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

Submitted by

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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

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“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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