Online Methods

Bacterial strains, plasmids and growth conditions

All bacteriological culture media were obtained from Oxoid or Difco. For mating procedures and extraction of plasmid and genomic DNA, *C. difficile* strains were cultured in BHIS broth[29], supplemented with 0.1% L-cysteine HCl and 0.375% glucose, or BHIS agar supplemented with 0.09% $FeSO_4$. Cefoxitin and thiamphenicol (Sigma) were used at 25 μg ml^{-1} and 10 μg ml^{-1}, respectively. *C. difficile* cultures were grown in an atmosphere of 10% H_2, 10% CO_2 and 80% N_2 at 37°C in a Coy anaerobic chamber. *E. coli* strains were derivatives of either DH5α (Life Technologies) or S17-1[30]. *E. coli* strains were cultured aerobically at 37°C in 2xYT agar or broth[31]. Where appropriate, *E. coli* cultures were supplemented with chloramphenicol, erythromycin or ampicillin, at 30 μg ml^{-1}, 150 μg ml^{-1}, 100 μg ml^{-1}, respectively.

Isolation and manipulation of nucleic acids

Plasmid DNA was isolated from *E. coli* strains grown overnight in 5 ml of broth, with appropriate antibiotic selection, using Qiaprep spin miniprep columns (Qiagen), following the manufacturer's instructions. *E. coli* transformations were performed using the Pipes method[32]. *C. difficile* genomic DNA was prepared as previously described[13]. Standard methods were used for the digestion, modification, ligation, and analysis of plasmid and genomic DNA and PCR products[33]. Oligonucleotide primers used in this study are listed in Supplementary Information. Nucleotide sequencing reactions were carried out using a PRISM™ Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's instructions. Sequence detection was carried out on an Applied Biosystems 3730S Genetic Analyser by Micromon at Monash University and sequences were analyzed using Sequencher™ 3.1 software (Gene Codes Corporation).

QRT-PCR analysis of PaLoc gene expression

The procedure was carried out essentially as before[34]. In brief, RNA was extracted from 40 ml of *C. difficile* cultures grown to an OD_{600} of approximately 0.3 for *tcdC* gene expression analysis, and from 10 ml of *C. difficile* cultures grown to an OD_{600} of approximately 1.8 for all other PaLoc gene expression analysis, using the Ribopure Bacteria kit (Ambion Inc.), according to the manufacturer's instructions. To remove contaminating genomic DNA, the purified RNA was treated with the DNA-free kit (Ambion Inc.), according to the

manufacturer's instructions. Reverse transcription reactions were then performed using 2 µg of purified RNA, 0.5 µM primer, and the Omniscript Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Prior to real-time PCR analysis all RT reactions were diluted 2-fold and 5-fold. Reactions were then performed in a final volume of 25 µl with SYBR Green PCR master mix (Applied Biosystems), 2 µl of diluted RT reactions and 120 nM primers using an Eppendorf Realplex^4 Mastercycler. Triplicate reactions were performed in multiple experiments using RNA from three biological replicates. The data were normalised to *C. difficile rpoA* RNA levels. Primers used for QRT-PCR are listed in Supplementary Information.

Construction of toxin A- and toxin B-specific recombination vectors

Oligonucleotide primers JRP2342 and JRP2343 were used to amplify the 566 bp region corresponding to nt 1071-1637 of *tcdA* (Accession number M30307). The corresponding region of *tcdB* (Accession number X53138) was amplified using JRP2344 and JRP2345. Since *Eco*RI restriction sites had been incorporated into the primers, this enzyme was used to clone the products into the corresponding site of the shuttle vector pJIR1456[35] (Accession number U90554) to construct the *tcdA*- and *tcdB*-targeted recombination vectors pJIR3051 and pJIR3050, respectively.

Isolation of chromosomal *C. difficile* mutants

To construct *C. difficile* strains with mutations resulting from insertion of the recombination vectors in the *tcdA* or *tcdB* genes, the vectors pJIR3051 and pJIR3050 were introduced into *E. coli* strain S17-1, respectively. Strain S17-1 carries a chromosomal copy of the broad host-range plasmid RP4 and although it is not able to self-transfer it provides all the functions required to mobilize co-resident IncP *oriT* plasmids. The *oriT*-containing plasmids pJIR3051 and pJIR3050 were therefore transferred by conjugation to *C. difficile* strain JIR8094, as previously described[13]. In the final step, the growth from each non-selective plate was resuspended in 1 ml of BHI diluent (0.37% BHI, 0.01% sodium thioglycollate) and 100 µl spread onto BHIS agar supplemented with thiamphenicol and cefoxitin, the latter antibiotic being used as a counter selection against *E. coli*. The plates were incubated anaerobically at 37°C for at least 72 h, until colonies were observed. The colonies then were subcultured onto the same medium and incubated for a further two to three days. Subcultures that showed bacterial growth were subcultured twice more, then analyzed by PCR, Southern blotting and plasmid rescue experiments.

Southern hybridization analysis of *C. difficile* strains

Genomic *C. difficile* DNA was digested with *Xba*I, subjected to agarose gel electrophoresis and then transferred to a Nylon H+ Hybond membrane (Amersham)[33]. Southern hybridization analysis was then carried out on the resultant membrane using standard methods[36]. DNA probes were digoxygenin (DIG)-labeled by random PCR labeling, following the manufacturer's instructions (Roche). The blots were hybridized with probes specific for *tcdA* (the 566 bp PCR product of primers JRP2342/JRP2343), *tcdB* (the 566 bp JRP2344/JRP2345 PCR product), and *catP* (0.73 kb product amplified from pJIR1456 using primers JRP2142/JRP2143[13]).

Partial purification of toxins from culture supernatants

The toxins were partially purified by ammonium sulphate precipitation from culture supernatants. Briefly, *C. difficile* was grown overnight in 20 ml of 2TY broth (3.0% tryptone, 2.0% yeast extract and 0.1% sodium thioglycollate) and approximately 3 ml transferred to 90 ml of the same medium. The cultures were then grown for 3 days, after which time the cells were pelleted by centrifugation (10,000 x g, 15 min, room temperature). The supernatants were then filter sterilized (0.45 mm filters) and solid ammonium sulphate was added with stirring at room temperature, to reach 70% saturation, followed by incubation for 20 min at 4^{o}C. The precipitated protein was collected and dissolved in 2 ml of water and dialyzed overnight at 4^{o}C in PBS, with four changes of buffer.

Toxin A-specific Western Blots

Partially purified toxin proteins were subjected to SDS-PAGE and transferred by electrophoresis to nitrocellulose membranes (Whatman), as previously described[34]. The membranes were incubated for 1 h in PBS containing 5% dry milk powder and then incubated overnight with affinity purified polyclonal goat antibodies specific for *C. difficile* toxin A (Techlab). Toxin A-bound antibodies were then detected with peroxidase-conjugated anti-goat antibodies (Chemicon) and the Western lightning chemiluminescence reagent kit (Perkin-Elmer), according to the manufacturer's instructions.

Vero and HT-29 cell cytotoxicity assays

To prepare the supernatants used in the toxin assays, *C. difficile* was grown as before in 90 ml of TY broth for 3 days, and the cells pelleted by centrifugation as before. The supernatants were filter sterilized and stored on ice prior to use. Vero and HT-29 cells were cultured in minimum essential medium (MEM alpha medium: GIBCO^{TM}, Invitrogen) or

McCoy's medium (5A medium modified: GIBCO^{TM}, Invitrogen), respectively, containing 10% heat inactivated fetal calf serum (HI FCS), 100 units/ml penicillin and 100 μg/ml streptomycin in culture flasks at 37°C in 5% CO_2. The cells were grown to a confluent (Vero) or semi-confluent (HT-29) monolayer and subcultured by incubation in 1 to 2 ml of 0.1% trypsin in 1 mM EDTA. The cells were counted and resuspended in fresh medium at a concentration of 0.25×10^5 (Vero) or 5.0×10^5 (HT-29) cells/ml. One ml of the cell suspension was seeded into each well of 24-well plates. The plates were incubated for 20 to 24 h and the culture medium removed, after which cells were washed with PBS. Serial two-fold dilutions of the *C. difficile* culture supernatants were made in PBS and 100 µl added to each well, followed by 400 µl of MEM or McCoy's medium containing 1% HI FCS. Negative controls were treated with 500 µl of fresh medium. The plates were incubated at 37°C in 5% CO_2. The morphological changes were observed by microscopy after 24 h. The cytopathic effect (CPE) was determined on a scale from 0 to +4 in comparison to the negative control wells. The end-point was scored as the last dilution at which 100% or 4+ CPE was observed. The assays were performed in triplicate on independent culture supernatants. An Olympus 1X71 inverted microscope was used to visualise the cells at 10X and 20X magnifications.

Toxin neutralization assays

For neutralization of toxin activity, affinity purified polyclonal antibodies specific to toxin A (goat anti-TcdA, Techlab) and toxin B (rabbit anti-TcdB, tgcBIOMICS) were used. Neutralization assays were performed by incubating filtered supernatants with appropriately diluted antibodies for 90 min at room temperature before the addition of the treated supernatant to Vero or HT-29 cell monolayers, together with growth medium supplemented with 1% HI FCS. As a negative control, culture supernatants were treated with a polyclonal antibody reactive against the ErmB RNA methylase.

Anaerobic *C. difficile* adherence assays

Quantification of *C. difficile* adhesion to host cells was achieved by an anaerobic bacterial adherence assay that used a derivative of the Caco-2 human intestinal epithelial cell-line, Caco-2BBE (C2BBE). C2BBE host cells express the human sodium-glucose transporter SGLT1 and elaborate a brush-border when cultured in monolayers. C2BBE cells were cultured in high-glucose (25 mM) Dulbecco's Minimal Eagle Medium (DMEM), 10% fetal bovine serum, 20 mM HEPES, 100 units ml^{-1} penicillin, and 100 mg/ml streptomycin at 37°C in the presence of 5% CO2. Cells between passages 25 and 45 were grown as

confluent monolayers (1.2 x 10^6 cells) in 6-well plates, and transferred to antibiotic and serum-free DMEM 24 hours prior to adherence assays. For the assays, C2BBE plates were introduced into the anaerobic chamber just before use, serum-free medium was removed and exponentially-growing *C. difficile* strains applied at a multiplicity of infection of 20 in a volume of 2 ml. All bacterial strains used were washed and resuspended in completely anaerobic DMEM with 25 mM $CaCl_2$ prior to incubation with host cells. Adherence was allowed to proceed under anaerobic conditions for 40 min. Host cells and adherent bacteria were then washed twice with 1 ml of completely anaerobic phosphate-buffered saline (PBS), scraped, serially diluted and plated to enumerate adherent *C. difficile*. Each experiment was performed in quadruplicate, and repeated at least three times in its entirety. The percent adherence was calculated as the ratio of recovered *C. difficile* to input *C. difficile* multiplied by 100. The SPSS software package was used for statistical analyses. Significance (P<0.01) was determined using ANOVA to enable comparison between multiple groups of continuous numerical data. The Least Significant Difference test was used for post hoc analyses.

Hamster experiments

C. difficile strains were tested in groups of 10 specific pathogen-free, 90-100 g Syrian hamsters. To minimize contamination, hamsters were housed in individual isolator cages fitted with air filters on their lids. Cages, food, water bottles, water, and bedding were autoclaved, and personnel handling the animals wore disposable gowns, masks, hats, shoe covers, and gloves. Each experimental group was housed in a separate room to prevent cross-contamination with different strains of *C. difficile*[23,37].

To prepare spores, *C. difficile* strains were streaked heavily and grown anaerobically at 37°C on anaerobic blood agar plates (BBL anaerobic blood agar plates, Columbia base, Fisher L21928) to confluency, with an incubation time of 5 to 6 days to induce sporulation. The organisms were then harvested with disposable loops, placed into 10 ml of PBS, washed in PBS, and heat-shocked at 56°C for 10 min to kill surviving vegetative cells. The spores were centrifuged and resuspended in Dulbecco's modified Eagle medium (DMEM), aliquoted, and frozen at -80°C. The frozen spores were then quantified before use by plating 10-fold serial dilutions of the spores onto taurocholate-fructose-agar plates. The agar base was taurocholate-cefoxitin-cycloserine-fructose-agar (TCCFA), but without cycloserine and cefoxitin[23]. Spores were diluted in DMEM prior to inoculation.

For each test strain, hamsters were given clindamycin orogastrically (30 mg/kg), to render them susceptible to *C. difficile* infection (day zero). On study day five hamsters received 1000 cfu of *C. difficile* spores by gastric inoculation. Hamsters were then monitored twice daily for 5 days and daily thereafter. Animal bedding was changed and fecal pellets were collected daily. Specimens were inoculated onto selective TCCFA plates and incubated anaerobically at 37°C to determine if they were colonized with *C. difficile*. Fecal pellets were collected daily for 12 days, then weekly until termination of the study (up to 30 days). *C. difficile* isolates from at least three colonized hamsters per group were typed by restriction endonuclease analysis (REA) to confirm the identity of the infecting *C. difficile* isolate[38]. Hamsters groups were assessed for colonization, time interval between challenge with *C. difficile* and colonization and time interval between colonization and death. Note that this study was approved by the Institutional Animal Care and Use Committee (IACUC) at the Hines VA Hospital.

The wild type parent strain JIR8094 and two independently derived toxin A (*tcdAΩ1* and *tcdAΩ2*, JIR8253 and JIR8263, respectively) and toxin B mutants (*tcdBΩ1* and *tcdBΩ2*, JIR8253 and JIR8263, respectively) were each analyzed in groups of 10 hamsters. During the hamster experiments with the second group of independent mutants, fecal pellets from all of the *C. difficile*-colonized hamsters were analyzed for stability of the toxin A or toxin B mutation. Fecal samples taken after colonization were homogenized in 3.75 ml of PBS and serially diluted and subcultured onto TCCFA. For each hamster, 100 *C. difficile* colonies were then individually patched onto BHIS agar containing cefoxitin and thiamphenicol, to confirm that the *catP* antibiotic resistance marker from the recombination plasmid was still present and onto BHIS agar containing cefoxitin as a positive control for growth. Hamster fecal pellets, homogenized in 0.75 ml PBS were also analyzed for the presence of toxin A, using a toxin A-specific ELISA (TechLab) and for the presence of toxin B using a human foreskin fibroblast cytotoxicity assay (human foreskin fibroblast cell monolayers, Diagnostic Hybrids; anti-*C. difficile* toxin, Trinity Biotech).

For the toxin A ELISA, 0.2 g of homogenized fecal pellet sample was added to 0.2 ml of diluent and mixed. The samples were clarified by centrifugation and the assay performed according to the manufacturer's instructions. The OD_{450nm} was determined using a microtiter plate reader (Bio-Rad). Samples were considered positive if their OD_{450nm} was at least 0.19, and negative if their OD_{450nm} was less than 0.05; there were no intermediate results. For the toxin B cytotoxicity assay, 0.1 g of homogenized fecal sample was mixed

with 0.5 ml of diluent and mixed. The samples were clarified by centrifugation, filtered through 0.45 µm pore filters (Millipore), and the assay performed according to the manufacturer's directions. The cytotoxic effect was recorded at 24 h and all positive results were confirmed by neutralization with antitoxin. Results were recorded as negative (-), weak cytotoxicity (+; less than 10% cell rounding), or high cytotoxicity (+++; greater than 80% cell rounding), there were no intermediate results. REA typing was performed and analyzed as previously described[37].

Additional References

[31] Miller, J. H., *Experiments in molecular genetics*. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1972).

[32] Inoue, H., Nojima, H., and Okayama, H. High efficiency transformation of *Escherichia coli* with plasmids. *Gene* **96**, 23-28 (1990).

[33] Sambrook, J. and Russell, D. W., *Molecular cloning: a laboratory manual.* (Cold Spring Harbor Laboratory Press., Cold Spring Harbor, NY., 2001).

[34] Carter, G. P. *et al.* Binary toxin production in *Clostridium difficile* is regulated by CdtR, a LytTR family response regulator. *J. Bacteriol.* **189**, 7290-7301 (2007).

[35] Lyras, D. and Rood, J. I. Conjugative transfer of RP4-*oriT* shuttle vectors from *Escherichia coli* to *Clostridium perfringens*. *Plasmid* **39**, 160-164 (1998).

[36] Lyras, D. and Rood, J. Transposition of Tn*4451* and Tn*4453* involves a circular intermediate that forms a promoter for the large resolvase, TnpX. *Mol. Microbiol.* **38**, 588-601 (2000).

[37] Razaq, N. *et al.* Infection of hamsters with historical and epidemic BI types of *Clostridium difficile*. *J Infect.Dis.s* **196**, 1813-1819 (2007).

[38] Clabots, C. R. *et al.* Development of a rapid and efficient restriction endonuclease analysis typing system for *Clostridium difficile* and correlation with other typing systems. *J. Clin. Microbiol.* **31**, 1870-1875 (1993).