2 Materials and methods

2.1 Materials

2.1.1 Bacteria and plasmids

Table 2.1 describes the bacteria and plasmids used in this study.

Table 2.1. Strains and plasmids used in this study.

Strain or plasmid	**Characteristics**	**Source**
Strains		
E. coli CA434	Electrocompetent conjugation donor	(195)
Top10	Electrocompetent *E. coli* for plasmid and amplicon cloning	Invitrogen
Rosetta(DE3) pLys	Competent *E. coli* for protein expression	Novagen
C. difficile 630	Virulent and multidrug resistant PCR-ribotype 012, isolated from a patient with pseudomembranous colitis in Zurich, Switzerland (1985)	(10)
C. difficile 630Δ*erm*	Erythromycin sensitive derivative of *C. difficile* 630	(196)
C. difficile R20291	Hypervirulent and epidemic PCR-ribotype 027, isolated from a hospital outbreak in Stoke Mandeville, UK (2004-2005)	(11)

630Δ*erm*Δ*spo0A*	*C. difficile* 630Δ*erm spo0A*::*erm*	This study
R20291Δ*spo0A*	*C. difficile* R20291 *spo0A*::*erm*	This study
630Δ*erm*Δ*spo0A*+p*spo0A*	Complemented 630Δ*erm*Δ*spo0A* mutant	This study
R20291Δ*spo0A*+p*spo0A*	Complemented R20291Δ*spo0A* mutant	This study
Plasmids		
pMTL007	First generation ClosTron plasmid with *catP* marker and intron containing *erm* RAM. Intron expression is induced using IPTG	(186)
pMTL007C-E2	Second generation ClosTron plasmid with *catP* marker and intron containing *erm* RAM. Contains a constitutive *fdx* promoter to direct intron expression	(190)
pRPF101	*E. coli* – *C. difficile* shuttle vector	This study
p*spo0A*	pRPF101 containing the 825 bp *spo0A* coding region (and upstream promoter)	This study
pWKS1245	Plasmid for the production of full-length Spo0A protein with a C-terminus His_6-tag	This study

2.1.2 Oligonucleotides

Oligonucleotides used in this study were designed by Primer*3* (0.4.0) software, synthesised by Sigma Aldrich, and are described in Table 2.2. All were dissolved in 10 mM TE buffer to a concentration of 40 pmoles/µl. Oligonucleotide pairs were designed with approximately the same G/C content, length and annealing temperature (T_m).

Table 2.2. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5'-3')
Intron retargeting and screening	
spo0A-178|179a-IBS	AAAAAAGCTTATAATTATCCTTATTATTCCATCTAG TGCGCCCAGATAGGGTG
spo0A-178|179a-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCC ATCTAGTTAACTTACCTTTCTTTGT
spo0A-178|179a-EBS2	TGAACGCAAGTTTCTAATTTCGGTTAATAATCGATA GAGGAAAGTGTCT
RAM-F	ACGCGTTATATTGATAAAAATAATAATAGTGGG
RAM-R	ACGCGTGCGACTCATAGAATTATTTCCTCCCG
EBS universal primer	CGAAATTAGAAACTTGCGTTCAGTAAAC
spo0A F	GCTAAGGATGGAATTGAAGCA
spo0A R	GCTCCTAGATTTATTGCGCTTT
spo0A complementation	
spo0A_A1F	(ATAT)GTCGACGGTGCAATAACTCATGTTTTTAGAG

spo0A_A2Rb	(ATAT)GTCGACGACTCTCATATTTAAACCTCCAC
DNA screening primers	
CD1498 F	GATTGCAGATGCATGTGGTT
CD1498 R	TTGGAGAGCAAGAACAGCAA
CD1455 F	GATGCAGAGGCAATTTCACA
CD1455 R	GCTAGAAGGATGCACGAAGG
CD0011 F	CCAGCTTTGCAACACCAACT
CD0011 R	GGCTATGGAGGCTTCTTATGG
CDadk F	TTACTTGGACCTCCAGGTGC
CDadk R	GCAGCCTTAGGAAGTGGAAA
C. difficile spo0A for protein purification	
oWKS-1122	TTTCATATGGGGGATTTTTAGTGG
oWKS-1123a	TGCTCGAGTTTAACCATACTATGTTCTAGT

2.1.3 Mice

All experiments were carried out using 5-to-9 week old specific-pathogen-free mice from colonies maintained at the Wellcome Trust Sanger Institute. All mice were of C57BL/6 wild type genetic background. Mice were housed in sterile cages with *ad libitum* access to food

and water. All animal infections were performed in accordance with the UK Home Office Animals (Scientific Procedures) Act of 1986.

2.2 Methods

2.2.1 Bacterial culture

All *C. difficile* were grown for 24 to 48 h at 37°C under anaerobic conditions in a MACS MG-500 anaerobic workstation (Don Whitley Scientific). *C. difficile* was routinely cultured in Wilson's broth (197) with agitation (80 rpm) or on CCEY agar (Bioconnections) supplemented with cycloserine (250 μg/ml; Bioconnections), cefoxitin (8 μg/ml; Bioconnections) and 0.1% taurocholate (Sigma Aldrich). *E. coli* were grown at 37°C using Luria-Bertani (LB) broth with agitation (200 rpm) or LB agar with appropriate selection. For the enumeration of spores, *C. difficile* cultures were mixed with 100% ethanol (1:1 ratio) for 1 h at room temperature to kill vegetative cells, pelleted, washed in PBS and cultured as above.

2.2.2 *In vivo* methods

Infection monitoring, faeces and organ collection and plating were performed by L. J. Pettit. Mouse infections, bleeding and sacrifices were performed by Dr S. Clare (WTSI, Cambridge), with assistance from L. J. Pettit.

All procedures and mouse handling were performed aseptically in a biosafety cabinet to contain spore-mediated transmission. A clinical scoring system was employed to track the condition of the mice, and symptoms including abnormal/hunched gait, piloerection, lethargy and emaciation were monitored. Moribund mice or mice displaying overt signs of disease were sacrificed humanely in accordance with approved protocols. Mice were anaesthetised with isoflourane (IsoFlo) prior to surgical procedures or interventions, and were sacrificed via cervical dislocation.

2.2.2.1 Mouse infections

Mice were pre-treated with drinking water containing clindamycin (250 mg/L; Apollo Scientific) for 7 d. Clindamycin was then withdrawn for 24 h and donors were infected via oral gavage or transmission, as indicated.

2.2.2.1.1 Oral gavage with C. difficile

C. difficile cultures were grown overnight in broth as described above, after which the culture was diluted 1/4 in PBS. Mice were then orally inoculated (with anaesthetic) with 200 µl of the bacterial suspension using a sterilised blunt-tipped gavage needle. When infected via oral gavage, mice received 10^7 vegetative cells and 10^5 spores (R20291 and 630Δ*erm* parental

strains) or 10^7 vegetative cells (R20291Δ*spo0A* and 630Δ*erm*Δ*spo0A* mutant derivatives), as determined by plate counts of the inocula.

2.2.2.1.2 Enumeration of viable C. difficile from mouse faeces

C. difficile was enumerated from fresh faeces as previously described (98). Briefly, mice were placed under sterilised beakers and faeces were collected. Samples were homogenised in phosphate-buffered saline (PBS; 100 mg faeces/ml PBS), serially diluted in PBS and plated on CCEY agar with appropriate supplementation. This was always performed within 30 min of excretion. For the enumeration of spores, samples were mixed with 100% ethanol (1:1 ratio) for 1 h at room temperature to kill vegetative cells, pelleted, washed in PBS and cultured as above.

2.2.2.1.3 Competitive index infections

Mice (n = 5) were infected via oral gavage with 10^7 CFU in 0.2 ml PBS of an overnight culture in 0.2 ml PBS containing equal proportions of parental *C. difficile* and the respective isogenic *spo0A* mutant. In order to determine the inoculum dose and ratio of parent to *spo0A* mutant derivative, the inocula were grown on both CCEY plates (as a non-selective media) and CCEY supplemented with 20 mg/ml lincomycin (which selects for the *ermB* gene inserted into the *spo0A* gene of both mutant derivatives). Faecal samples were then diluted as above

and plated on CCEY agar and CCEY agar supplemented with lincomycin. The competitive index (CI) was determined by dividing the ratio of mutant to parent bacteria using the following formula:

CI = (*spo0A* mutant/parental strain)$_{output}$/(*spo0A* mutant/parental strain)$_{input}$.

All data were logarithmically converted prior to averaging and statistical analysis. The Mann-Whitney test was applied to the $\log_{10}$ values of the CI ratios to determine the statistical significance of the results.

2.2.2.1.4 Transmission experiments

Donor mice (n = 5) were infected via oral gavage as described above. To measure transmission efficiency, naïve recipient mice (n = 5) were pre-treated with clindamycin as above and used in one of four types of transmission assays, as described in Table 2.3 and Figure 2.1. Following transmission, recipient mice were individually housed and the transmission efficiency was determined after 4 d by the isolation of the same strain of *C. difficile* in the faeces that was used to infect the respective donor mice.

Table 2.3. Summary of transmission routes.

Type of transmission (1 h)	**Characteristics**
Mingling	Donor and recipient mice were able to freely touch and interact, with no restriction in movement. Potential for coprophagy
Contact	Donor and recipient mice were separated by a single porous barrier, and were able to touch each other. No potential for coprophagy
Airborne	Donor and recipient mice were separated by a double porous barrier, and were unable to touch each other. No potential for coprophagy
Environmental	Recipient mice were housed in a contaminated donor cage, which had been left for 24 h under ambient oxygen conditions. Donor faeces were removed to prevent transmission via coprophagy

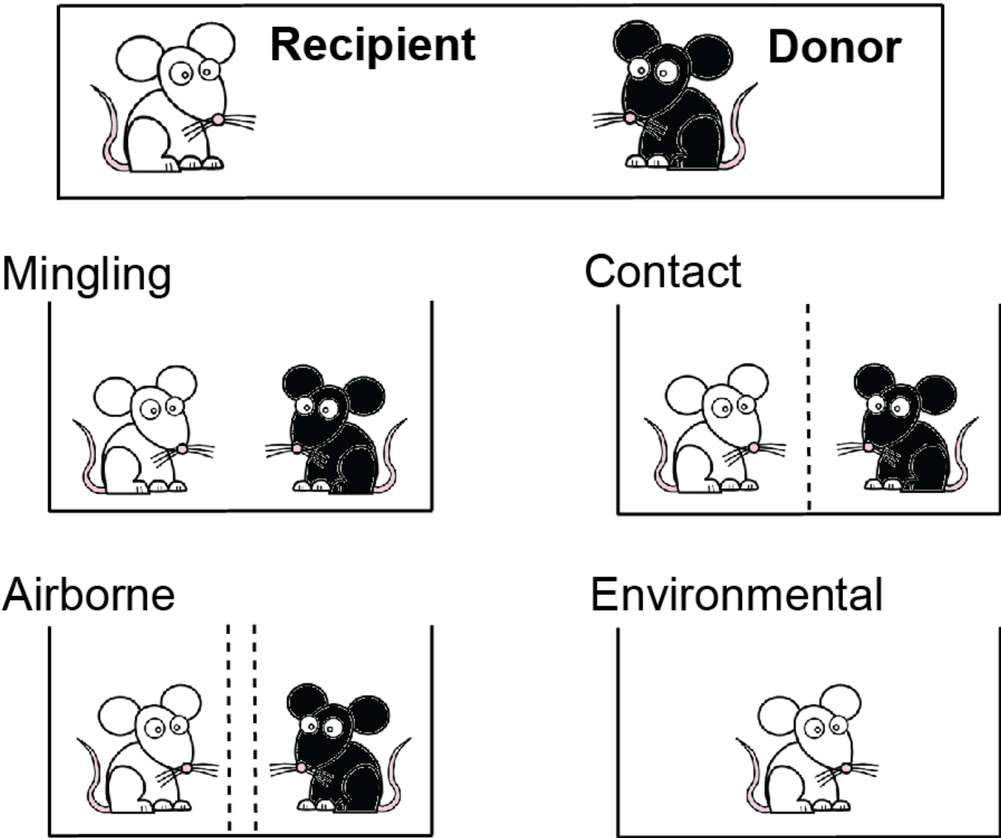

Figure 2.1. Schematic diagram demonstrating the experimental models used to investigate the role of the *C. difficile spo0A* gene in host transmission. Donor mice infected with comparable levels of *C. difficile* R20291, 630Δ*erm* or their equivalent *spo0A* mutant derivatives were exposed to susceptible naïve recipient mice via distinct transmission routes. Dashed vertical lines represent porous barriers between donor and recipient mice. A definition of the transmission routes is given in Table 2.3.

2.2.2.2 Antibody generation and serum extraction

Antibodies were generated via subcutaneous delivery of *C. difficile* 630Δ*erm*Δ*spo0A* into mice (for anti-vegetative cell sera; *n* = 5) or *C. difficile* 630Δ*erm* pure spores into rabbits (for anti-spore sera; *n* = 2). The latter was performed by Cambridge Research Biochemicals, UK. Animals received 100 µl of a ~10^7 CFU/ml culture in each flank, followed by a series of boost immunisations and test bleeds. To generate anti-Spo0A antibodies, mice (*n* = 4) received 30 µl (10 µg) purified Spo0A protein via intranasal delivery (15 µl/nare). Additionally, mice received *E. coli* heat-labile toxin (LT) to potentiate the immune response to Spo0A. Identical boosts were administered on days 7 and 21, without LT. Whole blood was collected, centrifuged at full speed (13,000 rcf) for 15 min, and sera were collected and stored at 4ºC.

2.2.3 Tissue methods

2.2.3.1 Paraffin embedding and sectioning of caecum tissue

For pathological analysis, mice were anaesthetised with isoflourane, sacrificed via cervical dislocation and surface sterilised with 70% ethanol. Caecum tissue (0.5 cm tubular sections) was then carefully excised, opened and incubated in 4% paraformaldehyde for 24 h at room temperature. Tissues were processed in a Shandon Excelsior Tissue Processor (Fisher Scientific) and paraffin wax embedded. Sections (5 µm) were cut using a RM2125 microtome (Leica) and slide mounted.

2.2.3.1.1 Histology

Paraffin wax embedded sections were processed for haematoxylin and eosin staining, as previously described (98). Briefly, sections were de-paraffinised and rehydrated stepwise as follows: Histoclear (Fisher Scientific; 2 min), two washes of 100% ethanol (2 min each), 90% ethanol, 70% ethanol (2 min) and water (5 min). Sections were stained with Mayer's haematoxylin (2 min) followed by washing in water and 1% ethanol to remove excess dye, followed by staining with eosin (5 min), and repeated washing as above. Sections were then dehydrated as follows: 70% ethanol (2 min), 90% ethanol (2 min), 100% ethanol (2 min) and Histoclear (2 min). Finally, slides were mounted with DPX resin, dried and visualised using a LSM510 Meta confocal microscope (Carl Zeiss Ltd.).

2.2.4 Microscopy

2.2.4.1 Indirect immunofluorescence

Cultures of *C. difficile* vegetative cells and spores were washed in PBS and seeded onto microscope slides. Briefly, slides were then fixed in acetone and blocked with 1% BSA in PBS. Wells were incubated with polyclonal rabbit sera (raised against pure wild-type *C. difficile* 630Δ*erm* pure spores) and polyclonal mouse sera (raised against 630Δ*erm*Δ*spo0A* vegetative cells), washed in PBS and reacted with Cy3-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG (each 1:1000) in a humified chamber, in the dark.

Slides were then mounted in ProLong Gold with DAPI (Invitrogen), sealed and immunofluorescence was assessed using a LSM510 Meta confocal microscope.

2.2.4.2 Preservation for transmission electron microscopy

Tissue preservation and visualisation was performed by David Goulding (WTSI, Cambridge), assisted by L. J Pettit.

Mice were sacrificed and surface sterilised as above. Following excision, ceacum tissue (0.5 cm tubular sections) was immediately incubated in a primary fixative (2% paraformaldehyde, 2% glutaraldehyde 0.1% magnesium chloride, 0.05% calcium chloride in 0.1 M sodium cacodylate buffer [pH 7.4]) for 2 h on ice. Tissue was then washed 3x in sodium cacodylate buffer supplemented with magnesium and calcium chlorides, after which they were incubated in 1% osmium tetroxide in sodium cacodylate buffer for 1 h. Following washing, the tissue was incubated with 1% tannic acid (a mordant) for 1 h, washed with 1% sodium sulfate for 10 min, and dehydrated in an ethanol series for 30 min each (20%, 30%, 50%, 70%, 90%, 95%) additionally staining with 2% uranyl acetate at the 30% ethanol wash stage, followed by a final dehydration stage in 100% ethanol (3x for 20 min each). The tissue was incubated 2x in propylene oxide for 15 min each, then for 1 h in a 1:1 mixture of propylene oxide and Epoxy resin (Epon-812; Sigma Aldrich) for 1 h, and finally in pure Epon-812 resin overnight. Ceacum tissue was embedded and cured in Epon-812 in a flat-moulded tray at 65°C. 50 nm ultrathin sections were cut, loaded onto Formvar-carbon-coated grids (Agar Scientific),

contrast stained with uranyl acetate and lead citrate and ultimately visualised on a FEI 120 kV Spirit Biotwin transmission electron microscope (TEM) fitted with a F415 digital Teitz camera.

2.2.4.3 Flagellar negative staining

Negative staining and visualisation was performed by David Goulding (WTSI, Cambridge), assisted by L. J. Pettit.

Grids were prepared by briefly submerging slides into Formvar (0.1%) in dry chloroform. Formvar-carbon-coated support films were then floated onto distilled water, after which grids were placed onto the film before lifting onto parafilm and air-drying. Fresh bacterial colonies were picked, suspended in ammonium acetate and loaded onto the film side of the grid. An equal volume of ammonium molybdate (1%) was added to the film and immediately drained with filter paper. Samples were allowed to air-dry and were visualised via TEM as described above.

2.2.4.4 Tissue processing for ImmunoGold Electron Microscopy

Tissue processing and visualisation was performed by David Goulding (WTSI, Cambridge), assisted by L. J. Pettit.

Caecum tissue was excised and fixed, opened and incubated in 4% paraformaldehyde, 0.2% gluteraldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min at 37ºC, followed by room temperature for 2 h. Samples were then washed in PBS and low-temperature embedded in Lowicryl HM20 resin (Agar Scientific). Briefly, tissue was dehydrated in an ethanol series for 30 min each (30% ethanol at 4ºC, 50% ethanol at 1ºC, 70% ethanol at -20ºC, 90% ethanol at -20ºC, 100% ethanol at -30ºC, and finally100% ethanol at -50ºC) and impregnated with a Lowicryl-ethanol series for 1 h each (1/3, 1/1, 3/1) followed by an overnight incubation with pure Lowicryl. Samples were then embedded and UV polymerised at -50ºC. 50 nm ultrathin sections were cut, loaded onto Formvar-carbon-coated grids and then blocked with 0.02 M glycine in PBS for 10 min followed by fetal calf serum (10%) for 1 h. Tissues were then labelled with anti-spore and anti-vegetative cell antisera (1/200 dilution), washed in PBS x3 and probed with protein A-gold conjugates (Sigma Aldrich) for 20 min as appropriate. Following thorough washing in PBS, tissues were contrast stained with uranyl acetate and lead citrate, and visualised via TEM as described above. IGEM-localised *C. difficile* (and background bacterial cells) were then counted and recorded.

2.2.5 Molecular methods

2.2.5.1 Polymerase Chain Reaction (PCR)

For general amplification of template DNA, a standard *Taq* polymerase PCR reaction with Platnium PCR Supermix (Invitrogen) was used. When products were to be cloned, high

fidelity PCR was performed with *Pfu* DNA polymerase (Promega) or Go*Taq*® PCR mix (Promega). All amplifications were performed in accordance with the manufacturer's instructions, in a total volume of 50 µl. Reactions were performed using 0.2 ml thin wall PCR tubes (ABgene) on a DNA Engine DYAD Thermal Cycler (MJ Research). Template quantity was dependent on the source, but was typically 100 ng for genomic DNA, and up to 50 ng for plasmid DNA. For negative controls, template DNA was omitted. DNA amplifications were performed using the following thermal cycling conditions as a guide: 95°C for 2 min × 1; 95°C for 30 sec, 50°C* for 30 sec, 68°C for 2 min** × 35 cycles; and 68°C for 10 min*** × 1.

* Annealing temperature is dependent primers T_m and was calculated as

** Elongation parameters dependent on product size (approximately 1 min/kb) and DNA polymerase used

*** Extension parameters are dependent on type of DNA polymerase used

2.2.5.2 Cloning PCR amplicons

Amplicons were cloned using TOPO TA Cloning Kits (Invitrogen), according to the manufacturer's instructions. Briefly, 2 µl of PCR product was incubated at room temperature for 25 min with 1 µl salt and 1 µl vector in a final volume of 6 µl. DNA was then transformed via electroporation as described in section 2.2.5.7.

2.2.5.3 Plasmid DNA extraction

Plasmid DNA was isolated using QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions. Briefly, 3 ml of *E. coli* culture was grown overnight in LB supplemented with chloramphenicol (20 μg/ml), and was harvested by centrifugation. Plasmid DNA was then adsorbed onto a QIAprep membrane, washed and eluted in nuclease-free water.

2.2.5.4 Restriction digestion

All restriction endonucleases were purchased from New England Biolabs. Digestion was performed according to manufacturer's instructions for 2 h at 37°C. All plasmids were designed to enable cleavage with *SalI*, *HindIII* or *BsrG1*. Briefly, 20 units of enzyme were used per μg plasmid DNA, along with 3 μl of the appropriate 10x buffer made up to a final volume of 30 μl with water. Following digestion, samples were examined by gel electrophoresis. In addition, digests to be sub-cloned were extracted with phenol:chloroform:isoamyl alcohol (25:24:1; Sigma Aldrich) and dephosphorylated with Shrimp Phosphatase (Promega) to prevent self-ligation. Samples were heated to 70°C for 1 h to inactivate the phosphatase enzyme prior to ligation.

2.2.5.5 DNA ligation

Ligations of *spo0A* and pRPF101 vectors were performed using T4 DNA ligase (Roche), according to the manufacturer's instructions. Briefly, reactions were carried out in a total volume of 10 µl in the presence of ligation buffer, and were incubated at 14°C overnight. Following ligation, plasmids were cloned into electrocompetent *E. coli* Top10. Plasmids in which ligation and transformation had been successful were then transformed into electrocompetent *E. coli* CA434, which were used as conjugation donors.

2.2.5.6 Preparation of electrocompetent cells

An overnight *E. coli* CA434 culture was diluted 1/100 into 30 ml LB broth and grown as described to OD_{600} = 0.5. Bacteria were then harvested by centrifugation (4000 rpm, 10 min), washed x3 in ice cold 10% glycerol and pelleted as above. The pellet was resuspended in 10% glycerol to an approximate density of 10^{10} CFU/ml, and stored at -80°C.

2.2.5.7 Transformation via electroporation

Electroporation was used for the transformation of *E. coli* Top10 or *E. coli* CA434 with plasmid DNA. Electrocompetent cells were aliquoted (50 µl) into pre-chilled 0.2 cm electroporation cuvettes (Equibio), to which approximately 50 ng plasmid DNA was added.

Cells were then electroporated using a Bio-Rad Gene Pulsar^{TM} (Bio-Rad) as follows: 2.5 kV, 25 µF, 600 Ωhms. Following electroporation, cells, were immediately transferred to 400 µl pre-warmed recovery media (SOC; Invitrogen), and incubated with agitation at 37^{0}C for 2 h. Cells were then incubated overnight on LB supplemented with chloramphenicol (20 µg/ml) or kanamycin (50 µg/ml) as required, after which plasmid DNA was extracted, digested as described above and examined by gel electrophoresis.

2.2.5.8 Conjugation

Donor (*E. coli* CA434) and recipient (*C. difficile* R20291 or 630) strains were grown overnight with appropriate antibiotic supplementation, as described. Donor cells (1 ml) were then *gently* pelleted (4,000 rpm, 1 min), to which 200 µl of recipient cells were added and *gently* resuspended. The latter was performed in an anaerobic environment. The resulting slurry was plated as 20 µl spots onto non-selective BHI agar, and incubated for 24 – 48 h. Colonies were screened by plating onto cycloserine (250 µg/ml) and cefoxitin (8 µg/ml) to counter-select for *E. coli* CA434, and thiamphenicol (15 µg/ml) to select for the *catP* marker.

2.2.5.9 ClosTron mutagenesis

The *C. difficile* 630Δ*erm*Δ*spo0A* and *C. difficile* R20291Δ*spo0A* mutants were made by Dr L. F. Dawson (London School of Hygiene and Tropical Medicine, London) and Dr R. P. Fagan

(Imperial College, London), respectively, prior to the commencement of this study.

ClosTron technology was used to make targeted mutants in the *spo0A* gene of *C. difficile* strains R20291 (PCR ribotype 027) and 630Δ*erm* (PCR ribotype 012), as previously described (186, 190). Figures 2.2 and 2.3 describe the fundamentals of ClosTron mutagenesis. Briefly, the group II Ll.LtrB intron was retargeted to *spo0A* by splicing by overlapping extension (SOE) PCR, as previously described (186). The retargeted intron, an antisense insertion between nucleotides 178/179, was then cloned into the *HindIII* and *BsrGI* sites of pMTL007 (*C. difficile* 630Δ*erm*) or pMTL007C-E2 (*C. difficile* R20291) and transformed into the *E. coli* conjugation donor strain CA434.

C. difficile strains 630Δ*erm* and R20291 were mated with the *E. coli* donor harboring the retargeted pMTL007 or pMTL007C-E2, respectively, as described above. Transconjugants were selected for in the presence of thiamphenicol (15 µg/ml; Sigma Aldrich). For pMTL007, mobilisation of the intron to the *spo0A* gene of *C. difficile* 630Δ*erm* was induced using IPTG (1 mM) as previously described (186). pMTL007C-E2 is a version of pMTL007, in which the promoter of the group II intron is modified for a constitutive *fdx* promoter (190), and as such does not require IPTG induction to direct intron expression to the *spo0A* gene of *C. difficile* R20291.

Potential transconjugants were then serially diluted onto plates containing lincomycin (15 µg/ml; *C. difficile* R20291; Sigma Aldrich) or erythromycin (5 µg/ml; *C. difficile* 630Δ*erm*; Sigma Aldrich) to select for chromosomal intron insertion. Replica plating with lincomycin

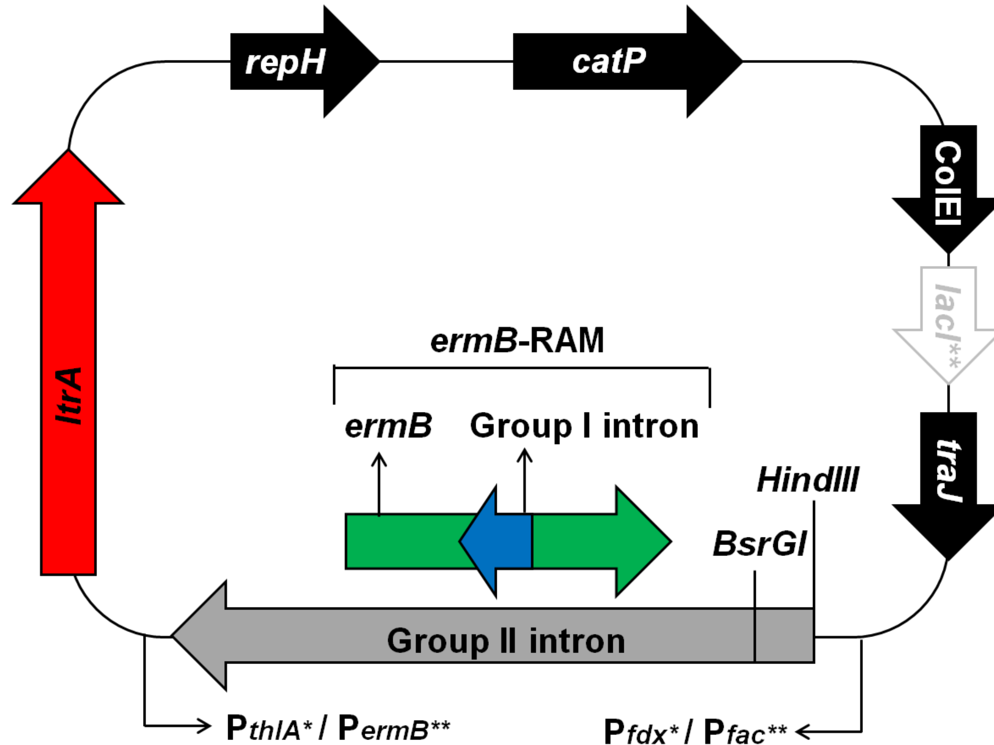

Figure 2.2. Schematic representation of ClosTron mutagenesis plamids. ClosTron plasmids share a common backbone of a group II intron (gray), within which lies a RAM based on the erythromycin resistance gene, *ermB* (green). The *ermB*-RAM is itself inactivated by a group I intron from the phage T4 *td* gene (blue), and is only activated upon its retroposition. *ltrA*, an intron encoded protein (IEP) is critical for intron mobility, RNA splicing, recognition of target site DNA and reverse transcription. *HindIII* and *BsrGI* restriction sites represent the insertion site of the retargetted group II intron. Also included are the replication gene, *repH*, the clostridial *catP* gene conferring chloramphenical/thiamphenicol resistance, the Gram-negative ColEI replicon and conjugal transfer region, *traJ*. Plasmid pMTL007 has a *fac* promoter derived from the *Clostridium pasteurianum* ferridoxin gene, which contains a *lacI* operator sequence to repress transcription from *fac*. Transcription from *fac* is induced by IPTG. Plasmid pMTL007C-E2 comprises a constitutive *fdx* promoter derived from *Clostridium sporogenes*, such that *lacI* is not required in this plamid. Intron *ermB* expression is driven by a native *ermB* promoter or a stronger *thlA* promoter derived from the *Clostridium acetobutylicum* thiolase gene, in plasmids pMTL007 and pMTL007C-E2, respectively. * Specific to plasmid pMTL007C-E2, ** Specific to plasmid pMTL007.

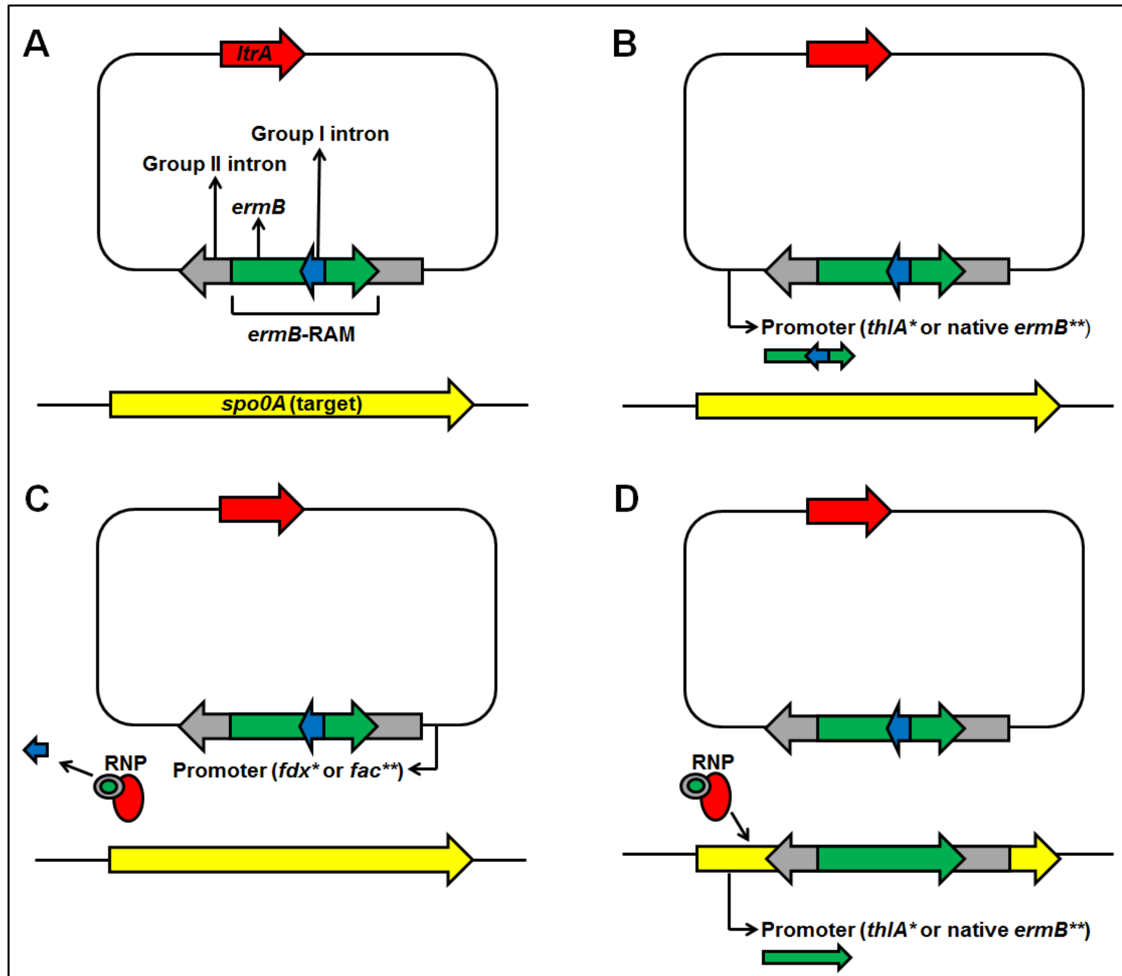

Figure 2.3. Schematic representation of the ClosTron mutagenesis system. A) ClosTron plasmids as described in Figure 2.2. Present are a group II intron (gray), an *ermB*-RAM (green), inactivated by a group I intron (blue), and *ltrA*, an intron encoded protein (IEP). **B)** Transcription of *ermB* produces a transcript containing the group I intron. However, it is in the incorrect orientation, such that self-catalytic splicing cannot occur and *ermB* remains innactive. **C)** On transcription of the group II intron (from the opposite strand), it binds LtrA producing a ribonuclear protein complex (RNP). Since the group I intron is now in the correct orientation, it is spliced out on transcription. **D)** LtrA recognises target site DNA and inserts the group II intron RNA into the host chromosome, and initiates reverse transcription for complementary DNA synthesis. As the group I intron has already spliced out, a functional *ermB* gene resides in the target gene of integrants. * Specific to plasmid pMTL007C-E2, ** Specific to plasmid pMTL007.

(15 µg/ml) or erythromycin (5 µg/ml) and thiamphenicol (15 µg/ml) was performed to select for the restored *ermB* retrotransposition-activated marker (RAM) that signals integration into the genome, and loss of the *catP* gene encoding thiamphenicol resistance carried on the plasmid.

2.2.5.9.1 Screening and verification of mutants

Mutants were screened by PCR and sequencing to confirm the chromosomal integration of the intron within the desired genes and loss of plasmids pMTL007 and pMTL007C-E2.

Briefly, three PCRs were performed to screen putative mutants using the following oligonucleotides (Table 2.2): i) RAM-F and RAM-R, to screen for loss of the group I intron, which insertionally inactivated the *ermB* RAM prior to chromosomal integration of the group II intron; ii) a gene specific primer (spo0A F) and the group II intron specific EBS universal primer, to screen for insertion of the intron into the desired location in the genome; and iii) gene specific forward and reverse primers (spo0A F and R) that flank the insertion site. Additionally, sequencing was performed across the junction of the gene to intron using gene specific primers and the EBS universal primer to verify insertion site.

2.2.5.10 Genetic complementation of the *spo0A* mutation

To complement the *spo0A* mutations, *C. difficile* - *E. coli* shuttle vector pRPF101 was constructed using the ColE1 replicon from pBlueScript II SK (+) (Agilent Technologies), *catP* from pJIR418 (198), the *C. difficile* pCD6 replicon (195) and oriT from pJB665 (199). The wild-type *C. difficile* 630 *spo0A* gene and upstream promoter region was amplified using oligonucleotide pair spo0A_A1F and spo0A_A2Rb (Table 2.2), which were designed to enable cleavage with *SalI*, and cloned into pRPF101 yielding p*spo0A*. Since the amino acid sequence of Spo0A is identical in both *C. difficile* R20291 and 630Δ*erm*, only one plasmid was constructed for the complementation of both strains.

p*spo0A* was initially transformed into *E. coli* CA434 followed by conjugation into the *spo0A* mutant derivatives as described above, generating strains R20291Δ*spo0A*+p*spo0A* and 630Δ*erm*Δ*spo0A*+p*spo0A*, respectively. Potential transconjugants were screened as above. Plasmid transfer was verified by p*spo0A* isolation and restriction analysis.

2.2.5.11 TcdA and TcdB quantification

C. difficile cultures were growth in Wilson's broth as described for 30 h, pelleted by centrifugation and the supernatant was removed for TcdA and TcdB quantification. Total and spore counts were determined to ensure equal numbers of vegetative cells in the cultures.

For TcdA quantification, microtitre plates were coated with capture antibody by adding 50 µl/well of a 2 µg/ml solution of anti-TcdA (tgcBiomics, GmbH) in PBS, and incubating overnight at 4ºC. Plates were then washed x3 in 0.05% Tween20 in PBS (PBS-T) and blocked with 200 µl 1% BSA in PBS for 2 h at room temperature. Purified TcdA (tgcBiomics) was diluted in 1% BSA-PBS (50 µl/well) and used to construct a standard curve. Culture filtrates (50 µl/well) were diluted as above in order to generate readings within the linear range of the standard curve. Plates were then incubated at room temperature for 2 h, followed by washing in PBS-T as above. The detection antibody (rabbit anti-*Clostridium difficile* TcdA; antibodies-online, GmbH) was diluted 1:5000 in 1% BSA-PBS, added to wells (50 µl/well) and incubated for 2 h at room temperature. After washing, polyclonal swine anti-rabbit IgG conjugated to horseradish peroxidase (HRP; Dako) was diluted 1:1000 in 1% BSA-PBS, added to the wells (50 µl/well) and incubated for 2 h at room temperature. Finally, plates were washed and 100 µl 3, 3′, 5, 5′-tetramethylbenzidine (TMB; Sigma-Aldrich) substrate was added for 30 min at room temperature in the dark. 50 µl 0.5 M H_2SO_4 was added to stop the reaction.

TcdB quantification was determined using a TcdB-specific ELISA kit performed according to manufacturer's instructions (tgcBiomics, GmbH). Briefly, microtitre plates pre-coated with anti-TcdB capture antibody were co-incubated with either *C. difficile* culture filtrates or purified TcdB (as described above) and anti-*Clostridium difficile* TcdB-HRP, and incubated for 1 h at 37ºC. Plates were then washed and 100 µl TMB substrate was added for 30 min at room temperature in the dark. The reaction was stopped with 50 µl 0.5 M H_2SO_4. Absorbance

of all plates was measured at 450 nm on a FLUOStar Omega (BMG Labtech). Data for TcdA and TcdB are from three independent experiments performed in triplicate.

2.2.5.12 Butyrate quantification

Butyrate quantification was performed in triplicate by Dr S. Duncan (Rowett Institute of Nutrition and Health, Aberdeen). Briefly, spent culture supernatants of exponentially growing *C. difficile* 630Δ*erm* strains were acidified, converted to *t*-butyldimethylsilyl derivatives as previously described (200) and quantified by capillary gas chromatography.

2.2.6 RNA methods

2.2.6.1 RNA stabilisation

Three biological replicates of *C. difficile* RNA was stabilised using *RNA*Protect Bacteria Reagent (Qiagen) according to manufacturer's instructions. Briefly,the *C. difficile* culture (~10^{10} cells) from exponentially growing cells was diluted 1/3 in pre-reduced *RNA*Protect and incubated under anaerobic conditions for 30 min. Bacteria were then harvested via centrifugation (4,500 rpm, 30 min) after which the supernatant was removed and the pellet was dried fully before storage at -80°C.

2.2.6.2 RNA extraction

RNA was isolated in a 2-step process. Initially, stabilised pellets underwent chemical and mechanical lysis using a FastRNA Pro Blue Kit (MP biomedicals) and FastPrep ribolyser, according to the manufacturer's recommendations. RNA quantification and intergrity was determined using both a ND-1000 (NanoDrop Technologies) and 2100 Bioanalyser (Agilent). The product of the first step was then transferred to a SV RNA Isolation Purification Kit (Promega), and RNA was isolated from the spin column assembly (washing) step, according to the manufacturer's instructions. RNA was quantified as described above.

2.2.6.3 DNA removal

DNA was removed from RNA samples using TURBO DNase (Ambion). Briefly, 2 units of DNase were used per 10 µg RNA in a final volume of 50 µl. This was incubated at 37°C for 30 min, after which a further 1 unit of DNase per 10 µg RNA was added to the reaction, followed by a second 37°C incubation for 30 min. DNAse was inactivated according to the manufacturer's recommendations. Samples were precipitated overnight at -80°C in 2.5 volumes of 100% ethanol, harvested by centrifugation (13,000 rpm, 1 h at 4°C), washed in ice cold 70% ethanol, harvested as before, resuspended in nuclease-free water (Ambion) and stored at -80°C. Samples were screened for the presence of DNA using primer pairs CD1498, CD1455, CD0011 and CDadk.

2.2.6.4 Reverse transcription of isolated RNA

RNA was reverse transcribed to complementary DNA (cDNA) as follows. All reagents were purchased from Invitrogen unless otherwise stated. 20 µg RNA was incubated with 3 µg random hexamers and RNaseOUT ribonuclease inhibitor in a total volume of 16.4 µl, at 70°C for 10 min and then cooled on ice. For cDNA synthesis, 6 µl First Strand buffer, 0.6 µl dNTP mix (25 mM each dATP, dCTP, dGTP, dATP), 0.4 µl actinomycin D (1.2 mg/ml), 3 µl DTT (0.1 M) and 2 µl Superscript III were added to a total volume of 33 µl. Samples were then incubated for 2 h at 42°C, following which RNA was hydrolysed with 1.5 µl NaOH (1 M) for 20 min at 70°C. Finally, samples were neutralised with 1.5 µl HCL (1M) and cDNA was purified using a G50-Sephadex column (Sigma-Aldrich), according to manufacturer's instructions. Samples were screened for the presence of cDNA using primer pairs MM_1498, MM_1455, MM_0011 and MM_adk.

2.2.7 DNA sequencing

2.2.7.1 Library construction and Illumina HiSeq cDNA sequencing

Samples for Illumina sequencing were submitted to the project co-ordinator, Dr David Harris (WTSI, Cambridge). Libraries were prepared, sequenced and passed through a quality-control pipeline under the direction of Dr Michael Quail (WTSI, Cambridge). All enzymes were

purchased from NEB and were used according to manufacturer's instructions except were stated.

Briefly, libraries were constructed by shearing the purified cDNA using a Covaris LE220 focused ultrasonicator to give fragments in the range of 150-250 bp. This was followed by an end-repair incubation with T4 DNA polymerase, Klenow polymerase and T4 polynucleotide kinase (to phosphorylate blunt-ended fragments) for 30 min at 20°C. cDNA samples were then 3' adenosine-tailed via the addition of Klenow exo- and dATP for 30 min at 37°C to reduce concatamerisation. Illumina adaptors (containing complementary sites to oligonucleotide anchors on the flow cell surface and primer sites for sequencing) were then ligated onto the cDNA repaired ends, and ligated fragments were electrophoretically separated from any unligated adapters based on size-selection. Fragments were then isolated via gel extraction. Libraries were amplified via PCR (18 cycles), quantified and denatured with 2 M NaOH to generate single stranded cDNA for sequencing. Samples were then loaded onto an Illumina flow cell to which the samples hybridise to the lawn of complementary oligonucleotide primers. Flow cell primers were then extended for 75 sequencing cycles, ultimately yielding clusters of clonally amplified cDNA templates. All steps were performed according to the manufacturer's recommendations.

2.2.7.2 Transcript mapping

Transcript mapping was performed by the pathogen informatics team as part of the ssRNA-

Seq computational analysis pipeline (WTSI, Cambridge). All sequence reads were aligned to the *C. difficile* 630 genome as a reference, using BWA (201) with a quality parameter (-q) of 15. Reads that did not align to the genome were discarded. Directional coverage plots were generated with mpileup, using:

```
run-mpileup +verbose +loop 600 +maxjobs 200 +config my.conf
+mail your@email -o outdir/ 2>&1 | tee -a my.log.
```

These data could then be read into Artemis (202) using the command “Graph, Add User Plot”.

2.2.7.3 ssRNA-Seq transcript normalisation

Due to variations in sequencing depths, the total number of Illumina read counts were not identical between samples. To adjust for this and to reduce the systematic technical variation, all data were normalised using the DEseq “R” package for Bioconductor, and was accessed using:

```
source("http://www.bioconductor.org/biocLite.R")
biocLite("DESeq").
```

This uses a scaling factor based on the median of the ratios between the read count per gene and the geometric mean per gene. Normalised data were additionally transformed on a variance stabilised scale to yield homoscedastic data. This was then $\log_2$ transformed with a

false discovery rate (FDR) of 10% to adjust for multiple hypothesis testing. Data were then filtered for a *P* value of ≤ 0.01.

2.2.8 Protein methods

2.2.8.1 Spo0A purification and generation of anti-Spo0A antibodies

Spo0A purification was performed by Dr W. K. Smits (Leiden University Medical Centre, Leiden). Briefly, plasmid pWKS1245 was used to generate full-length Spo0A carrying a C-terminal His_6-tag, using primer pairs oWKS-1122 and oWKS-1123a. This was then cloned into *E. coli* Rosetta(DE3)pLys (Novagen), and transformants were selected for using chloramphenicol (34 μg/ml). Protein production was induced using 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG), after which cells were harvested, washed in PBS and stored at -80°C prior to Spo0A purification.

Protein purification was performed at 4°C. Briefly, cells were disrupted in lysis buffer (2 mM phenylmethylsulfonyl fluoride [PMSF], 10mM imidazole, 5mM β-mercaptoethanol, 300 mM NaCl, 50 mM NaH_2PO_4, pH 7.9), and lysates were incubated with 50% TALON His-Tag resin slurry (Clonech) for 1 h. The resin was allowed to settle on a Poly-Prep column (BioRad), and was washed with buffer (20 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH 7.9). Spo0A was eluted, assayed for purity and yield, and was dialysed against the final buffer (50mM Tris-HCl pH8, 1mM EDTA, 0.5mM DTT). Purified Spo0A concentrations were

determined using Bradford Reagent (BioRad), according to the manufacturer's instructions. Anti-Spo0A antibodies were generated and collected as described in section 2.2.2.2.

2.2.8.2 Protein preparation and cell lysis antibodies

Three biological replicates and three technical replicates of both *C. difficile* 630Δ*erm* and *C. difficile* 630Δ*erm*Δ*spo0A* cultures were prepared for mass spectrometry. Briefly, cells (~10^{10}) from exponentially growing *C. difficile* were harvested by centrifugation, resuspended in 300 µl EBT reducing lysis buffer (8 M urea, 2 M thiourea, 4% sodium dodecyl sulphate [SDS], 20 mM tris(2-carboxyethyl)phosphine [TCEP]) and incubated at 70°C for 10 min. Cells were then mechanically disrupted using acid-washed glass beads (size 425-600 µm; Sigma Aldrich) and a FastPrep ribolyser. The lysate supernatant was collected from the glass beads, centrifuged (14,000 rpm, 30 min) and separated from the resulting DNA pellet. Finally, samples were alkylated with a final concentration of 5 mM idoacetamide (IAA; Sigma-Aldrich), and diluted in lithium dodecyl sulfate (LDS) loading buffer (Pierce).

2.2.8.3 One-dimensional gel electrophoresis of proteins

Samples with an equivalency to ~$2x10^7$ cells per lane were loaded onto a pre-cast 1 mm, 12% polyacrylamide gel (Invitrogen), and electrophoresed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions in MOPS running

buffer (Invitrogen). All gels were run for 1 h at 200 V. These parameters were experimentally verified as providing the optimal resolution of *C. difficile* proteins. Following separation, proteins were fixed for 30 min (40% methanol, 2% acetic acid).

2.2.8.4 Western blotting

Proteins resolved by SDS-PAGE were electrophoretically transferred to a nitrocellulose membrane run for 1 h at 30 V. Protein transfer was visualised by staining in Ponseau-S Red (Sigma Aldrich) for 2 min, and membranes were blocked in blocking buffer (5% milk powder in 0.1% PBS-T) for 1 h at room temperature. Membranes were then probed with a specific primary antibody (1/10,000) overnight at 4°C, washed, and detected with an appropriate HRP-conjugated secondary antibody (1/10,000) for 1 h at room temperature. Proteins were revealed by chemiluminescence detection according to the Amersham ECL system (GE Healthcare), as per the manufacturer's instructions.

2.2.8.5 Coomassie staining

To visualise the proteins resolved by SDS-PAGE, gels were stained overnight with Coomassie Brilliant Blue (BioRad), prepared according to manufacturer's instructions. The gel was then de-stained in 30% methanol until the background stain was reduced, but protein bands were visible. The gel was then scanned and points of excision were marked.

2.2.8.6 Protein digestion and peptide extraction

Protein bands were carefully excised, cut into 1 mm^3 pieces, placed into wells of a 96 well plate (18 wells per lane of gel), and washed repeatedly in 40% acetonitrile/50 mM triethylammonium bicarbonate (TEAB) until completely de-stained. Gel pieces were then dehydrated in 100% acetonitrile, and digested with 0.15 µg trypsin/well (sequencing grade; Roche) (203) for 2 h at 37°C followed by 25°C overnight. For peptide extraction, gel pieces were repeatedly dehydrated in 50% acetonitrile/% formic acid to extract all peptides, and the resultant trypsin digests were collected in a clean 96 well plate and concentrated using a SpeedVac until the plate was dry.

2.2.8.7 Isotopomeric dimethyl labeling

Prior to mass-spectrometry peptides were chemically tagged with isotopomeric dimethyl labels (204). Specifically, primary amines exposed by tryptic digestion were converted to dimethylamines via a reaction with different combinations of formaldehyde and cyanoborohyde isotopes, resulting in 4 Da mass shifts (Table 2.4). This allows samples to be multiplexed and facilitates quantification and direct comparison of protein expression profiles between mutant and parental *C. difficile*.

Briefly, samples were dissolved in 100 µl 100 mM TEAB, after which 2 µl of the appropriate formaldehyde isotope (4%) was added, followed by 2 µl of the corresponding

Table 2.4. Mass shifts and dimethyl labels resulting from isotope-labelled formaldehyde and cyanoborohydride

	Label		
	"Light"	**"Intermediate"**	**"Heavy"**
Formaldehyde isotope	CH_20 Formaldehyde	CD_20 Deuterated	$^{13}CD_20$ Deuterated and ^{13}C-labelled
Cyanoborohyride isotope	$NaBH_3CN$ Cyanoborohyride	$NaBH_3CN$ Cyanoborohyride	$NaBD_3CN$ Cyanoborodeuteride
Mass increase/label (Da)	+ 28.0313	+ 32.0564	+ 36.0757

cyanoborohyriode isotope (600 mM), see Table 2.4. The reaction was incubated for 1 h with agitation in a fume hood to label all available primary amines, quenched with 2 µl ammonium (8 % v/v) and acidified with 5 µl formic acid (5% v/v). Wells of corresponding differentially labelled samples were pooled prior to mass spectrometry, and stored at -20°C prior to quantification. To prevent biases associated with labeling, different biological and technical replicates of mutant and parental *C. difficile* peptides were labelled with different isotopes. The differentially labelled samples were then combined and simultaneously analysed by liquid

chromatography with tandem mass spectrometry detection (LC-MS/MS), whereby the 4 Da mass shifts were used to compare relative peptide abundance.

2.2.8.8 LC-MS/MS analysis

Protein sequencing and database searching was performed by Dr Lu Yu (WTSI, Cambridge). Briefly, dimethyl labelled peptides were analysed with an on-line nanoLC-MS/MS on an Ultimate 3000 RSLCnano System (Dionex) coupled to a LTQ Orbitrap Velos (Thermo Fisher) hybrid mass spectrometer equipped with a nanospray source. The system was controlled by Xcalibur 2.1 (Thermo Fisher) and DCMSLink 2.08 (Dionex). Only 1/3 of total volume of each sample was submitted for analysis. On the RSLCnano, samples were first loaded and desalted on a PepMap C18 trap (0.3 mm id x 5 mm, 5µm, Dionex) at 10 µL/min for 15 min, then peptides were separated on a PepMap RSLC column with 2 µm particle size (Dionex) at a 75 µm id x 50 cm column over a 120 min linear gradient of 4–32% CH_3CN/0.1% FA at a flow rate at 300 nL/min.

The LTQ Orbitrap Velos was operated in the "Top 10" data-dependent acquisition mode. The 10 most abundant and multiply-charged precursor ions in the MS survey scan in the Orbitrap (m/z 400 – 1500, with the lock mass at 445.120025) were dynamically selected for CID fragmentation (MS/MS) in the LTQ Velos ion trap. The ions must have a minimal signal above 2000 counts. The preview mode of FT master scan was disabled. The Orbitrap resolution was set at 60,000 at m/z 400 with one microscans. The isolation width for the

precursor ion was set at 2 Th. The normalised collision energy was set at 35% with activation Q at 0.250 and activation time for 10 msec. The dynamic exclusion mass width was set at ±20ppm and exclusion duration for 1 min. To achieve high mass accuracy, the Automatic Gain Control (AGC) were set at $1x10^6$ for the full MS survey in the Orbitrap with a maximum injection time at 100 msec, and 5000 for the MS/MS in the LTQ Velos with a maximum injection time at 300 msec.

The raw files were analyzed with MaxQuant Software (v. 1.2.2.5; http://maxquant.org) for protein quantification (205). The Andromeda search engine was used to search the MS/MS spectra using the following parameters: trypsin/P with maximum 2 missed cleavages sites; peptide mass tolerance at first search was set at 20 ppm; MS/MS fragment mass tolerance at 0.49 Da, and top 6 MS/MS peaks per 100 Da and a minimum peptide length of 6 amino acids were required. The mass accuracy of the precursor ions was improved by the time-dependent recalibration algorithm of MaxQuant. Fixed modification for carbamidomethyl and variable modifications for deamidated (NQ) and oxidation (M) were used, and a maximum of three labelled amino acids per peptide were allowed. The protein databases were extracted from annotated genome databases of *C. difficile* 630 *(November 2008)* and the contaminate database were supplemented by MaxQuant.

False discovery rates (FDR) were estimated based on matches to reversed sequences in the concatenated target-decoy database. The maximum FDR at 1% was allowed for proteins and peptides. Peptides were assigned to protein groups, a cluster of a leading protein(s) plus additional proteins matching to a subset of the same peptides. Protein groups with posterior

error probability (PEP) value over 0.01 or containing matches to reversed database or contaminants were discarded. Protein ratios of different labelled status were calculated after summing the corresponding peptide ions' intensity for a specific protein. A minimum of two peptides per protein was required.

2.3 Statistical analysis

Statistical significance of results was determined as follows; CI ratios were analysed using a non-parametric two-tailed Mann-Whitney test. In all other cases, a two-tailed Student's *T*-test was used. In all cases, a P value of < 0.05 was considered to be significant. Tests were performed using GraphPad Prism 5 graphing and statistical software (GraphPad Software, Inc.)

2.3.1 R

R was used for the differential analysis of transcriptome data. This is a freely available statistical programming language (http://www.r-project.org/).