

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

Submitted by
Andrea Kovacs-Simon

to the University of Exeter as a thesis for the degree of Doctor of Philosophy in
Biological Sciences
June 2013

This thesis is available for Library use on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University

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. difficile* 630 Δ *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. difficile* was investigated by several different assays

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

List of Contents

Abstract	iv
List of Contents	vi
List of Figures	xi
List of Tables	xv
List of Appendices	xvi
Declaration	xvii
Acknowledgements	xviii
Abbreviations	xix
Publication	xxii
1. Chapter One: Introduction	1
1.1. The <i>Clostridium</i> genus	2
1.2. <i>Clostridium difficile</i>	3
1.2.1. Typing	4
1.3. <i>Clostridium difficile</i> infection (CDI)	4
1.3.1. Symptoms	4
1.3.2. Risk factors	6
1.3.3. Epidemiology	7
1.3.4. Diagnosis	8
1.3.5. Treatment and prevention	10
1.4. Pathogenesis of <i>C. difficile</i>	12
1.4.1. Antibiotics	12
1.4.2. Life cycle of <i>C. difficile</i>	13
1.4.3. Virulence factors	15
1.4.3.1. Toxins	15
1.4.3.2. Surface proteins	17
1.4.3.3. Other virulence factors	21
1.5. Bacterial lipoproteins	23
1.5.1. Biosynthesis	23
1.5.2. Localisation	26
1.5.3. Functions	29
1.5.4. Antimicrobial targets	35

1.6. This study	35
1.6.1. Objectives	35
1.6.2. Thesis overview	36
2. Chapter Two: Materials and Methods	37
2.1. General materials	38
2.2. Bacterial strains and growth conditions	39
2.3. Preparation and manipulation of DNA and RNA	42
2.3.1. Plasmid design	42
2.3.2. Oligonucleotide design	43
2.3.3. DNA extraction and purification	46
2.3.4. Quantification of DNA	46
2.3.5. Polymerase Chain Reaction (PCR)	46
2.3.6. Restriction enzyme digestion of DNA	47
2.3.7. Agarose gel electrophoresis	47
2.3.8. Gel extraction of DNA	47
2.3.9. DNA ligation	47
2.3.10. DNA sequencing	48
2.3.11. Southern blot	48
2.3.12. Reverse transcriptase PCR (RT-PCR)	49
2.4. Manipulation of <i>E. coli</i>	49
2.4.1. Transformation of <i>E. coli</i>	49
2.4.1.1. Preparation of CaCl ₂ -competent <i>E. coli</i> cells	49
2.4.1.2. Transformation of CaCl ₂ -competent <i>E. coli</i>	50
2.4.2. Expression of recombinant proteins in <i>E. coli</i> C43(DE3)	50
2.5. Generation of <i>C. difficile</i> mutants	51
2.5.1. ClosTron system	51
2.5.2. Conjugation	53
2.5.3. Isolation of the integrants (mutants)	54
2.6. Protein techniques	54
2.6.1. Preparation of the cellular and extracellular protein fractions of <i>C. difficile</i>	54
2.6.2. SDS polyacrylamide gel electrophoresis (SDS-PAGE)	56
2.6.3. Western blotting	57
2.6.4. Two-dimensional gel electrophoresis (2DGE)	57

2.6.5.	Mass spectrometric analysis	58
2.6.6.	Chemical biology experiment (`Click` chemistry)	59
2.6.7.	Purification of recombinant proteins from <i>E. coli</i> C43(DE3)	60
2.6.8.	Differential scanning fluorometry (DSF)	61
2.6.9.	Native PAGE	61
2.6.10.	Production of antibodies to the CD0873 protein	62
2.6.11.	Protein microarray	62
2.7.	Phenotypic assays	63
2.7.1.	Determination of the growth rate of <i>C. difficile</i>	63
2.7.2.	Scanning electronmicroscopy (SEM)	64
2.7.3.	Recovering <i>C. difficile</i> spores	64
2.7.4.	Antimicrobial susceptibility testing	64
2.7.5.	Caco-2 cell adherence assays	65
2.7.5.1.	Bacterial binding assays	65
2.7.5.2.	Protein binding assays	67
2.7.6.	Cell viability assay	67
2.7.7.	Mouse CDI model	68
2.7.8.	Immunofluorescence microscopy	69
2.8.	Bioinformatical methods	70
2.8.1.	Identification of putative lipoproteins	70
2.8.2.	Other bioinformatical methods	70
3.	Chapter Three: Investigation of lipoprotein biosynthesis in <i>C. difficile</i>	72
3.1.	Introduction	73
3.1.1.	Identification of lipoproteins by bioinformatics	73
3.1.2.	Lipoprotein processing in Gram-positive bacterial species	74
3.1.3.	Aim of this study	75
3.2.	Results	75
3.2.1.	Identification of the putative lipoproteins of <i>C. difficile</i>	75
3.2.2.	Disruption of the lipoprotein biogenesis in <i>C. difficile</i>	76
3.2.3.	Lipoproteins are differentially distributed in the <i>lgt</i> mutant of <i>C. difficile</i>	83
3.2.4.	Investigation of the lipoprotein processing in <i>C. difficile</i>	90
3.3.	Discussion	97

4. Chapter Four: Immunoreactive lipoproteins of <i>C. difficile</i>	100
4.1. Introduction	101
4.1.1. Toxin and non-toxin antigens of <i>C. difficile</i>	101
4.1.2. Lipoprotein antigens of bacterial pathogens	102
4.1.3. Aim of this study	103
4.2. Results	103
4.2.1. Hamster antibody responses to <i>C. difficile</i> proteins	103
4.2.2. Building of a protein microarray	104
4.2.3. Serum samples and experimental controls of the protein microarray	109
4.2.4. Human antibody responses to <i>C. difficile</i> lipoproteins	113
4.3. Discussion	116
5. Chapter Five: Role of the Lipoprotein diacylglycerol transferase in the virulence of <i>C. difficile</i>	119
5.1. Introduction	120
5.1.1. Lipoprotein processing and virulence	120
5.1.2. Stress factors in the gut	122
5.1.3. Aim of this study	123
5.2. Results	123
5.2.1. <i>In vitro</i> growth of <i>C. difficile</i> is not affected by mutation of the <i>lgt</i> gene	123
5.2.2. Heat-resistant colony formation by <i>C. difficile</i> is not affected by <i>lgt</i> mutation	125
5.2.3. Effect of <i>lgt</i> mutation on the growth of <i>C. difficile</i> under intestinal stress conditions	126
5.2.4. Inactivation of the <i>lgt</i> gene does not affect the cell morphology of <i>C. difficile</i>	128
5.2.5. Lipoprotein processing by Lgt is required for <i>in vitro</i> adherence of <i>C. difficile</i>	129
5.2.6. Mouse CDI experiment	132
5.2.6.1. Generation and <i>in vitro</i> characterisation of a <i>tetM</i> mutant of <i>C. difficile</i>	132
5.2.6.2. Effect of <i>lgt</i> mutation on colonisation of <i>C. difficile</i> in mice	136
5.2.6.3. Confirmation of the bacterial strains recovered from mice	141
5.3. Discussion	142

6. Chapter Six: Lipoprotein CD0873 is a putative adhesion of <i>C. difficile</i>	147
6.1. Introduction	148
6.1.1. Colonisation by <i>C. difficile</i>	148
6.1.2. Lipoproteins and ABC-type transporters involved in colonisation of bacterial species	149
6.1.3. Aim of this study	150
6.2. Results	150
6.2.1. <i>In silico</i> identification of the CD0873 lipoprotein as a putative adhesin of <i>C. difficile</i>	150
6.2.2. Interaction of the recombinant CD0873 protein with metal ions and amino acids	154
6.2.3. Generation and complementation of a CD0873 mutant of <i>C. difficile</i>	162
6.2.4. Effect of CD0873 mutation on the growth of <i>C. difficile</i>	166
6.2.5. The CD0873 lipoprotein is exposed to the bacterial cell surface	173
6.2.6. Cytoadherence of <i>C. difficile</i> is inhibited by anti-CD0873 antibodies	175
6.2.7. Effect of CD0873 mutation on the adherence of <i>C. difficile</i> to Caco-2 cell line	177
6.2.8. The CD0873 lipoprotein binds to Caco-2 cells	179
6.2.8.1. Binding of the recombinant CD0873 protein to Caco-2 cells	179
6.2.8.2. Binding of the native CD0873 lipoprotein to Caco-2 cells	181
6.2.9. The CD0873 lipoprotein is expressed in <i>C. difficile</i> clinical isolates	183
6.3. Discussion	186
7. General conclusion	190
7.1. Key findings of this work	191
7.2. Final discussion and future perspectives	193
7.3. Epilogue	197
Appendices	201
References	210
Publication	232

List of Figures

Chapter One

Figure 1.1. Effect of antibiotic treatment on the normal gut flora and on the likelihood of the CDI.

Figure 1.2. Schematic overview of *C. difficile* infection (CDI).

Figure 1.3. Biosynthesis of the bacterial lipoproteins.

Figure 1.4. Localisation of bacterial lipoproteins.

Chapter Two

Figure 2.1. Map of the pMTL007C-E2 Clostron plasmid.

Chapter Three

Figure 3.1. Overview of the inactivation of the *lgt*, *lspA* and *lspB* genes of *C. difficile* 630 Δ *erm*.

Figure 3.2. Confirmation of the *lgt* mutant *C. difficile* 630 Δ *erm* by PCR and Southern blot analysis.

Figure 3.3. Map of the pMTL84151 modular plasmid.

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*.

Figure 3.5. Confirmation of the *lspA* and *lspB* mutants of *C. difficile* 630 Δ *erm* by PCR analysis.

Figure 3.6. Effect of *lgt* mutation on the extracellular and membrane proteome of *C. difficile*.

Figure 3.7. Comparison of the detergent phase proteins extracted from the wild-type and the *lgt* mutant *C. difficile* by 2DGE.

Figure 3.8. Protein lipidation is reduced in the *lgt* mutant *C. difficile*.

Figure 3.9. Detection of the CD0873 lipoprotein in the hydrophobic fraction of the wild-type *C. difficile* cell lysate.

Figure 3.10. Processing of the CD0873 lipoprotein is affected in the *lgt* and *lspA* mutant *C. difficile*.

Figure 3.11. Effect of the *lspA* and *lspB* mutations on the cleavage of the lipoprotein signal peptides in *C. difficile*.

Chapter Four

Figure 4.1. Immunoblot of *C. difficile* proteins using hamster sera.

Figure 4.2. Map of the pNIC28-Bsa4 LIC vector.

Figure 4.3. PCR amplification of the genes of *C. difficile* lipoproteins for recombinant protein production.

Figure 4.4. Expression and purification of the recombinant lipoproteins of *C. difficile* in *E. coli*.

Figure 4.5. IgG seroreactivity of the purified histidine tag with human serum samples.

Figure 4.6. IgG seroreactivity of the recombinant lipoproteins of *C. difficile* with human serum samples.

Chapter Five

Figure 5.1. Comparison of the growth of the wild-type, *lgt* mutant and complemented *lgt* mutant *C. difficile* in BHI medium.

Figure 5.2. Development of heat-resistant CFU by the wild-type, *lgt* mutant and complemented *lgt* mutant *C. difficile*.

Figure 5.3. Determination of the minimum inhibitory concentration (MIC) of bile salts against the wild-type and the *lgt* mutant *C. difficile*.

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.

Figure 5.5. Scanning electron microscopy of the wild-type, *lgt* mutant and complemented *lgt* mutant *C. difficile*.

Figure 5.6. Adherence of *C. difficile* to Caco-2 cells is reduced when *lgt* is inactivated.

Figure 5.7. Development of heat-resistant CFU by the wild-type, *lgt* mutant and *tetM* mutant *C. difficile*.

Figure 5.8. Competition growth curve of the wild-type, *lgt* mutant and *tetM* mutant of *C. difficile*.

Figure 5.9. Schematic overview of the mouse CDI competition experiment.

Figure 5.10. Effect of Lgt inactivation on the faecal shedding of *C. difficile* by mice.

Figure 5.11. Effect of Lgt inactivation on the colonisation of *C. difficile* in mice.

Chapter Six

Figure 6.1. Hypothetical models for the structure of the CD0873 protein from *C. difficile* 630.

Figure 6.2. SDS-PAGE of the recombinant CD0873 protein.

- Figure 6.3.** Determination of the stability of the recombinant CD0873 protein in various buffers.
- Figure 6.4.** Determination of the stability of the recombinant CD0873 protein in the presence of metal cations.
- Figure 6.5.** The recombinant CD0873 protein is destabilized in the presence of ferrous and ferric ions.
- Figure 6.6.** Determination of the stability of the recombinant CD0873 protein in the presence of amino acids.
- Figure 6.7.** Effect of protease treatment on the decomposition of the recombinant CD0873 protein.
- Figure 6.8.** Construction and PCR confirmation of the CD0873 mutant *C. difficile*.
- Figure 6.9.** Map of the pRPF144 plasmid.
- 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*.
- Figure 6.11.** The CD0873 lipoprotein is not expressed in the CD0873 mutant of *C. difficile*.
- Figure 6.12.** Comparison of the growth of the wild-type and CD0873 mutant *C. difficile* in BHI medium.
- Figure 6.13.** Effect of the presence of iron on the growth of the wild-type and the CD0873 mutant *C. difficile* in CDM.
- Figure 6.14.** Effect of the presence of zinc on the growth of the wild-type and the CD0873 mutant *C. difficile* in CDM.
- 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.
- Figure 6.16.** The CD0873 lipoprotein is exposed to the surface of *C. difficile*.
- Figure 6.17.** Determination of the viability of the Caco-2 cells in the presence of anti-CD0873 sera.
- Figure 6.18.** Adherence of *C. difficile* to Caco-2 cells is inhibited by CD0873 antibodies.
- Figure 6.19.** Mutation of the CD0873 gene results in reduced adherence of *C. difficile* to Caco-2 cells.
- Figure 6.20.** The recombinant CD0873 protein binds 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.

Figure 6.22. Homologues of the CD0873-CD0875 ABC-type transporter from *C. difficile* 630 in other *C. difficile* strains.

Figure 6.23. The CD0873 homologues are expressed in *C. difficile* clinical isolates.

Figure 6.24. Binding of the CD0873 homologues from *C. difficile* clinical isolates to Caco-2 cells.

List of Tables

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

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

Table 2.2. Strains used in this study.

Table 2.3. Plasmids used in this study.

Table 2.4. List of oligonucleotides used in this study.

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

Table 3.2. Distribution of lipoproteins in the Cdi::WT and Cdi::lgt strains.

Table 4.1. CDI patient information.

Table 4.2. Details of the serum samples collected from the CDI patients.

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

Table 4.4. Recognition of the recombinant lipoproteins by human sera (summary).

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

Table 5.2. PCR identification of the bacteria isolated from infected mice.

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

Table 6.2. Statistical analysis of the results of the CDM growth experiments (summary).

List of Appendices

Appendix I

List of the putative lipoproteins of *C. difficile* 630 and their predicted roles (Chapter 3).

Appendix II

Quantification of the lipoproteins in the protein extracts of the wild-type and the *lgt* mutant *C. difficile* (Chapter 3).

Appendix III

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

Appendix IV

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

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.

Acknowledgements

In the last four years I have learnt a lot and my PhD course has been a great experience. Many people have helped and supported me during this period and I would like to thank them all.

I would like to first offer many thanks to Rick Titball for giving me the opportunity to study in his group and for all his help he gave me and knowledge he passed on to me during this project. I would also like to express my special thanks to Steve Michell for his guidance, continued enthusiasm and for being an excellent supervisor.

I am also very grateful to all the co-workers, without them much of this work would not have been possible. I am thankful to Nic Harmer for his time and letting me use his equipments. I wish to thank Nigel Minton and the European Union 7th Framework Programme for funding my studentship. I would also like to thank everyone in the Bacterial Pathogenesis Research Group for the great environment and for their help whenever needed.

I owe massive thanks to my mum who gave me so much support despite being far away from me. Finally, I offer my deepest thanks to my husband, Denes, for his patience, encouragement and being with me through the good and bad times.

Thank you all

Abbreviations

AAD	antibiotic-associated diarrhoea
ABC	ATP-binding cassette
ADP	adenosine-diphosphate
AFLP	amplified fragment length polymorphism
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	<i>C. difficile</i> -associated diarrhoea
CDI	<i>C. difficile</i> infection
CDM	chemically defined medium
CdtLoc	<i>C. difficile</i> 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
Lint	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 <i>Staphylococcus aureus</i>
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
μ l	microliter
μ m	micrometer
μ M	micromolar

Publication

Kovacs-Simon, R. W. Titball and S. L. Michell. Lipoproteins of bacterial pathogens. *Infect Immun*, 2011. 79(2): p. 548-61.

Cover image of *Infection and Immunity*. February 2011, volume 79, issue 2.