A mariner-Based Transposon System for In Vivo Random Mutagenesis of Clostridium difficile

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Running title: Random mutagenesis of C. difficile.

Keywords: Clostridium, difficile, mariner, transposon

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ABSTRACT

Understanding the molecular basis of Clostridium difficile infection (CDI) is a prerequisite to the development of effective counter measures. Although there are methods for constructing gene-specific mutants of C. difficile, currently there is no effective method for generating libraries of random mutants. In this study, we developed a novel mariner-based transposon system for in vivo random mutagenesis of C. difficile R20291, the BI/NAP1/027 epidemic strain at the centre of the CDI outbreaks in Stoke Mandeville, UK in 2003-4 and 2004-5. Transposition occurred at a frequency of $4.5(\pm0.4) \times 10^{-4}$ per cell to give stable insertions at random genomic loci, which were only defined by the nucleotide sequence ‘TA’. Furthermore, mutants with just a single transposon insertion were generated in an overwhelming majority (98.3% in this study). Phenotypic screening of a C. difficile R20291 random mutant library yielded a sporulation/germination defective clone with an insertion in the germination specific protease gene cspBA, and an auxotroph with an insertion in the pyrimidine biosynthesis gene pyrB. These results validate our mariner-based transposon system for use in forward genetic studies of C. difficile.
INTRODUCTION

*Clostridium difficile* infection (CDI) is widely recognised as the leading cause of healthcare associated diarrhea in North America and Europe. Infection usually follows antibiotic treatment, which disrupts the native gastrointestinal microflora and thus allows *C. difficile* to proliferate. The emergence of so-called “epidemic” or “hyper-virulent” strains of *C. difficile* over the last 5 to 10 years has compounded an already serious problem. Classed as BI/NAP1/027, these epidemic strains are believed to cause a more severe disease and lead to increased mortality and relapse rates (11, 20, 24).

Understanding the genetic and molecular basis of CDI will be a crucial step in the development of effective counter measures. Methods for directed gene inactivation in *C. difficile* have recently been described (7, 21). This has opened the way for reverse genetic studies, in which the exact role of a specific gene, hypothesized to be important in a given phenotype, can be elucidated experimentally. By way of contrast, forward genetic studies aim to identify the genetic basis of a particular phenotype, without making any assumptions about the genes involved. In forward genetic studies, transposons are often used to generate libraries of random insertion mutants. Libraries are then screened to identify mutants which are defective in a particular phenotype. Identification of the gene or genes which have been inactivated by transposon insertion, then implicates them as having a role in that particular phenotype. Recently, just such an approach was used to identify a novel toxin regulatory locus in *C. perfringens* (29). This study elegantly demonstrated the power of forward genetic studies in bacterial pathogens.
A number of transposon mutagenesis systems have been described for gram-positive bacteria (2, 3, 15, 16, 29, 32). Two different systems have recently been developed for use in *C. perfringens* (15, 29). Both are *in vitro* mutagenesis systems which rely on being able to transform the recipient organism. As such, they are not suitable for use in *C. difficile* because, in the laboratory, recombinant DNA can only be transferred into *C. difficile* via conjugation at present. The conjugative transposons *Tn*916 and *Tn*5397 have been studied in *C. difficile*, but both have been found to either have a strong target-site preference or yield multiple insertions in individual clones (9, 30). Therefore, neither is well suited to generating libraries of random *C. difficile* mutants.

We reasoned that a *mariner*-based transposon mutagenesis system would be an effective tool for generating libraries of random *C. difficile* mutants. The *mariner* transposable element Himar1 has been shown to insert randomly into the genomes of many bacterial species (3, 6, 16, 17, 32). The cognate Himar1 transposase is the only factor required for transposition, which occurs via a “cut-and-paste” mechanism (13, 14). The transposon itself is defined by inverted terminal repeats (ITR’s) at either end and inserts into a ‘TA’ target site. This is highly appropriate for an organism with a low GC content such as *C. difficile*. In this study, we have developed a novel *mariner*-based transposon system for *in vivo* random mutagenesis of *C. difficile*. Moreover, we have exemplified the system in *C. difficile* R20291, the BI/NAP1/027 epidemic strain at the centre of the CDI outbreaks in Stoke Mandeville, UK in 2003-4 and 2004-5. This new genetic tool opens the way for forward genetic studies of *C. difficile*. 
MATERIALS AND METHODS

Bacterial strains, growth conditions and plasmid transfer

*Escherichia coli* TOP10 (Invitrogen) and *E. coli* CA434 (31) were cultured in Luria-Bertani (LB) medium, supplemented with erythromycin (50 µg/ml) or chloramphenicol (25 µg/ml), where appropriate. *C. difficile* R20291 was sourced from Jon Brazier (Anaerobe Reference Laboratory, Cardiff, UK). It is the BI/NAP1/027 epidemic strain isolated from the CDI outbreaks in Stoke Madeville, UK in 2003-4 and 2004-5. Routine culture of *C. difficile* R20291 was carried out in BHIS medium (Brain Heart Infusion supplemented with 5 mg/ml yeast extract and 0.1% w/v L-Cysteine) (26). Tryptose-Yeast (TY) medium (3% w/v Bacto-tryptose, 2% w/v yeast extract and 0.1% w/v thioglycolate, adjusted to pH 7.4) was used to enhance expression from the *tcdB* promoter of *C. difficile* (5). *C. difficile* media was supplemented with D-cycloserine (250 µg/ml), cefoxitin (8 µg/ml), lincomycin (20 µg/ml) and/or thiamphenicol (15 µg/ml) where appropriate. For solid media, agar was added to a final concentration of 1.0% (w/v). All *C. difficile* cultures were incubated in an anaerobic workstation at 37°C (Don Whitley, Yorkshire, UK).

Transposon mutants were screened for a sporulation/germination null (spo/ger<sup>-</sup>) phenotype following a five day incubation period in BHIS medium, to allow sporulation to occur (27). Cultures were heat-treated (60°C for 30 min) to kill vegetative cells and plated onto BHIS agar supplemented with 0.1% w/v sodium taurocholate (Sigma), to induce spore germination. Wild-type *C. difficile* R20291 was used as a spo/ger<sup>+</sup> control and a spo0A knock-out mutant was used as a spo/ger<sup>-</sup> control (7). Mutants with a spo/ger<sup>-</sup> phenotype were identified by failure to grow again after heat-treatment.
Transposon mutants were screened for auxotrophy on *C. difficile* Minimal Medium (CDMM). CDMM was made according to a recipe described by Karlsson *et al* (10) with modifications made for ease of preparation. Briefly, separate stock solutions of amino acids (5×), salts (10×), glucose (20×), trace salts (50×), iron (100×) and vitamins (100×) were made by dissolving the appropriate components in dH$_2$O, as detailed in Table 1. Each stock solution was made fresh and filter sterilized (0.2 µm pore size) prior to use. CDMM was then made by mixing the appropriate volume of each stock solution together with sterile dH$_2$O (Table 1). For solid CDMM, stock solutions were mixed together with molten agar in water (cooled to 50°C in a water bath following autoclaving), to give a final concentration of 1% w/v agar. Wild-type *C. difficile* R20291 was used as a non-auxotroph control and a *pyrF* knock-out mutant was used as an auxotroph control (7). Auxotrophic mutants were identified by failure to grow on CDMM.

**General molecular biology techniques**

Plasmids were isolated using the plasmid mini-prep kit (Qiagen). DNA was purified from agarose gels using the QIAquick gel extraction kit (Qiagen). Genomic DNA was isolated from *C. difficile* cultures using phenol-chloroform (25), following sequential treatment with lysosyme (10 mg/ml in PBS, 37°C for 30 min) and 10% w/v SDS (65°C for 30 min) to break down cell walls and lyse cells, respectively. Unless stated otherwise, enzymes were sourced from New England Biolabs (NEB) and PCR’s were carried out using Phusion™ High-Fidelity DNA polymerase (NEB). All PCR primers used in this study are detailed in Table 2. *E. coli* strains were transformed by electroporation using a Gene-Pulser (Bio-Rad), as recommended by the manufacturer.
Southern blot analysis was performed using the DIG High-Prime DNA labelling and
detection kit (Roche) as instructed by the manufacturer. All DNA sequencing was
carried out by Geneservice, UK.

Conjugations

Plasmids were transferred to *C. difficile* R20291 by conjugation as described
previously (23), with minor modifications. Briefly, 1 ml of *E. coli* CA434 overnight
culture harbouring plasmid was washed in PBS and transferred to the anaerobic
workstation. The *E. coli* pellet was re-suspended in a 150 µl volume of overnight *C.
difficile* culture and spotted onto BHIS agar. Following 24 h incubation, the
conjugation mating mixture was harvested into 500 µl PBS and plated onto BHIS agar
supplemented with antibiotics which only permitted growth of *C. difficile*
transconjugant clones. Transconjugant colonies were picked and re-streaked following
48 to 72 h incubation.

Plasmid stability assays

Plasmid segregational stability was determined as described previously (8). Briefly,
*C. difficile* transconjugants were cultured for 12 h in BHIS supplemented with
antibiotic to select for the plasmid. The culture was washed twice in PBS, to remove
the antibiotic, then used to inoculate a fresh un-supplemented BHIS culture at 1% v/v.
This denoted the start (i.e., 0 h) of the stability experiment. Un-supplemented culture
was then diluted 1% v/v into fresh medium every 12 h. At 24, 48 and 72 h the culture
was plated to enumerate total colony forming units (cfu) and thiamphenicol resistant
cfu. Plasmid stability per generation was calculated as \( n\sqrt{R} \) where \( R \) was the
proportion of cells in the culture retaining the plasmid, at the last time-point it could
be determined, and \( n \) was the number of \( C. \) difficile generations passed by this time, in the absence of antibiotic selection. Assuming that cultures reached maximum cell-density (i.e., 100\%) in each 12 h period, and given that an inoculum of 1\% v/v was used for each sub-culture, we took the number of generations per 12 h period to be 6.64 (because \( 1 \times 2^{6.64} = 100 \)).

**Construction of transposon delivery vectors**

The *mariner* transposon, consisting of the *catP* gene and the transcriptional terminator sequence from the ferrodoxin (*fdx*) gene of *C. pasteurianum*, flanked by inverted terminal repeats, was constructed by PCR using primers ITR-F1 and ITR-R1, and pMTL5402F (7) as template. The resulting product was cloned as a *SnaBI/PmeI* fragment into *EcoRI* (Promega) digested pMTL80241 (8) and sequenced in situ, using the M13 universal sequencing primers, to give plasmid pMTL80241::miniTn(*catP*). The hyper-active *Himar1* C9 transposase gene (12) was PCR amplified without a promoter, using primers HmrC9-F1 and HmrC9-R1, and pMarA (16) as template. The resulting product was cloned as a *NdeI/AseI* fragment into *NdeI* digested pMTL80241::miniTn(*catP*) and sequenced in situ using primers M13R, HmrC9-F1 and HmrC9-R1, to give pMTL80241::*Himar1* C9-miniTn(*catP*). The transposase gene and *catP* transposon sequence were then excised together on a single *PstI* restriction fragment and cloned into *SfiI* digested pMTL82250 (8) to give pMTL-SC0. Finally, pMTL-SC1 was generated by cloning the *tcdB* promoter of *C. difficile* R20291 into pMTL-SC0, in order to drive expression of *Himar1* C9. To do this, a 326 bp fragment comprising the intergenic sequence between *tcdD* and *tcdB* was amplified using primers PtdB-F1 and PtdB-R1, and R20291 genomic DNA as template. The resulting PCR product was cloned as a *NotI/NdeI* fragment into
similarly digested pMTL-SC0 and sequenced using the M13R primer, giving rise to pMTL-SC1 (Fig. 1).

Isolation of transposon mutants

The mariner plasmids were transferred into C. difficile R20291 by conjugation. Transconjugants were initially selected on BHIS supplemented with cycloserine, cefoxitin and lincomycin, then picked and re-streaked onto TY medium supplemented with the same antibiotics, in order to enhance expression from the tcdB promoter which was driving expression of the Himar1 C9 transposase in pMTL-SC1. After 72 h, all growth was harvested into PBS, serial dilutions were made and plated onto BHIS supplemented with cycloserine, cefoxitin and thiamphenicol, to select for the transposon-based catP marker. Individual colonies, visible after 12 – 16 h, were picked and re-streaked onto the same medium twice for further analysis and/or phenotypic screening.

Inverse-PCR and DNA sequence analysis

Genomic DNA was isolated from individual transposon mutants and digested overnight with HindIII at a concentration of 200 ng/µl. The HindIII restriction endonuclease was heat-inactivated (65°C for 30 min) and DNA was diluted to a concentration of 5 ng/µl in a reaction with T4 DNA ligase, to favour self-ligation (and thus circularization) of restriction fragments. Ligation reactions were incubated at ambient temperature for 1 h then the T4 ligase was heat-inactivated (65°C for 30 min). Inverse-PCRs were carried out in 50 µl volumes using the KOD Hot Start DNA polymerase Maser Mix kit (Novagen), with 100 ng of ligated DNA and primers catP-INV-F1 and catP-INV-R1, which face out from the transposon based catP sequence.
Inverse-PCR products were run out on a 0.8% w/v agarose gel, purified with the QIAquick gel purification kit (Qiagen) and sequenced using primer catP-INV-R2 (Table 2). To identify the genomic location of transposon insertions, sequence data was analysed using GENtle (http://gentle.magnusmanske.de/) and compared to the genome sequence of *C. difficile* R20291 (Refseq: NC_013316, Genbank: FN545816) (28) using Artemis (http://www.sanger.ac.uk/Software/Artemis/).
RESULTS AND DISCUSSION

Construction of a mariner-based transposon system for *C. difficile*

As a first step towards constructing a transposon mutagenesis system it was necessary to identify a suitable vehicle for delivering a transposon into the chromosome of *C. difficile*. In other bacteria, both suicide (33) and conditional (3, 16, 17, 32) plasmid vectors have been used for this purpose. However, the low frequency of DNA transfer achieved by conjugation from *E. coli* to *C. difficile* means that use of a suicide vector would be unfeasible for constructing mutant libraries and no conditional vectors have been described for *C. difficile* to date.

Autonomously replicating, but segregationally unstable plasmids have been used as ‘pseudo-suicide’ vectors in specific-gene inactivation methods for *C. difficile* (7, 21). Therefore, we proposed to use a similar approach. To identify a suitable pseudo-suicide vector to deliver our mariner-transposon system, we assessed the conjugation frequency and segregational stability of four gram-positive plasmid replicons in *C. difficile* R20291, all of which are readily available and have been reported previously (8). Each replicon was tested in an identical shuttle vector context, consisting of the chloramphenicol / thiamphenicol resistance gene *catP*, the gram-negative replicon ColE1, the conjugal transfer function *traJ* and a *lacZα* gene harbouring a multiple cloning site. The plasmid based on the *C. difficile* replicon pCD6 transferred with the highest frequency and displayed the greatest stability in R20291, followed by the plasmids based on the *C. botulinum* replicon pBP1 and the *C. butyricum* replicon pCB102, respectively (Table 3). We were unable to transfer the plasmid based on the *B. subtilis* pIM13 replicon. It was notable that cells of *C. difficile* R20291 harbouring either the pBP1 or the pCB102-based plasmid took 48 – 72 h to form colonies on...
thiamphenicol plates, whereas those with the pCD6-based plasmid formed visible colonies after 24 h. Although pCB102 was the most unstable replicon of those we were able to transfer, we selected the pBP1 replicon as our pseudo-suicide plasmid replicon for transposon delivery. This was because, although the pBP1 replicon was slightly more stable than the pCB102 replicon (1.3% per generation), it could be conjugated into R20291 at a frequency almost 8-fold greater than that of pCB102.

Having selected the pBP1 replicon as our transposon delivery vehicle, the mariner plasmids pMTL-SC0 and pMTL-SC1 were constructed as described in Materials and Methods. These plasmids are identical except that expression of the Himar1 C9 transposase gene is driven by the C. difficile toxin B promoter in pMTL-SC1, whereas there is no promoter driving its expression in pMTL-SC0 (Fig. 1). As such, pMTL-SC0 served as the no transposase control plasmid. In addition to the pBP1 pseudo-suicide replicon (repA and orf2), the mariner plasmids pMTL-SC0 and pMTL-SC1 each harbour the antibiotic resistance gene ermB, the gram-negative replicon ColE1 and the conjugal transfer function traJ in their backbone. The transposon itself consists of the antibiotic resistance gene catP and a transcriptional terminator (Ω), flanked by inverted terminal repeats (ITR1 and ITR2). The whole mariner element (i.e., the Himar1 C9 transposase and the catP transposon) can be excised as a SbfI fragment so it is easily transferred to alternative vector contexts.

Isolation and analysis of transposon mutants

The mariner plasmids pMTL-SC0 and pMTL-SC1 were transferred separately into C. difficile R20291 by conjugation. Transconjugants were selected on BHIS supplemented with cycloserine, cefoxitin and lincomycin, then sub-cultured on TY
medium as described in Materials and Methods. This was done in an attempt to enhance expression from the \textit{tcdB} promoter which was driving the \textit{Himar1 C9} transposase in pMTL-SC1 (5). Transconjugant clones were finally sub-cultured onto BHIS medium under selection for the transposon based \textit{catP} marker. After 12 – 16 h of incubation thiamphenicol resistant (Tm\textsuperscript{R}) colonies were visible at a frequency of $4.5(\pm0.4) \times 10^{-4}$ (calculated as the ratio of Tm\textsuperscript{R} cfu to total cfu) for the pMTL-SC1 transconjugant cultures. In contrast, no Tm\textsuperscript{R} colonies were visible for the pMTL-SC0 (‘no transposase control’) transconjugant cultures. We postulated that the pMTL-SC1 derived Tm\textsuperscript{R} colonies were the result of one or more independent transposition event(s). To test this hypothesis 17 randomly selected Tm\textsuperscript{R} colonies, all derived from the same conjugation, were isolated for further analysis.

PCR analysis with primers \textit{catP-F1} and \textit{catP-R1} (Table 2) revealed that the transposon based \textit{catP} sequence was still present in the genomic DNA of all 17 clones isolated for further analysis (Fig. 2A). In contrast, PCR analysis with primers HmrC9-F1 and HmrC9-R1 (Table 2) revealed that the plasmid based \textit{Himar1 C9} transposase gene was no longer present in any of the 17 clones (Fig. 2B). This indicated that the transposon had mobilized from the plasmid and that pMTL-SC1 (harbouring the \textit{Himar1 C9} transposase) had subsequently been lost from the cells, thus immobilizing the transposon \textit{in situ}. To ensure that pMTL-SC1 had not integrated into the \textit{C. difficile} R20291 chromosome via homologous recombination at the P(tcdB) locus, a third PCR was carried out on the same genomic DNA templates, using primers PtcdB-Fs1 and PtcdB-Rs1. These primers flank the chromosome-based 326 bp P(tcdB) sequence which is common with pMTL-SC1. The results revealed that no such
integration event had occurred (Fig. 2C), providing further evidence that one or more independent transposition events had occurred.

To establish whether or not each of the 17 pMTL-SC1 derived TmR clones were the result of independent transposition events, Southern blot analysis was carried out. Genomic DNA was isolated from wild-type C. difficile R20291 and each of the TmR clones, digested with HindIII, resolved on a 0.8% w/v agarose gel and transferred to a Hybond H+ nitrocellulose membrane. Probing the membrane for the transposon-based catP sequence revealed that the transposon sequence was present on a different size restriction fragment in each of the TmR clones and confirmed its absence in wild-type R20291 genomic DNA (Fig. 3). Furthermore, 16 of the 17 clones analysed had a single transposon insertion while only one (clone 12) appeared to have a double insertion. These results indicated that each of the 17 TmR clones did indeed arise from independent transposition events and suggested that the mariner-based catP transposon inserted randomly into the genome of C. difficile R20291.

To further test the “randomness” of our mariner-based transposon we successfully sequenced 60 independent transposon insertions, including those of the 17 clones which had been analysed by Southern blot. All the TmR clones sequenced had a single transposon insertion, with the exception of one (clone 12) which had already been found to have a double insertion by Southern blot. Transposon insertions were distributed throughout the genome of C. difficile R20291, with no evidence for a preferred target site (Fig. 4). Furthermore, insertions were found to be stable through at least 10 serial sub-cultures, in the absence of selection (data not shown). Characteristic of Himar1-based transposons, all insertions occurred at a ‘TA’ di-
nucleotide target site, which was duplicated at the point of insertion. Overall, there were 28 insertions in the plus (+) strand and 32 in the minus (−) strand. Moreover, 45 of the 60 insertions sequenced (75%) were located within protein coding sequences. This is within the range that would be expected for a random mutagen considering that 81% of the *C. difficile* R20291 genome is protein coding. Collectively, these data provide good evidence that our *mariner*-based transposon system is an effective tool for generating libraries of random *C. difficile* mutants.

**Phenotypic screens and identification of transposon insertions**

Finally, to demonstrate the use of our *mariner*-based transposon system for forward genetic studies, we generated and screened a *C. difficile* R20291 mutant library for sporulation/germination (spo/ger−) mutants and auxotrophic mutants. We identified one spo/ger− mutant which failed to grow on BHIS supplemented with 0.1% w/v taurocholate following heat treatment (60°C for 30 min), and one auxotroph which failed to grow on *C. difficile* minimal medium (CDMM). Inverse-PCR was carried out to identify the genes which had been interrupted in these mutants (Table 4). The spo/ger− mutant was found to have an insertion in a germination specific protease gene (*cspBA*), which has been shown to be essential for spore germination in *C. perfringens* (22). The auxotroph mutant was found to have an insertion in the gene encoding the aspartate carbamoyltransferase catalytic chain (*pyrB*). A search of the Kyoto Encyclopaedia of Genes and Genomes (KEGG: http://www.genome.jp/kegg/kegg2.html) revealed that this mutant is auxotrophic for uracil. These results validate the use of our *mariner*-based transposon system for forward genetic studies of *C. difficile*. 
In summary, we have successfully developed a novel mariner-based transposon system for in vivo random mutagenesis of *C. difficile* and exemplified its use in the epidemic BI/NAP1/027 strain R20291. The transposon inserted into the genome in a random fashion, generating mutants with just a single insertion in an overwhelming majority (98.3% in this study). This is superior to the conjugative transposons *Tn916* and *Tn5397*, both of which either display a strong target site preference or yield multiple insertions with a high frequency in *C. difficile* (9, 30). This new genetic tool opens the way for forward genetic studies of *C. difficile.*
ACKNOWLEDGEMENTS

Thanks to John Heap for useful discussions throughout this work and to Laura Whitehorn for technical assistance. The authors wish to acknowledge the financial support of the MRC (G0601176) and the European Union (HEALTH-F3-2008-223585).
REFERENCES


**Table 1. Clostridium difficile Minimal Medium (CDMM)**

<table>
<thead>
<tr>
<th>Stock solutions</th>
<th>Concentration in stock solution (mg/ml)</th>
<th>Final concentration in CDMM (mg/ml)</th>
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</thead>
<tbody>
<tr>
<td><strong>Amino acids (5×)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cas-amino acids</td>
<td>50</td>
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<tr>
<td>L-Tryptophan</td>
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<td>L-Cysteine</td>
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<td><strong>Salts (10×)</strong></td>
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<td>Na₂HPO₄</td>
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<td>NaHCO₃</td>
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<tr>
<td>KH₂PO₄</td>
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<tr>
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<tr>
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<td><strong>Trace salts (50×)</strong></td>
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<td>CaCl₂.2H₂O</td>
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<tr>
<td>Pyridoxine</td>
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*a 1 litre volume of CDMM was made by mixing 200 ml of 5× amino acids, 100 ml of 10× salts, 50 ml of 20× glucose, 20 ml of trace salts, 10 ml of 100× iron and 10 ml of 100× vitamins with 610 ml sterile dH₂O.*
### Table 2. Oligonucleotide primers

<table>
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<th>Primer Name</th>
<th>Sequence (5’ – 3’)</th>
<th>Use (restriction sites)</th>
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<tr>
<td>PtcdB-F1</td>
<td>tgcggccgcTTAATGAAATTTAAAAGAATATTT</td>
<td>Amplify tcdB promoter (NotI)</td>
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<tr>
<td>PtcdB-R1</td>
<td>acatatgATTTTTCTCTTTTACATAATAATTTTT</td>
<td>Amplify tcdB promoter (NdeI)</td>
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<td>HmrC9-F1</td>
<td>ttatgtttcatatgGAAAAAGGAATTTCGTGTTTT</td>
<td>Amplify Himar1 C9 transposase gene (NdeI) / PCR screening for Himar1 C9 transposase sequence</td>
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<td>HmrC9-R1</td>
<td>tatgtttcatatgTTATCAACATAGTTCCCTCAAG</td>
<td>Amplify Himar1 C9 transposase gene (AseI) / PCR screening for Himar1 C9 transposase sequence</td>
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<td>ITR1-F1</td>
<td>tatgtttacatagttgtgataaagccccggtctgacaacatg</td>
<td>Amplify catP gene to yield transposon sequence (SnaBI/MfiI)</td>
</tr>
<tr>
<td>ITR-R1</td>
<td>cagatgtttaaacaggttggctgataagtccccggtctgacaacatg</td>
<td>Amplify catP gene to yield transposon sequence (Pmel/HindIII)</td>
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<td>PtcdB-Fs1</td>
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<td>PCR screening of chromosomal tcdB promoter</td>
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<tr>
<td>PtcdB-Rs1</td>
<td>TTGTTCAATCATTTTCTCTGAAACAGAAATCCTGTTTAATATGGA</td>
<td>PCR screening of chromosomal tcdB promoter</td>
</tr>
<tr>
<td>catP-F1</td>
<td>GGCAAGTGTCTAAAGAGTTATAAGTACCGGAG</td>
<td>PCR screening for transposon based catP gene and Southern probe synthesis</td>
</tr>
<tr>
<td>catP-R1</td>
<td>TGAGTTAATCTATTACATATCTTCTGCAATTCG</td>
<td>PCR screening for transposon based catP gene and Southern probe synthesis</td>
</tr>
<tr>
<td>catP-INV-F1</td>
<td>TAAATCATTTTTAGACAGATATAGAAATGGAAGC</td>
<td>Inverse PCR</td>
</tr>
<tr>
<td>catP-INV-R1</td>
<td>TATGTTAAGATCTGGATACATCCTCTCATATA</td>
<td>Inverse PCR</td>
</tr>
<tr>
<td>catP-INV-R2</td>
<td>TATTTGTGTGATATCTCCATTTAAGGCATTGCTC</td>
<td>Sequencing of inverse PCR products</td>
</tr>
</tbody>
</table>

*Bases in capitals are complementary to the target sequence. Underlined bases indicate recognition sequences of the corresponding restriction endonucleases listed in the final column. Bases in boldface are the mariner ITR sequences. Bases in italics indicate the fdx terminator sequence of *C. pasteurianum.*
Table 3. Plasmid replicon performance in *C. difficile* R20291

<table>
<thead>
<tr>
<th>Gram +ve replicon</th>
<th>Organism replicon was isolated from (Reference[s])</th>
<th>Replicon context</th>
<th>Conjugation frequency into R20291</th>
<th>Segregational stability in R20291</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBP1</td>
<td><em>C. botulinum</em> (8)</td>
<td>pMTL82151</td>
<td>$2.61 \pm 0.04 \times 10^7$</td>
<td>$57.2 \pm 0.7$</td>
</tr>
<tr>
<td>pCB102</td>
<td><em>C. butyricum</em> (4, 18)</td>
<td>pMTL83151</td>
<td>$3.40 \pm 1.90 \times 10^8$</td>
<td>$55.9 \pm 1.1$</td>
</tr>
<tr>
<td>pCD6</td>
<td><em>C. difficile</em> (23)</td>
<td>pMTL84151</td>
<td>$4.48 \pm 0.47 \times 10^7$</td>
<td>$76.0 \pm 0.7$</td>
</tr>
<tr>
<td>pIM13</td>
<td><em>B. subtilis</em> (1, 19)</td>
<td>pMTL85151</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Each gram positive replicon was tested in an identical shuttle vector context consisting of the chloramphenicol / thiamphenicol resistance gene *catP*, the gram-negative replicon ColE1, the conjugal transfer function *traJ* and a *lacZα* multiple cloning site (8).*  
*Conjugation frequencies were calculated as no. of transconjugant colonies per cfu of *E. coli* donor.*  
*Segregational stabilities were calculated as % stability per generation as described in Materials and Methods.*  
*The pIM13-based plasmid pMTL85151 could not be transferred into *C. difficile* R20291.*
**Table 4.** Transposon insertion sites in *C. difficile* R20291 mutants

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Transposon insertion site</th>
<th>Location in genome</th>
<th>Transposon orientation</th>
<th>ORF interrupted</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>spo/ger</td>
<td>tggaacacgtaatgcaactata - Tn - tataactgtgtagcagcag</td>
<td>2517626</td>
<td>-</td>
<td><strong>capBA</strong> (CDR20291_2147)</td>
<td>Putative germination specific protease</td>
</tr>
<tr>
<td>Auxotroph</td>
<td>tccctttatagctttctata - Tn - tataactgtgtagcagcag</td>
<td>238739</td>
<td>-</td>
<td><strong>pyrB</strong> (CDR20291_0185)</td>
<td>Aspartate carbamoyltransferase catalytic chain</td>
</tr>
</tbody>
</table>

*Target site duplication in boldface*
**Figure legends**

**Figure 1.** Vector map of plasmid pMTL-SC1. Expression of the hyper-active mariner transposase gene Himar1 C9 was driven by the *C. difficile* toxin B promoter, P(tcdB). The control plasmid pMTL-SC0 was identical, except there was no promoter driving expression of the transposase. The plasmid backbone consisted of the pBP1 replicon of *C. botulinum* (repA and orf2), the MLSB antibiotic resistance gene ermB, the Gram-negative replicon ColE1 and the conjugal transfer function traJ. The whole mariner element (i.e., transposase gene and catP mini-transposon) can be excised as a SbfI fragment. The transcriptional terminators (Ω) are identical in sequence to those found immediately downstream of the fdx gene of *C. pasteurianum* and the CD0164 open reading frame of *C. difficile* 630. This vector conforms to the pMTL80000 modular system for *Clostridium* shuttle plasmids (8).

**Figure 2.** PCR screens of 17 randomly selected pMTL-SC1 derived TmR clones. Genomic DNA prepared from each clone was screened for the transposon-based catP gene (A), the plasmid-based Himar1 C9 transposase gene (B) and for an un-interrupted chromosomal tcdB promoter sequence (C). Lanes: M, 1 kb ladder (Promega); P, pMTL-SC1; wt, wild-type *C. difficile* R20291; 1-17, pMTL-SC1 derived TmR clones 1 to 17.

**Figure 3.** Southern hybridization analysis of pMTL-SC1 derived TmR clones. Genomic DNA samples were digested with *Hind*III. The membrane was probed for the transposon-based catP sequence. Lanes: wt, wild-type *C. difficile* R20291; 1 – 17 pMTL-SC1 derived TmR clones 1 to 17.
Figure 4. Genetic map of mariner-transposon insertions. Sixty independent transposon insertions were sequenced. Insertions in the plus (+) orientation are marked on the circle exterior. Insertions in the minus (-) orientation are marked on the circle interior. Numbers indicate the precise point of insertion according to genome sequence data for C. difficile R20291 (Refseq: NC_013316, Genbank: FN545816) (28).