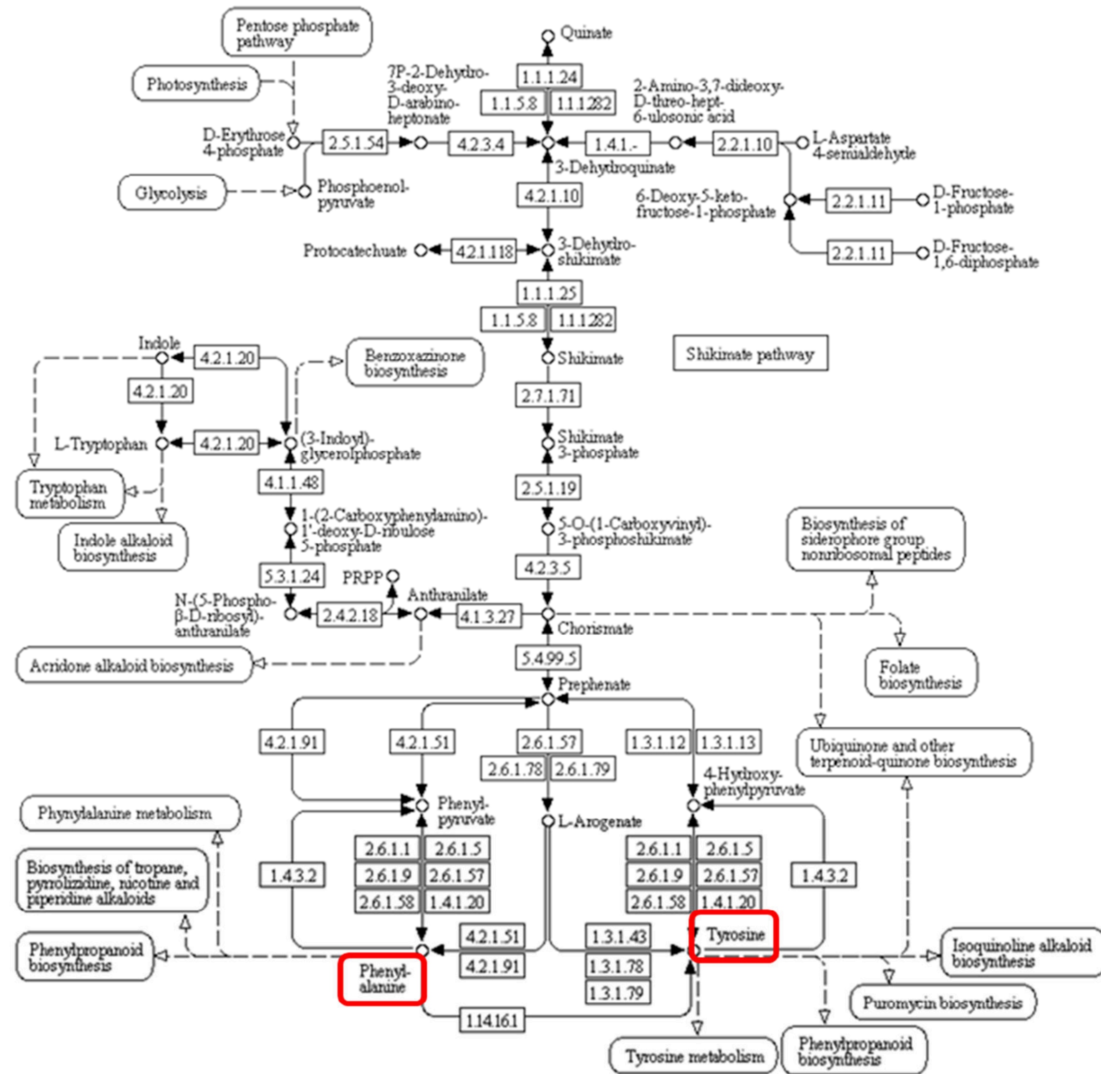
List of the buffers used for screening to determine the stability of the recombinant CD0873 protein (Chapter 6).

No.	Buffer component (100 mM)	pH	NaCl concentration (mM)	Additive (concentration)
1	Sodium acetate-AcOH	5.0	150	
2	Pyridine-HCl	5.5	150	
3	Sodium cacodylate-HCl	6.0	150	
4	MES-NaOH	6.0	150	
5	Imidazole-HCl	7.0	150	
6	PIPES-NaOH	7.0	150	
7	MOPS-NaOH	7.2	150	
8	HEPES-NaOH (reference)	7.5	150	
9	Tricine-NaOH	8.0	150	
10	Tris-HCl	8.1	150	
11	Bicine-NaOH	8.3	150	
12	Glycine-NaOH	9.0	150	
13	BORAX-Boric acid	9.0	150	
14	CHES-NaOH	9.5	150	
15	Ethanolamine-NaOH	9.5	150	
16	CAPS-NaOH	10.0	150	
17	HEPES-NaOH	7.5		
18	HEPES-NaOH	7.5	50	
19	HEPES-NaOH (reference)	7.5	150	
20	HEPES-NaOH (reference)	7.5	150	
21	HEPES-NaOH	7.5	500	
22	HEPES-NaOH	7.5	150	Glycerol (5 %)
23	HEPES-NaOH	7.5	150	Glycerol (10 %)
24	HEPES-NaOH	7.5	500	Glycerol (5 %)

Taken from Niesen *et. al* (2007) (Niesen *et al.*. 2007)

Appendix IV

Biosynthesis of tyrosine and phenylalanine in the Shikimate pathway (Chapter 6).



http://www.genome.jp/kegg-bin/show_pathway?scale=1.0&query=clostridium&map=map00400&scale=&auto_image=&show_description=hide&multi_query

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MINIREVIEWS

Lipoproteins of Bacterial Pathogens[▽]

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Bacterial lipoproteins are a set of membrane proteins with many different functions. Due to this broad-ranging functionality, these proteins have a considerable significance in many phenomena, from cellular physiology through cell division and virulence. Here we give a general overview of lipoprotein biogenesis and highlight examples of the roles of lipoproteins in bacterial disease caused by a selection of medically relevant Gram-negative and Gram-positive pathogens: *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, *Borrelia burgdorferi*, and *Neisseria meningitidis*. Lipoproteins have been shown to play key roles in adhesion to host cells, modulation of inflammatory processes, and translocation of virulence factors into host cells. As such, a number of lipoproteins have been shown to be potential vaccines. This review provides a summary of some of the reported roles of lipoproteins and of how this knowledge has been exploited in some cases for the generation of novel countermeasures to bacterial diseases.

Lipid modification of bacterial proteins facilitates the anchoring of hydrophilic proteins to hydrophobic surfaces through the hydrophobic interaction of the attached acyl groups to the cell wall phospholipids. The addition of acyl moieties effectively provides a membrane anchor allowing the protein to function effectively in the aqueous environment (86). Bacterial lipoproteins have been shown to perform various roles, including nutrient uptake, signal transduction, adhesion, conjugation, and sporulation, and participate in antibiotic resistance, transport (such as ABC transporter systems), and extracytoplasmic folding of proteins (2, 91, 105, 131, 170). In the case of pathogens, lipoproteins have been shown to play a direct role in virulence-associated functions, such as colonization, invasion, evasion of host defense, and immunomodulation (75, 82, 88). In Gram-negative bacteria, two of the three lipoprotein biosynthetic enzymes appear to be essential for viability (55, 63, 123, 177), while in some Gram-positive bacteria, they have been shown to be dispensable (95). Consequently, mutations in enzymes involved in the pathway of lipoprotein processing are lethal in Gram-negative bacteria, but many Gram-positive bacteria tolerate these mutations, exhibiting only slight growth defects (12, 64, 133, 177). One reason for the viability of Gram-positive mutants could be that the precursors of some essential lipoproteins have the same functionality as the mature lipoproteins (95, 187). However, in many Gram-positive bacteria, the presence of lipoproteins is necessary for virulence (106, 175). Previous reviews of lipoprotein biosynthesis and/or their roles (75, 170) emphasize that there is still much to be discovered concerning the biosynthetic pathways and functions of lipoproteins. Since the latter reviews, there has been a significant increase in the number of proteins reported to be lipoproteins, directly from biochemical studies, and predicted to be lipoproteins, indirectly from sequenced genomes. Further study of this group of bacterial proteins will contribute to a better understanding of their roles and mechanisms of action, supporting their use in the development of countermeasures against bacterial pathogens.

LIPOPROTEIN BIOSYNTHESIS

In both Gram-negative and Gram-positive bacteria, lipoproteins are initially translated as preprolipoproteins (Fig. 1), which possess an N-terminal signal peptide of around 20 amino acids with typical characteristic features of the signal peptides of secreted proteins (76). A conserved sequence at the C region of the signal peptides, referred to as lipobox, [LVI][ASTVI][GAS]C, is modified through the covalent attachment of a diacylglycerol moiety to the thiol group on the side chain of the indispensable cysteine residue (7). This modification is catalyzed by the enzyme lipoprotein diacylglyceryl transferase (Lgt), resulting in a prolipoprotein (Fig. 1) consisting of a diacylglycerol moiety linked by a thioester bond to the protein. Lgt is a basic (pK_a of ~10) protein and transfers negatively charged phospholipids, phosphatidylglycerol in particular, as its lipid substrate (151). Bacterial lipid modification is suggested to be initiated at the cytoplasmic side of the membrane rather than in the cytoplasm (159), but due to insufficient information about Lgt, this is only speculation. Lgt is a critical enzyme in the generation of bacterial lipoproteins, although most of its characteristics are still poorly understood. Comparisons of the Lgt sequences from phylogenetically distantly related microorganisms revealed several highly conserved regions that might be important for function (140). In site-directed mutagenesis studies, His-103 and Tyr-235 were found to be essential for Lgt activity (150). Although *lgt* is found as a single gene in most bacterial genomes, there are two putative *lgt* paralogues carried in some bacteria, such as *Coxiella burnetti*, *Bacillus cereus*, *Clostridium perfringens*, and *Streptomyces coelicolor* (75), but the reason why multiple Lgt enzymes are present in these strains is unknown.

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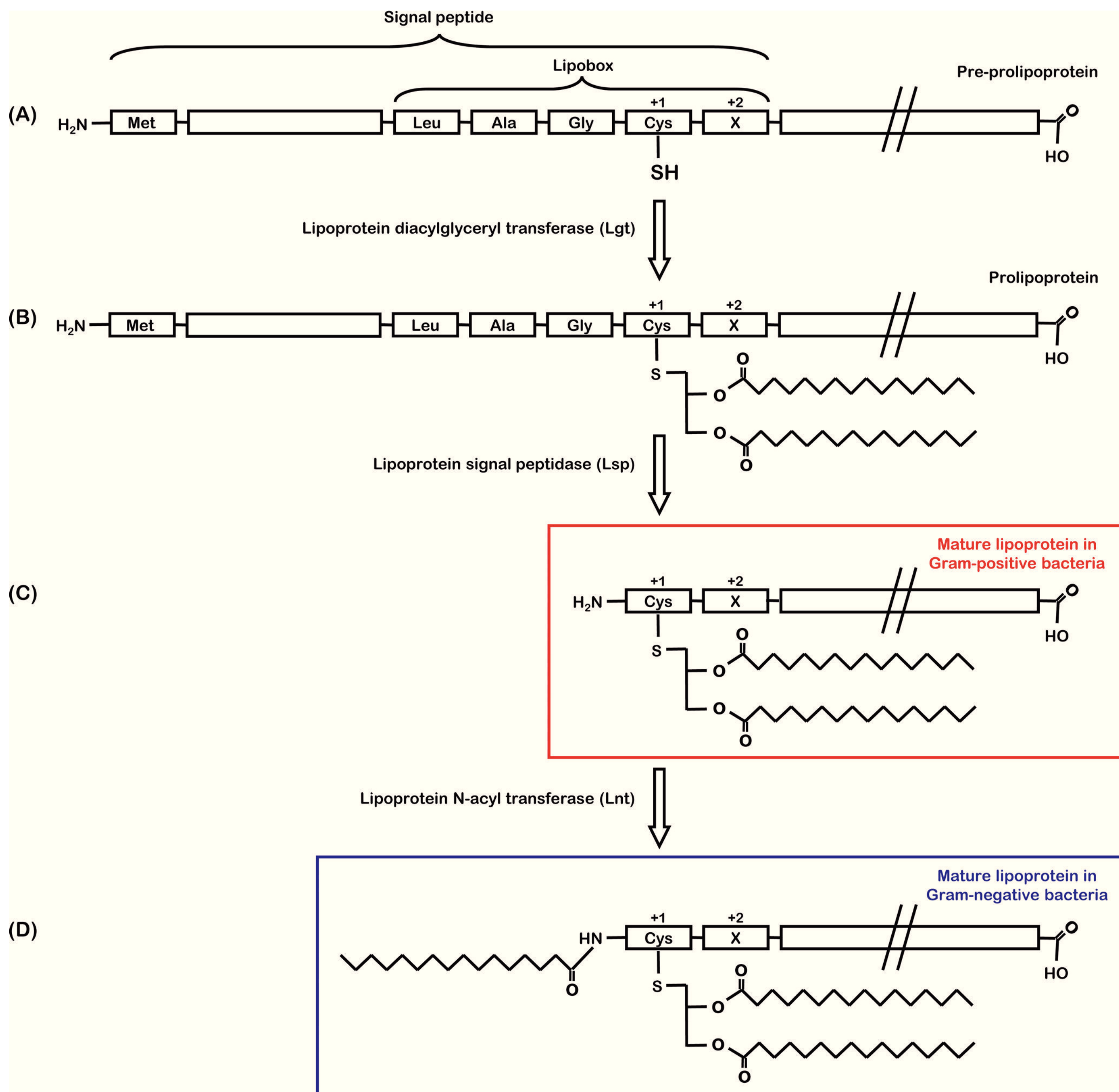

FIG. 1. Biosynthesis of bacterial lipoproteins. (A to C) Two-step biosynthetic pathway in Gram-positive bacteria. (A to D) Three-step biosynthetic pathway in Gram-negative bacteria. (A) The precursor of lipoproteins is the preprolipoprotein, with an N-terminal signal peptide possessing a characteristic consensus sequence of the lipobox. (B) During lipoprotein maturation, the thiol group of the invariant cysteine in the lipobox is modified by a diacylglyceryl moiety by lipoprotein diacylglyceryl transferase (Lgt), which serves as a membrane anchor. (C) After lipidation, lipoprotein signal peptidase (Lsp) cleaves the signal peptide, leaving the cysteine as the new amino-terminal residue forming the mature lipoprotein in Gram-positive bacteria. (D) In Gram-negative and some Gram-positive bacteria, the mature lipoprotein has an additional amide-linked fatty acid at the N-terminal cysteine residue attached by lipoprotein N-acyl transferase (Lnt). Amino acid residues at position +2, +3, and +4 have a role in membrane localization of Gram-negative bacterial lipoproteins.

After lipidation, lipoprotein signal peptidase (Lsp or SPase II) is responsible for cleaving the signal sequence of the lipidated prolipoprotein and leaves the cysteine of the lipobox as the new amino-terminal residue (181). This transmembrane enzyme (108) has five conserved sequence regions (178) and six functionally important residues (Asp-14, Asn-99, Asp-102, Asn-126, Ala-128, and Asp-129) and is a member of the aspartic protease family (40). Application of the reversible, noncompetitive inhibitor of Lsp, globomycin, has been important in studying the activity and function of Lsp enzymes (39), and a mechanism for cleavage of target proteins by Lsp has been proposed by Tjalsma et al. (178). Similarly to the case with *lgt*,

only a single *lsp* gene is present in most bacteria, although some organisms possess two putative *lsp* paralogues (for example, *Listeria monocytogenes* and *Nocardia farcinica*). However, the roles of these paralogues are unknown (75, 144). Interestingly, a second peptidase, called Eep (enhanced expression of pheromone), a metallopeptidase, has been found in *Enterococcus faecalis* and is involved in the cleaving process of signal peptides of some lipoproteins (3). Lsp was originally thought to cleave only lipid-modified precursors. In a selection of *Eubacteria* (e.g., *L. monocytogenes* and *Streptococcus agalactiae*), however, it has been demonstrated that lipidation is not a prerequisite for the activity of Lsp (12, 70).

In Gram-negative bacteria and some Gram-positive bacteria, the cleaved prolipoprotein undergoes an additional modification by attachment of an amide-linked acyl group to the N-terminal cysteine residue by lipoprotein N-acyl transferase (Lnt) (63). Lnt has been shown to be able to utilize all available phospholipids from phosphatidylglycerol through phosphatidylethanolamine to cardiolipin as acyl donors (77). BLAST search analysis for homologues of *Escherichia coli* Lnt revealed that Lnt is widely present in Gram-negative bacteria. Some indications for N acylation in low-GC Gram-positive bacteria have been reported (89), but there is no evidence for the presence of Lnt in this group, raising the question of which enzyme catalyzes the N acylation of lipoproteins in these bacteria or if it indeed occurs. In contrast, *lnt* homologues have been identified in all classes of high-GC Gram-positive bacteria (189). Recently, Lnt activity was identified in mycobacteria, and the responsible genes were also assigned (183). There are two Lnt homologues in *Streptomyces coelicolor*, but their genes failed to rescue an *E. coli lnt*-deficient strain (189), and the function of these enzymes thus remains unclear. The previously held notion that lipoproteins of Gram-positive bacteria are diacylated and those of Gram-negative bacteria are triacylated has therefore ceased to be so clear-cut. Indeed, the situation may be more diverse in that a single species may contain both forms of lipoproteins, since it has been shown that *Mycoplasma gallisepticum* possesses both di- and triacylated proteins (80–81). In common with the aminoacylation of the amino-terminal cysteine of bacterial lipoproteins, it has been demonstrated that the eukaryotic signaling protein Sonic hedgehog is also acylated at its amino-terminal cysteine. Notwithstanding the characteristics of this modification relative to that performed by Lnt, i.e., formation of an acyl-amine linkage, the eukaryotic enzyme that facilitates this amino-terminal acylation has no similarity to its prokaryotic counterpart (25).

Both the diacylglyceryl group and the amino-terminal acyl group are derived from membrane phospholipids and provide tight anchorage of the lipoprotein to the membrane (66). Although the structure of the acyl chains is thought to be predominantly from membrane phospholipids, there are reports on variations in the structure of these acyl chains (22). Furthermore, in *Borrelia burgdorferi*, an unusual lipid component has been found in lipoproteins where one of the ester-linked fatty acids is replaced by an acetyl group (13). Since some lipoproteins are surface exposed, this raises the possibility that the acyl moieties and modifications may play more of a role than just anchoring the lipoproteins. The importance of bacterial lipoproteins, especially with respect to disease, is emphasized in the following sections of this review, which highlight the localization of lipoproteins and their effects on host cell interaction. As such, the enzymes involved in the biosynthesis of these posttranslationally modified proteins have been proposed to be targets for the development of novel antibacterial agents. This is in part due to the absence of any homologues in eukaryotes. However, lipidation of some proteins is observed in eukaryotic cells. These modifications are predominantly of two types, posttranslational palmitoylation of cysteine via a thioester linkage and the cotranslational amide-linked myristoylation of amino-termini glycine residues (49, 97), though the enzymes that mediate this are distinct from the enzymes that mediate bacterial lipidation.

LIPOPROTEIN LOCALIZATION

Following signal peptide-dependent translocation across the cytoplasmic membrane, lipoproteins can localize at various places in the cell (Fig. 2). It has been reported that *Escherichia coli* has more than 90 lipoproteins and that the majority of these are located at the periplasmic face of the outer membrane, with others present at the periplasmic face of the inner membrane (111). Although the lipid moiety is proposed to be responsible for attaching the protein to the membrane through hydrophobic interactions, whether inner membrane periplasmic, outer membrane periplasmic, or outer membrane external, in some cases it is not only the interaction of the acyl component that contributes to anchorage. The peptidoglycan-associated lipoprotein (Pal) of Gram-negative bacteria possesses a region that forms a binding pocket for the *m*-Dap residue (*meso*-diaminopimelate) of peptidoglycan. This binding pocket has conserved surface residues that interact with the peptide portion of peptidoglycan with the *m*-Dap residue, forming hydrogen bond and hydrophobic contacts to Pal (127). Pal is transcribed as part of an operon that also encodes the proteins TolB and TolA, which interact with the outer membrane-anchored Pal, forming a network linking the peptidoglycan layer with the inner and outer membranes (60).

The most abundant lipoprotein of Gram-negative bacteria, and the first discovered lipoprotein, Braun's lipoprotein, is the only known lipoprotein that is covalently linked to the cell wall. It has an unusual structure, with three monomers forming a coiled-coil trimer and the N-terminal domain embedding into the outer membrane. The protein binds to peptidoglycan via a peptide bond between the ε-amino group of the C-terminal lysine residue of the Braun lipoprotein and the α-carbonyl of *m*-Dap present in peptidoglycan (43). The Pal and Braun lipoproteins interact with each other and together contribute to the integrity of the cell wall.

In Gram-negative bacteria, mature lipoproteins are localized to various sites within the cell wall (Fig. 2). They are targeted to the periplasmic face of the inner or outer membranes by the lipoprotein localization machinery (Lol), which consists of a transmembrane protein complex (LolCDE), a periplasmic chaperone (LolA), and an outer-membrane receptor (LolB) (180). Lipoproteins destined for the outer membrane are translocated by the LolCDE complex, an ATP-binding cassette (ABC) transporter, and the periplasmic chaperone, LolA. The determining factor as to whether a lipoprotein is directed to the outer membrane by the Lol machinery or is retained at the inner membrane was initially

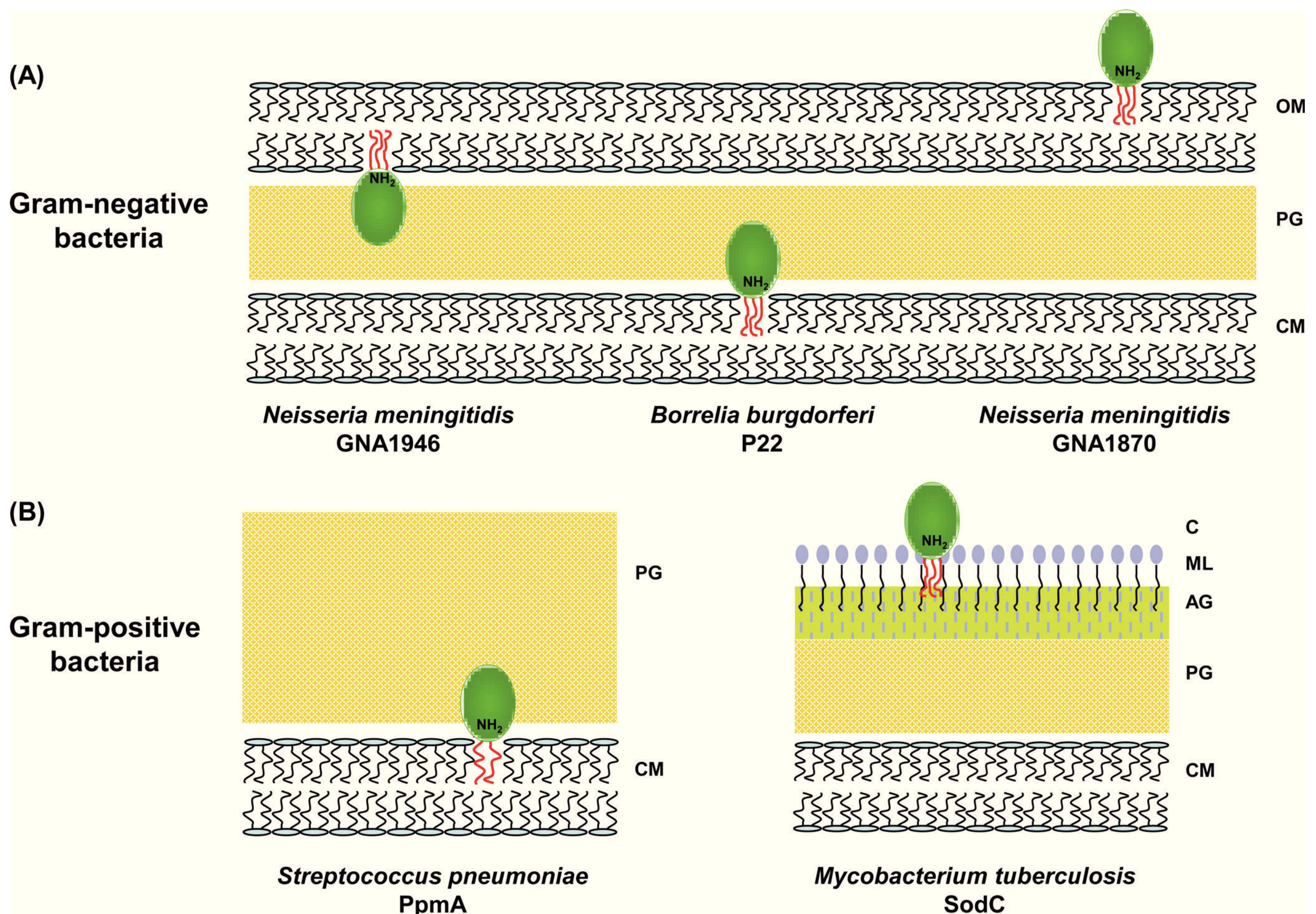

FIG. 2. Localization of bacterial lipoproteins. (A) In Gram-negative bacteria, lipoproteins are attached to the cytoplasmic membrane, the extracellular or peripheral side of the outer membrane. (B) In Gram-positive bacteria, lipoproteins are anchored to the extracellular surface of the cytoplasmic membrane and also to the unique mycolate-based lipid layer of the cell wall of *Mycobacterium tuberculosis*. OM, outer membrane; PG, peptidoglycan; CM, cytoplasmic membrane; AG, arabino-galactan; ML, mycolic acid layer; C, capsule-like material; N, N-terminal.

reported to be the identity of the amino acid adjacent to the conserved cysteine, known as the "+2" rule (200). This rule essentially requires an aspartate residue to be present at the +2 position for a protein to be retained at the inner membrane, while substitution with a different amino acid at this position results in translocation by the Lol machinery to the outer membrane. However, this rule is far from universal (162), with amino acid residues at positions +3 and +4 also having been shown to have a role in outer membrane localization in a number of Gram-negative bacteria(112, 163). Usually, LolCDE does not release lipoproteins, which lack an amino-linked acyl chain (54, 145). However, in a temperature-sensitive *lnt* mutant of *Salmonella enterica* serovar Typhimurium, lipoproteins without an amino-linked acyl chain are localized to the outer membrane, though how this occurs remains unknown (63).

Although in *E. coli* all of the known lipoproteins face the periplasm (21), in some Gram-negative bacteria, including pathogenic spirochetes, lipoproteins are present on the outer leaflet of the outer membrane (Fig. 2). However, little is known about the exact mechanism of how they are translocated across the outer membrane. In contrast to *E. coli* and many other Gram-negative bacteria, *Neisseria meningitidis* and several spirochetes do not possess the *tol-pal* gene cluster, nor do they have a complete Lol apparatus. It has been postulated that the lack of a complete Lol system in the spirochete *Borrelia burgdorferi* may be a cause of localization of several lipoproteins to the outer leaflet of the outer membrane of this bacterium (155). More recently, work on lipoproteins of *Borrelia* has led to the proposal that release from the inner membrane and outer membrane translocation are separate events and that there may be other amino-terminal determinants that direct outer membrane translocation relative to those that direct inner membrane retention or release (154). The role of the amino terminus of lipoproteins may be more than just to direct outer membrane localization. A recent study used nuclear magnetic resonance (NMR) to demonstrate that the amino terminus of the *Neisseria meningitidis* lipoprotein, LP2086, serves as an extended linker to display the protein at the extracellular surface (102–103).

In Gram-positive bacteria, the issue of localization is conceptually simpler due to their lack of an outer membrane, and it has been reviewed in detail by Hutchings et al. (75). The preprolipoprotein precursor is translocated across the cyto-

TABLE 1. Representative biological activities elicited by lipoproteins from bacterial pathogens

Role or function	Lipoprotein(s)	Strain	Reference(s)
Antigenicity	LpqH, LprG, PstS-1	*M. tuberculosis*	41, 51, 56, 73, 114, 148, 166, 179
	PsaA, PiaA, PiuA	*S. pneumoniae*	85, 125
	VlsE, Osp	*B. burgdorferi*	10, 199
	fHbp, LbpB, P47, GNA2132	*N. meningitidis*	5, 32, 100, 147, 160
Involved in colonization	PsaA, PpmA, SlrA	*S. pneumoniae*	34, 71, 101, 117
	DbpA, OspB, BmpA	*B. burgdorferi*	18, 113, 188
Components of transport systems	PstS-1, FecB	*M. tuberculosis*	182, 192
	PsaA, PiaA, PiuA, PspA	*S. pneumoniae*	23, 42, 65
	OppA, OspB	*B. burgdorferi*	96, 113
	LpbB, GNA1946, Tpb2	*N. meningitidis*	94, 134, 190, 201
Required for growth	ModA, SubI, LpqH	*M. tuberculosis*	28, 153, 166
	PsaA	*S. pneumoniae*	101
	OspB	*B. burgdorferi*	113
	DsbA, GNA33	*N. meningitidis*	1, 176
Protein folding	PpmA, SlrA	*S. pneumoniae*	71, 121
	DsbA	*N. meningitidis*	176
Signal transduction	LprF, LprJ	*M. tuberculosis*	167
Role in antibiotic resistance	PsaA	*S. pneumoniae*	117

plasmic membrane of bacteria, directed by the N-terminal signal peptide sequence, where it is modified by the biosynthetic enzymes discussed above. The resulting lipoproteins are thus being exposed to the extracellular environment, anchored to the outer leaflet of the plasma membrane via hydrophobic interactions. The majority of preprolipoproteins are exported in an unfolded conformation by the universally conserved Secretory (Sec) pathway (44) and obtain tertiary structure entirely after the Sec pore. In addition to the Sec pathway, many prokaryotic organisms possess other protein export pathways, including the twin-arginine translocation (Tat) pathway, which is responsible for the transport of folded proteins across the membrane (16). Translocation of lipoproteins by the Tat system in the high-GC Gram-positive bacteria (*Actinomycetes*) has also been indicated (195). Similarly, Giménez et al. represented the first experimental demonstration of a lipoprotein Tat substrate in the archaeon *Haloferax volcanii* (58). Tat-mediated transport of lipoproteins across the cytoplasmic membrane of Gram-negative bacteria has also been reported (186).

The cell wall of *Mycobacterium tuberculosis* contains an atypical outer lipid layer, consisting predominantly of mycolic acids, which serves as a potential anchoring point for lipoproteins (Fig. 2). This is supported by immunogold labeling experiments that demonstrate that mycobacterial lipoproteins are surface exposed (198). There is evidence for glycosylation of a number of mycobacterial lipoproteins (90, 107), although the role of glycosylation is not clear. It might contribute to maintaining the membrane association of the protein or protect against proteolytic cleavage (72). Finally, lipoproteins can be shed from the cell and may play an extracellular role, extending their ability to function farther away from the cell (196).

As described above, the pathway for lipoprotein biogenesis consists of three steps catalyzed by lipoprotein diacylglyceryl transferase (Lgt), lipoprotein or type II signal peptidase (Lsp or SPase II), and lipoprotein N-acyl transferase (Lnt). The accurate processing of lipoproteins is likely to be carried out by a close interaction between Lgt, Lsp, and the protein translocation apparatus. How the biosynthetic pathway couples to the translocation machinery nonetheless remains to be resolved (75). Moreover, recent publications regarding the existence of Tat translocation of lipoproteins reveal that linear lipoprotein precursors can not only be lipidated but also folded, which makes the understanding of the mode of modification even more unclear.

BIOLOGICAL PROPERTIES OF LIPOPROTEINS ASSOCIATED WITH VIRULENCE

Lipoproteins are required for virulence in some bacteria playing a variety of roles in host-pathogen interaction, from surface adhesion and initiation of inflammatory processes through to translocation of virulence factors into the host cytoplasm (Table 1). These roles of lipoproteins in virulence have typically been revealed in *lgt* and *lsp* mutants. In order to investigate the role of lipoproteins in virulence, studies relating to pathogenesis on *lgt* or *lsp* mutant bacteria have been carried out (35). For example, a reduction in fibronectin and laminin binding of Lsp mutants of *Streptococcus pyogenes* has been demonstrated, with the mutant showing reduced levels of adhesion and internalization (47). In many cases, mutations have led to attenuation of virulence in animal models of infection. For example, an *lgt* mutant of *Streptococcus pneumoniae* (133) was avirulent in a mouse model of infection, while disruption of *lgt* resulted in moderately attenuated virulence of *Streptococcus equi* (64), with 3 of 30 mice challenged with the Δ*lgt* strain exhibiting signs of disease. Loss of Lsp markedly reduced virulence of *M. tuberculosis*, (149), but no effect has been found on the virulence of *Streptococcus suis* (38). An *lsp* mutant of *L. monocytogenes* showed reduced virulence; the 50% lethal dose

(LD_{50}) value of the mutant was 10-fold lower than that of the wild-type (144). Importantly, an *lgt* mutant of *Staphylococcus aureus* showed a hypervirulent phenotype in a mouse infection model, accompanied with attenuated growth (168). An explanation for this increase in virulence could be that the nonlipidated lipoproteins are not recognized by the immune system and are thus unable to activate Toll-like receptor 2 (TLR2)-mediated signaling, a component of the innate immune response (24, 168). Bacterial lipopeptides are recognized by TLR2 in a complex manner. Initially, it was reported that diacylated lipoproteins were recognized by a heterodimer of both TLR2 and TLR6 and that triacylated lipopeptides, typically produced by Gram-negative bacteria, are recognized by a heterodimer of both TLR2 and TLR1 (171–172). However, in addition to the recent findings that some lipoproteins from Gram-positive bacteria are triacylated, it has also been shown that these triacylated proteins were native ligands of TLR2 that did not require either TLR6 or TLR1 (89). Although it has been shown that it is the acyl moiety of lipoproteins that interacts with TLR2, the question remains as to how these structures interact if the acyl chains are interacting hydrophobically with the bacterial membrane (83). Notwithstanding this, the discovery that bacterial lipoproteins are potent inducers of the host inflammatory responses adds a novel dimension to their role in pathogenesis, highlighting their potential to affect a wide range of mechanisms in virulence. The following section looks in detail at the roles of lipoproteins of four pathogens where the acyl-modified proteins of these bacteria have been shown to have several roles in pathogenesis.

Lipoproteins of *Mycobacterium tuberculosis*. The *M. tuberculosis* genome encodes 99 putative lipoproteins, ca. 2.5% of the predicted proteome, with many different functions verified either experimentally or by bioinformatical tools or both (29, 169). Some lipoproteins are solute binding proteins (SBPs), contributing to virulence as components of ABC transporters (PstS and ModA) (28, 191) or play a role in growth (SubI and GlnH) (153). PstSs are phosphate-binding receptors of a phosphate-specific transport system, and PstS-3 and PstS-1 have been shown to be promising vaccine candidates (37, 173). PstS-1 is a 38-kDa lipoglycoprotein involved in phosphate transport and has been demonstrated to be an apoptogenic molecule. It induces both the intrinsic and extrinsic pathway of the caspase cascade (148). In addition to PstS-1, three other lipoprotein antigens, namely, LpqH, LprG, and LprA, have also been shown to contribute to virulence by induction of either immunosuppressive responses and/or humoral and cellular responses. ModA functions as a molybdenum transporter and is potentially involved in virulence (28). A redox enzyme, the SodC (Cu, Zn superoxide dismutase) antioxidant, may participate in inhibition of the immune response in the phagolysosomal oxidative environment, and there is evidence of its lipid modification (36). The Mce family of proteins, whose members enable *M. tuberculosis* to invade mammalian cells, contains a high proportion of lipoproteins (33). However, how these lipoproteins mediate entry has not yet been established.

Similar to PstS, the 19-kDa antigen of *M. tuberculosis* is also a lipoglycoprotein and has been the focus of several studies because of its pleiotropic effects on the immune response. Glycosylation was found to be important for retaining the protein within the cell in association with the acyl component (197). The 19-kDa antigen is recognized by TLR2, activating immune responses at early time points of infection with an associated increase in the production of cytokines (e.g., interleukin 1β [IL-1β], IL-12p40, and tumor necrosis factor alpha [TNF-α] secretion) (197) and toxic intermediates, such as reactive oxygen radicals and reactive nitrogen intermediates (20). In contrast, in chronic infection, the 19-kDa antigen has been shown to inhibit antimicrobial mechanisms, including inhibition of gamma interferon (IFN-γ)-dependent responses, such as antigen processing (122). Following antigen presentation by major histocompatibility complex (MHC) class II molecules, $CD4^+$ T cells become activated and secrete cytokines, including IFN-γl which plays a central role in the host defense against tuberculosis by inducing transcription of more than 200 genes essential for antimicrobial mechanisms (51). Prolonged exposure of macrophages to the 19-kDa lipoprotein inhibits transcription of some genes regulated by IFN-γ, including MHC class II transactivator (CIITA), MHC II, interferon regulatory transcription factor I (IRF-I), and type I receptor for the Fc domain of IgG (CD64) (51, 130). Inhibition of these pathways is an efficient way to make macrophages unable to eradicate *M. tuberculosis*, and it is likely that it is not solely the 19-kDa lipoprotein of *M. tuberculosis* that is able to do so. With this mechanism, the 19-kDa lipoprotein evades immune responses by inhibiting MHC-II expression and antigen presentation, allowing the pathogen to persist in macrophages and maintain a chronic infection.

Although $CD4^+$ T cells are the more dominant participants of immunity against tuberculosis, the 19-kDa antigen can also modulate $CD8^+$ T cell responses, even if the mechanism of inhibition is different. In macrophages, the alternate MHC class I antigen presentation of *M. tuberculosis* occurs in a vacuole, not in the cytoplasm, consisting of phagosomal degradation of the antigen and then probably phagosomal binding of the antigenic peptides to MHC class I, leading to lysis of infected cells or cytokine production by the $CD8^+$ T cells. It has been shown that the 19-kDa antigen does not affect the levels of MHC-I and MHC-I mRNA but inhibits phagosome maturation and lysosomal protease delivery, resulting in decreased catabolism and presentation of antigens, resulting in inhibition of IFN-γ signaling (179).

Apoptosis is an important element of host defense mechanisms to kill intracellular pathogens. However, it remains unclear whether the pathogen or, rather, the host benefits from the apoptosis of infected cells. Apoptosis has been thought of as a silent event, although it has been shown that infection-induced apoptosis is accompanied by inflammation through production of proinflammatory mediators (e.g., IL-1β, which attracts neutrophil granulocytes to the site of infection), providing further immune reactions. It was shown that mycobacterial antigens carried by released apoptotic vesicles are presented by bystander dendritic cells through MHC-I molecules, leading to cross-priming of $CD8^+$ T cells (19). The 19-kDa antigen is involved in apoptosis, acting as a proapoptotic factor in monocytes and macrophages. The signaling is TLR2 mediated, dependent on caspase-8 but independent of caspase-9 (98).

The 19-kDa lipoprotein also stimulates neutrophil activation as determined by a decreased expression of L-selectin and an

increased expression of CR3 integrin and CR1. These phenotypic changes modulate functional changes in neutrophils, including adhesion and diapedesis abilities, and binding of opsonins. Furthermore, the 19-kDa antigen is indirectly involved in the late events of neutrophil activation, such as increased production of reactive oxygen species (ROS). While it does not have the capacity to induce the oxidative burst by itself, the 19-kDa antigen can enhance the production of ROS indirectly through priming for subsequent activation by *n*-formyl-Met-Leu-Phe (fMLP) (114).

Lipoproteins of *Streptococcus pneumoniae*. Five of the 42 predicted lipoproteins among the numerous virulence determinants (capsule, surface, and subsurface proteins) of *Streptococcus pneumoniae* are immunogenic and are suggested to be involved in virulence (15). Two of them, namely, PiaA (pneumococcal iron acquisition) and PiuA (pneumococcal iron uptake) (formerly Pit1A and Pit2A), are encoded on a pathogenicity island and are present in all typical *S. pneumoniae* strains (194). PiaA and PiuA are homologous ABC iron transporters (23), and anti-PiaA and -PiuA antibodies have been shown to promote opsonophagocytosis of *Streptococcus pneumoniae* (85). All serotypes of *S. pneumoniae* express PsaA (pneumococcal surface adhesion A), which has been demonstrated to play a major role in the pathogenesis of *S. pneumoniae* infection (17), while immunization with PsaA has been shown to be protective against carriage of *S. pneumoniae* but not against systemic disease (84). PpmA (putative proteinase maturation protein A) and SlrA (streptococcal lipoprotein rotamase A) are lipoproteins belonging to a family of peptidyl isomerases (PPIases) and have been demonstrated to contribute to the virulence of *S. pneumoniae* by promoting colonization (34, 71).

Similarly to the 19-kDa antigen of *M. tuberculosis*, PsaA also has pleiotropic effects on cellular physiology and might thus be a promising vaccine target. This 37-kDa antigen belongs to the lipoprotein receptor-associated antigen I (LraI) family and was previously thought to be an adhesin, although it is more likely to be involved in the regulation of adherence rather than being an adhesin itself. This question concerning the adhesin characteristics of PsaA has come from the finding that initial identification of some other proteins (FimA of *Streptococcus parasanguinis* and ScaA of *Streptococcus gordonii*) (4, 50, 64) from the LraI family of adhesins has seemed to be false (53, 79). However, in support of its role as an adhesin, application of antibodies against PsaA reduced the ability of pneumococci to adhere to nasopharyngeal epithelial cells (146), and in another study, upregulation of *psaA* has been found during attachment to pharyngeal epithelial cells (120). Consistent with these observations, PsaA mutants of *S. pneumoniae* showed impaired adherence to type II pneumocytes, and these strains were avirulent *in vivo* (17). Furthermore, PsaA exhibits invasion-like effects, and E-cadherin has been shown to be a potential eukaryotic receptor for PsaA (141). Contradictory to these findings is the fact that the structure of PsaA (93) and its membrane anchoring prevent the protein from reaching out of the at least 40-nm-thick Gram-positive bacterial cell wall and capsule (57, 84). The suggested regulatory role of PsaA in adhesion is supported by a study where the production of the adhesin, CbpA, was completely absent in a PsaA-deficient mutant (117). The *psaA* gene is found in the *psa* operon along with genes of other components of the transport system (ATP-binding protein and integral membrane protein) and encodes an ABC-type permease for Mn^{2+} (117), which makes the indirect role of the protein in adherence more relevant. Although a dual function of this lipoprotein is still not conclusively disproved, monofunctionality seems to be more accepted.

The cellular physiology of many pathogens relies on Fe(II), although some bacteria can grow independently of iron or require Mn(II) for both growth and pathogenesis (42, 78, 84, 116, 138). The Mn(II) concentration within the host is very low, and high-affinity manganese transporters are required for survival. In *S. pneumoniae*, PsaA might be the transporter which fulfils this function. Studies with bacteria containing mutations in their manganese transporters proved them to be highly sensitive to oxidative stress, thereby indicating that manganese is a key metal in protection from the oxidative environment (87, 185). Inactivation of the *psaA* gene makes *S. pneumoniae* hypersensitive to oxidative stress (184). H_2O_2 is produced by *S. pneumoniae* (165) and is thought to have role in virulence since H_2O_2 is toxic to alveolar epithelial cells (45). Another benefit of H_2O_2 apart from this is that it can eradicate other competing microorganisms in the upper respiratory tract (132). *S. pneumoniae* lacks catalase and protects itself from internally generated hydrogen peroxide and reactive radicals, produced by the Fenton reaction, which cause cellular damage (135) by detoxifying mechanisms such as those performed by SodA (superoxide dismutase), which requires Mn(II) (203). Virulence studies of *sodA* and *psa* mutant *S. pneumoniae* showed that a lack of SodA causes only partially impaired virulence while a lack of Psa makes the strain avirulent (101, 203), supporting the pleiotropic effect of Psa's complex role in adherence and detoxification.

PsaA stimulates immune responses through the production of antibodies (IgA, IgG, and IgM) by B cells (206) and by activation of $CD4^+$ T cells, resulting in expression of IL-4, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, and TNF-α (125). In addition, *in vivo* survival studies and human antibody (Ab) responses to pneumococcal infection revealed great immunogenicity of PsaA (141). In addition to being immunogenic, PsaA has been shown to contribute to the virulence of *S. pneumoniae* by being involved in adherence, growth, protection against oxidative stress, and competence by providing transport of the essential manganese. Vaccines against *S. pneumoniae* contain a mixture of serotype-specific capsule polysaccharides, but it is not possible to cover all serotypes given that new ones emerge from time to time. Being highly conserved among the *S. pneumoniae* serotypes from all over the world, PsaA may provide protection against a larger number of serotypes. Furthermore, when combined with another species-common immunogenic protein (PspA), its immunogenicity was shown to be enhanced (118).

Lipoproteins of *Borrelia burgdorferi*. The Gram-negative spirochete *Borrelia burgdorferi* is the causative agent of the emerging Lyme disease. *Borrelia* is transmitted to humans by the bite of infected ticks belonging to a few species of the genus *Ixodes* (26). Initial analysis of the genome sequence of *B. burgdorferi* identified 105 putative lipoproteins as predicted by the presence of a consensus lipobox in the first 30 amino acids (52). These represented more than 8% of the coding sequences, compared to 2.3% for *Neisseria meningitidis* (126). However,

given the plasticity of the consensus motif (lipobox) for acyl modification in spirochetes, a novel algorithm was generated that, when applied to the *B. burgdofreri* genome, identified 120 lipoproteins (161).

Intriguingly, for a bacterium that has a lifestyle that encounters diverse physiological niches, *B. burgdorferi* has no identifiable machinery for the synthesis of amino acids. It does, however, possess the oligopeptide ABC transporter (*opp* operon), which exhibits broad substrate specificity, probably compensating for its restricted coding potential by producing proteins that can import a wide variety of solutes (52). This peptide transport system is a member of the superfamily of ABC transporters and has a high degree of similarity to the oligopeptide permease (Opp) system of *Escherichia coli* (129). In *E. coli*, this system consists of a peptide binding protein (OppA), two transmembrane proteins (OppB and OppC), and two ATP binding proteins (OppD and OppF). In *B. burgdorferi*, the *opp* operon encodes two additional OppA-like proteins (OppA-2 and OppA-3). These *oppA* genes are part of a seven-gene operon that completes the components of a predicted peptide transport system. The three *oppA* homologues all encode lipoproteins, and this arrangement of proximal multiple tandem genes for peptide-binding proteins is unique among operons that encode peptide transporters (129). In addition to these three chromosomally encoded *oppA* genes, there are two further plasmid-carried homologues whose products, OppA-4 and OppA-5, are also lipoproteins (96). The observation that the five periplasmic binding proteins (OppA1 to -5) are lipoproteins suggests that they may be anchored to the outer surface of the cytoplasmic membrane as in Gram-positive bacteria, rather than being localized in the periplasmic space. The functional significance of this remains to be determined, although it is interesting to speculate that this may in some way be involved with the atypical architecture of the spirochete cell wall.

The ability of this spirochete to infect different hosts requires it to express different molecules at various stages of its life cycle, depending on its current environment. The differential expression of lipoproteins by *B. burgdorferi* is a characteristic of its transition to life inside the mammalian host (27). The 6.6-kDa lipoprotein of *B. burgdorferi* is one such protein that has been shown to be involved in the formation of outer membrane protein complexes and is consistently expressed within ticks (139). This pattern of expression is analogous that of to other *Borrelia* lipoproteins important in the vector, such as OspA and OspB (202). These findings suggest that the function of selected lipoproteins is likely to be important for the spirochete life cycle in ticks. In contrast, in murine models of host infection, these and other lipoproteins have been shown to be downregulated (156).

In addition to their variable expression, one of these lipoproteins, VlsE, is capable of antigenic variation and is a key virulence determinant in persistence of disease. The *vls* locus is comprised of an expression site (*vlsE*) encoding the 35-kDa lipoprotein VlsE and an operon of 15 unexpressed cassettes which have homology to the central region of VlsE. These cassette segments recombine with the expression locus in order to produce large numbers of distinct antigenic variants during infection (205). Recently, studies on a mutant strain of *B. burgdorferi* deficient for VlsE expression have demonstrated that while the strain is unable to persist in immunocompetent mice, it is able to persist in immunodeficient SCID mice, thus suggesting that VlsE is not required for persistent infection in the absence of an adaptive immune response and supporting the hypothesis that *vls* recombination occurs to evade the humoral immune response in the murine host (10). Other immunogenic lipoproteins of *B. burgdorferi* include the decorin binding protein DbpA, and this prompts the question as to why antibodies raised against this surface lipoprotein are not bactericidal. Nevertheless, it is clear that lipoproteins play a key role in evading the humoral immune response.

Lipoproteins of *Neisseria meningitidis*. Analysis of the genome sequences of the pathogen *Neisseria meningitidis* Z2491 (126), a serogroup A strain, and of the closely related meningococcal strains MC58 and FAM18 (serogroups B and C, respectively) (14, 174) reveals that all strains contain the genes encoding LolABCD (NMA0830, -1091, -1403, and -1402), a complex essential for targeting lipoproteins to the outer membrane (119). The annotation of *N. meningitidis* Z2491 (126) predicts that this genome encodes for 53 lipoproteins based on the presence of a signal peptide (115) and the presence of a prokaryotic membrane lipoprotein lipid attachment site (Prosite PS51527) (74). The following discusses the role of some of these lipoproteins in meningococcal disease.

Meningococcal lipoproteins that may play a role, although indirect, in the virulence of this pathogen include components of the type IV pili and those involved in the generation of bacterial disulfide bridges. The pathophysiology of meningitis caused by *N. meningitidis* involves the bacteria crossing the oropharyngeal mucosal barrier, surviving and replicating in the bloodstream, and finally crossing the blood-cerebrospinal fluid (CSF) barrier. *Ex vivo*, type IV pili have been shown to mediate adhesion of *N. meningitidis* to human endothelium and to the meninges, suggesting that they play a key role in the adherence of this pathogen in disease (67). The *N. meningitidis* lipoprotein PilP (NMA0651) is integral to the generation of type IV pili, since mutation of *pilP* results in a nonpiliated strain of meningococcus (8). In addition to pili, many pathogenic bacteria express a broad array of disulfide-bonded virulence factors, including secreted toxins and surface components. The formation of these bonds is mediated by the Dsb system, of which DsbA is the component that oxidizes the cysteine residues of the substrate molecule. *N. meningitidis* is unique in its possession of three homologues of this protein, two of which are lipoproteins. Deletion of any one of these homologues is compensated by the others, but deletion of the two lipoproteins results in the loss of functionality of the type IV pili (176).

Currently, vaccines against *N. meningitidis* are based on the polysaccharides of the O antigens of serogroups A, C, W-135, and Y, with glycoconjugate vaccines having these carbohydrates conjugated to a modified diphtheria toxin (164). However, such an approach is not applicable in the generation of a vaccine to serogroup B strains since their polysaccharide is nonimmunogenic because of its chemical identity to human neural surface antigens. Initial approaches to vaccines against *N. meningitidis* serogroup B focused on outer-membrane vesicles (OMVs) and iron-regulated outer membrane proteins (9, 30). These studies led to the identification of several immunogenic lipoproteins, including the antigen P47 (NMB0035) (5), lactoferrin binding protein B (LbpB) (NMB1541) (134), and

transferrin binding protein B (Tbp2) (NMB0460) (94). All of these proteins elicit an immune response, and sera against them are bactericidal *in vitro*. In alternative approaches to the development of a vaccine against *N. meningitidis* serogroup B, a reverse vaccinology approach identified a novel surface-exposed lipoprotein as genome-derived neisserial antigen GNA1870, or rLP2086 (NMA0586), which is conserved across many of the serogroups and is currently in phase III clinical trials as a component of a pentavalent vaccine, 5CVMB, for serogroup B meningitis (59, 104). Its inclusion in this vaccine is due to the profound effect this lipoprotein has on protecting the meningococcus from the immune system.

Complement is an important arm of innate immune defenses against invading pathogens. Complement activation leads to the deposition of C3 fragments (C3b and iC3b), which can enhance opsonophagocytosis of microbes. *N. meningitidis* binds to factor H, the main inhibitor of the alternative complement pathway, which enhances their ability to evade complement-dependent killing. This binding of factor H is mediated by the lipoprotein GNA1870 (also called factor H binding protein [fHBP]) (100). This was demonstrated by insertional inactivation of *gna1870*, which resulted in an abrogation of factor H binding directly to the bacterial surface, with a subsequent increased sensitivity of the meningococcus to complement-dependent killing in a serum bactericidal assay. This phenomenon mirrors the function of the *B. burgderfori* OspE lipoprotein, which also binds factor H (69). The ability to colonize the nasopharynx efficiently is also dependent on the ability of the meningococcus to evade host killing mechanisms, such as antimicrobial peptides. LL-37, one such peptide, is produced by upper-airway epithelial cells and has been shown to play a role in mucosal immunity against oral microflora (109). GNA1870 was found to counteract the antimicrobial effects of LL-37, probably through electrostatic interactions that prevent the peptide from accessing the bacterial surface (157). These observations demonstrate that this factor H binding protein from *N. meningitidis* plays a key role in evading attack from the innate immune system.

More recent bioinformatic approaches used to identify potential novel protein vaccine candidates against serogroup B *N. meningitidis* have identified several more lipoproteins (NMA1123, -1134, and -1091 [LolB] and NMB0033, -1163, -1946, and -2132 [sometimes NMB is replaced with GNA] [124, 137]) that are immunogenic and generate bactericidal antibodies as determined by a serum bactericidal assay. The functions of many of these proteins are unknown, though LolB is recognized by the immune system and was initially identified through early immunological screens of meningococcal libraries with a monoclonal antibody, H.8, that had been developed to selectively recognize pathogenic neisserial strains (31, 61). More recently, NMB2132 (renamed neisserial heparin binding antigen (NHBA), has been shown to bind heparin and induce bactericidal antibodies in humans (160).

SUMMARY

This review highlights the diverse roles that lipoproteins of bacterial pathogens play in infection, and as such, it can be postulated that there is much more to be elucidated about the potential role of these molecules as targets for the generation of novel antibacterial agents. Lipopeptides were first demonstrated to have a role in stimulating cytotoxic T-lymphocyte production around 20 years ago (110), and since then, lipoproteins of numerous pathogens have been promoted as vaccine candidates. In addition to the well-characterized lipoprotein vaccine antigens of *Neisseria* discussed above, bacterial lipoproteins have been shown to affect both the innate and acquired immune systems via such mechanisms as TLR2 signaling and the generation of cytotoxic T lymphocytes and bactericidal antibodies (11, 104, 204). As such, a number of lipoproteins from many pathogens have been evaluated as vaccine candidates (6, 48, 99, 136, 143, 152, 193). Many of these, however, have been administered in recombinant form, making it difficult to ascertain the contribution of the native acyl moiety to protection. There are two notable instances where lipoproteins have been shown to have a deleterious effect on protection. These are two immunodominant lipoproteins of *M. tuberculosis*, the 19-kDa and 27-kDa antigens, which, when overexpressed in *Mycobacterium bovis* BCG, cause a deleterious effect on protection (73, 142). In the case of the 19-kDa antigen, the overexpression is likely to be the cause of the deleterious phenotype, since immunization of mice with BCG, or purified 19-kDa antigen, leads to a protective phenotype, but whether it is overexpression of the lipid portion remains to be determined. In the case of the 27-kDa antigen, it is the protein rather than the lipid portion that is responsible for the antiprotective effect, since immunization with a nonacylated form of the protein led to an increase in *M. tuberculosis* multiplication following challenge relative to unimmunized mice. However, the acylated amino terminus of the 19-kDa antigen of *M. tuberculosis* has been used to create heterologous antigens that, when expressed in BCG, confer protection against pathogens such as *Listeria monocytogenes*, *Streptococcus pneumoniae*, *Borrelia burgdorferi*, and *Escherichia coli* (46, 62, 68, 92, 158). Thus, these immunogenic surface-exposed lipoproteins undoubtedly have an important role to play in the future development of vaccines against bacterial pathogens. Furthermore, the essentiality of certain lipoproteins and the enzymes involved in their biosynthesis and localization indicates that these proteins may be targets for the generation of novel antibacterials. This last point is highlighted by the finding that a novel screening approach of small molecules identified an inhibitor of LolA that may lead to a new generation of Gram-negative-specific antimicrobials (128) (128). Further understanding of the roles and mechanisms of synthesis of bacterial lipoproteins will aid these avenues of research.

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