Applied and Environmental Microbiology, Feb. 2010, p. 1103–1109 Vol. 76, No. 4
0099-2240/10/$12.00 doi:10.1128/AEM.02525-09

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

Stephen T. Cartman and Nigel P. Minton*

Centre for Biomolecular Sciences, School of Molecular Medical Sciences, Nottingham Digestive Diseases Centre, NIHR Biomedical Research Unit, The University of Nottingham, University Park, Nottingham NG7 2RD, United Kingdom

Received 18 October 2009/Accepted 9 December 2009

Understanding the molecular basis of *Clostridium difficile* infection is a prerequisite to the development of effective countermeasures. 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 center of the *C. difficile* outbreaks in Stoke Mandeville, United Kingdom, in 2003 to 2004 and 2004 to 2005. Transposition occurred at a frequency of 4.5 (±0.4) × 10^{-4} per cell to give stable insertions at random genomic loci, which were defined only 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*.

Clostridium difficile infection is widely recognized as the leading cause of health care-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 "hypervirulent" 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 *C. difficile* infection will be a crucial step in the development of effective countermeasures. 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 that 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 *Clostridium 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 at present, recombinant DNA can be transferred into *C. difficile* only via conjugation. The conjugative transposons Tn*916* and Tn*5397* have been studied in *C. difficile*, but both have been found either to have a strong target site preference or to 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 (ITRs) 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 demonstrated the system in *C. difficile* R20291, the BI/NAP1/027 epidemic strain at the center of the *C. difficile* outbreaks in Stoke Mandeville, United Kingdom, in 2003 to

* Corresponding author. Mailing address: Centre for Biomolecular Sciences, School of Molecular Medical Sciences, Nottingham Digestive Diseases Centre, NIHR Biomedical Research Unit, The University of Nottingham, University Park, Nottingham NG7 2RD, United Kingdom. Phone: 44 115 846 7458. Fax: 44 115 823 2120. E-mail: nigel.minton@nottingham.ac.uk.

▽ Published ahead of print on 18 December 2009.

2004 and 2004 to 2005. 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, United Kingdom). It is the BI/NAP1/027 epidemic strain isolated from the *C. difficile* outbreaks in Stoke Mandeville, United Kingdom, in 2003 to 2004 and 2004 to 2005. Routine culture of *C. difficile* R20291 was carried out in BHIS medium (brain heart infusion medium supplemented with 5 mg/ml yeast extract and 0.1% [wt/vol] L-cysteine) (26). Tryptose-yeast (TY) medium (3% [wt/vol] Bacto tryptose, 2% [wt/vol] yeast extract, and 0.1% [wt/vol] thioglycolate, adjusted to pH 7.4) was used to enhance expression from the *tcdB* promoter (P*tcdB*) of *C. difficile* (5). *C. difficile* medium 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 medium, agar was added to a final concentration of 1.0% (wt/vol). All *C. difficile* cultures were incubated in an anaerobic workstation at 37°C (Don Whitley, Yorkshire, United Kingdom).

Transposon mutants were screened for a sporulation/germination null (spo/ger$^-$) phenotype following a 5-day incubation period in BHIS medium to allow sporulation to occur (27). Cultures were heat treated (at 60°C for 30 min) to kill vegetative cells and plated onto BHIS agar supplemented with 0.1% (wt/vol) sodium taurocholate (Sigma) to induce spore germination. Wild-type *C. difficile* R20291 was used as a sporulation/germination-positive (spo/ger$^+$) control, and a *spo0A* knockout mutant was used as a spo/ger$^-$ control (7). Mutants with a spo/ger$^-$ phenotype were identified by failure to grow again after heat treatment.

Transposon mutants were screened for auxotrophy on *C. difficile* minimal medium. *C. difficile* minimal medium 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 distilled H_2O (dH_2O), as detailed in Table 1. Each stock solution was made fresh and filter sterilized (0.2-μm pore size) prior to use. *C. difficile* minimal medium was then made by mixing the appropriate volume of each stock solution together with sterile dH_2O (Table 1). For solid *C. difficile* minimal medium, 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% (wt/vol) agar. Wild-type *C. difficile* R20291 was used as a nonauxotroph control, and a *pyrF* knockout mutant was used as an auxotroph control (7). Auxotrophic mutants were identified by failure to grow to grow on *C. difficile* minimal medium.

General molecular biology techniques. Plasmids were isolated using a plasmid mini-prep kit (Qiagen). DNA was purified from agarose gels using a QIAquick gel extraction kit (Qiagen). Genomic DNA was isolated from *C. difficile* cultures using phenol-chloroform (25), following sequential treatment with lysozyme (10 mg/ml in phosphate-buffered saline [PBS] at 37°C for 30 min) and 10% (wt/vol) 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 PCRs 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 a digoxigenin (DIG) High-Prime DNA labeling and detection kit (Roche) as instructed by the manufacturer. All DNA sequencing was carried out by Geneservice, United Kingdom.

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 harboring plasmid was washed in PBS and transferred to the anaerobic workstation. The *E. coli* pellet was resuspended in a 150-μl volume of overnight *C. difficile* culture and spotted onto BHIS agar. Following 24 h of incubation, the conjugation mating mixture was harvested into 500 μl of PBS and plated onto BHIS agar supplemented with antibiotics, which permitted only growth of *C. difficile* transconjugant clones. Transconjugant colonies were picked and restreaked following 48 to 72 h of incubation.

Plasmid stability assays. Plasmid segregational stability was determined as described previously (8). Briefly, *C. difficile* transconjugants were cultured for 12 h in BHIS medium supplemented with antibiotic to select for the plasmid. The culture was washed twice in PBS to remove the antibiotic and then used to inoculate fresh, unsupplemented BHIS medium at 1% (vol/vol). This marked the start (i.e., 0 h) of the stability experiment. The unsupplemented culture was then diluted 1% (vol/vol) into fresh medium every 12 h. At 24, 48, and 72 h the culture was plated to enumerate total CFU and thiamphenicol-resistant (Tm^r) CFU. Plasmid stability per generation was calculated as $\sqrt[n]{R}$, where R is the proportion of cells in the culture retaining the plasmid at the last time point it could be determined, and n is 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% (vol/vol) was used for each subculture, we took the number of generations per 12 h period to be 6.64 (because $1 \times 2^{6.64} = 100$).

TABLE 1. *C. difficile* minimal medium[a]

Stock solution component	Concn in stock solution (mg/ml)	Final concn in CDMM (mg/ml)
Amino acids (5×)		
Casamino Acids	50	10
L-Tryptophan	2.5	0.5
L-Cysteine	2.5	0.5
Salts (10×)		
Na_2HPO_4	50	5
$NaHCO_3$	50	5
KH_2PO_4	9	0.9
NaCl	9	0.9
Glucose (20×)		
D-Glucose	200	10
Trace salts (50×)		
$(NH_4)_2SO_4$	2.0	0.04
$CaCl_2 \cdot 2H_2O$	1.3	0.026
$MgCl_2 \cdot 6H_2O$	1.0	0.02
$MnCl_2 \cdot 4H_2O$	0.5	0.01
$CoCl_2 \cdot 6H_2O$	0.05	0.001
Iron (100×)		
$FeSO_4 \cdot 7H_2O$	0.4	0.004
Vitamins (100×)		
D-Biotin	0.1	0.001
Calcium-D-pantothenate	0.1	0.001
Pyridoxine	0.1	0.001

[a] A 1-liter volume of *C. difficile* minimal medium (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 dH_2O.

Construction of transposon delivery vectors. The *mariner* transposon, consisting of the *catP* gene and the transcriptional terminator sequence from the ferrodoxin (*fdx*) gene of *Clostridium pasteurianum*, flanked by inverted terminal repeats, was constructed by PCR using primers ITR-F1 and ITR-R1 with plasmid pMTL5402F (7) as a template. The resulting product was cloned as an SnaBI/PmeI fragment into EcoICRI (Promega)-digested pMTL80241 (8) and sequenced *in situ*, using the M13 universal sequencing primers, to give plasmid pMTL80241::miniTn(*catP*). The hyperactive *Himar1* C9 transposase gene (12) was PCR amplified without a promoter using primers HmrC9-F1 and HmrC9-R1 with plasmid pMarA (16) as a template. The resulting product was cloned as an 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 sequences were then excised together on a single PstI restriction fragment and cloned into SbfI-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 PtcdB-F1 and PtcdB-R1 with R20291 genomic DNA as a template. The resulting PCR product was cloned as an NotI/NdeI fragment into similarly digested pMTL-SC0 and sequenced using the M13R primer, thus giving rise to pMTL-SC1 (Fig. 1).

TABLE 2. Oligonucleotide primers

Primer name	Sequence (5′–3′)[a]	Function [restriction site(s)]
PtcdB-F1	tgcggccgcTTAATGAATTTAAAGAAATATTTACAATAGA AATCAAATTTTAGAATTAAC	Amplify *tcdB* promoter (NotI)
PtcdB-R1	acatatgATTTTCTCCTTTACTATAATATTTTTATTGAATA TTTTTACATCTAAATGC	Amplify *tcdB* promoter (NdeI)
HmrC9-F1	tatgttcatatgGAAAAAAAGGAATTTCGTGTTTTGATAAA ATACTGTTTTCTGAAGGG	Amplify *Himar1* C9 transposase gene (NdeI)/PCR screening for *Himar1* C9 transposase sequence
HmrC9-R1	tatgttattaatTTATTCAACATAGTTCCCTTCAAGAGCGAT ACAACGATTATAACGACC	Amplify *Himar1* C9 transposase gene (AseI)/PCR screening for *Himar1* C9 transposase sequence
ITR1-F1	tatgtttacgta**taacaggttggctgataagtccccggtctgaca**caattgTTTTTCG GCAAGTGTTCAAGAAGTTATTAAGTCGGGAGTGC AGTCGAAGTGGG	Amplify *catP* gene to yield transposon sequence (SnaBI/MfeI)
ITR-R1	cagatgtttaaac**taacaggttggctgataagtccccggtctaaca***aaaaataagaagc ctgcatttgcaggcttcttatttttt*aagcttAGACAAACCTGAAGTTAAC TATTTATCAATTCCTGCAATTCGTTTACAAAACGG	Amplify *catP* gene to yield transposon sequence (PmeI/HindIII)
PtcdB-Fs1	GATAGAGAAATATCAGTGAAATTAAAAATATCTAGA CAAGCTGTTAATAAGG	PCR screening of chromosomal *tcdB* promoter
PtcdB-Rs1	ATTGCTACATATTCATCTTCCTGAACACGAAATCTTA CATTTGCC	PCR screening of chromosomal *tcdB* promoter
catP-F1	GGCAAGTGTTCAAGAAGTTATTAAGTCGGGAGTGCA GTCGAAGTGG	PCR screening for transposon based *catP* gene and Southern probe synthesis
catP-R1	TGAAGTTAACTATTTATCAATTCCTGCAATTCGTTTA CAAAACGG	PCR screening for transposon based *catP* gene and Southern probe synthesis
catP-INV-F1	TAAATCATTTTTAGCAGATTATGAAAGTGATACGCA ACGGTATGG	Inverse PCR
catP-INV-R1	TATTGTATAGCTTGGTATCATCTCATCATATATCCCC AATTCACC	Inverse PCR
catP-INV-R2	TATTTGTGTGATATCCACTTTAACGGTCATGCTGTAG GTACAAGG	Sequencing of inverse PCR products

[a] Bases in capitals are complementary to the target sequence. Underlining indicates recognition sequences of the corresponding restriction endonucleases listed in the final column. Boldface indicates the *mariner* ITR sequences. Italics indicate the *fdx* terminator sequence of *C. pasteurianum*.

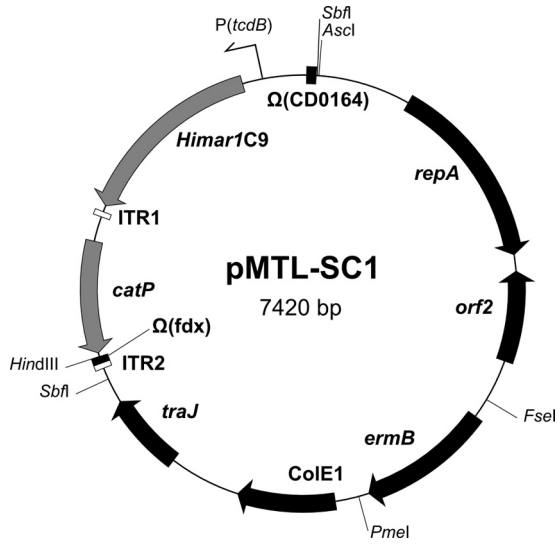

FIG. 1. Vector map of plasmid pMTL-SC1. Expression of the hyperactive *mariner* transposase gene *Himar1* C9 was driven by the *C. difficile* toxin B promoter, P*tcdB*. The control plasmid pMTL-SC0 was identical, except that there was no promoter driving expression of the transposase gene. The plasmid backbone consisted of the pBP1 replicon of *C. botulinum* (*repA* and *orf2*), the macrolide-lincosamide-streptogramin B 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 an SbfI fragment. The transcriptional terminators (Ω) are identical in sequence to those found immediately downstream of the *fdx* gene of *Clostridium pasteurianum* and the CD0164 open reading frame of *C. difficile* 630. This vector conforms to the pMTL80000 modular system for *Clostridium* shuttle plasmids (8).

Isolation of transposon mutants. The *mariner* plasmids were transferred into *C. difficile* R20291 by conjugation. Transconjugants were initially selected on BHIS medium supplemented with cycloserine, cefoxitin, and lincomycin and then picked and restreaked 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, and serial dilutions were made and plated onto BHIS medium supplemented with cycloserine, cefoxitin, and thiamphenicol to select for the transposon-based *catP* marker. Individual colonies, visible after 12 to 16 h, were picked and restreaked 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 favor self-ligation (and thus circularization) of restriction fragments. Ligation reaction mixtures were incubated at ambient temperature for 1 h, and 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 Master 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% (wt/vol) 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 were analyzed using GENtle (http://gentle.magnusmanske.de/) and compared to the genome sequence of *C. difficile* R20291 (Refseq number NC_013316; GenBank accession number 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 toward constructing a transposon mutagenesis system, it was necessary to identify a suitable vehicle for delivering a transposon into the chromosome of

TABLE 3. Plasmid replicon performance in *C. difficile* R20291

Gram-positive replicon	Source of replicon		Replicon context[a]	Conjugation frequency into R20291[b]	Segregational stability in R20291[c]
	Organism	Reference(s)			
pBP1	*C. botulinum*	8	pMTL82151	$(2.61 \pm 0.04) \times 10^{-7}$	57.2 (±0.7)
pCB102	*C. butyricum*	4, 18	pMTL83151	$(3.40 \pm 1.90) \times 10^{-8}$	55.9 (±1.1)
pCD6	*C. difficile*	23	pMTL84151	$(4.48 \pm 0.47) \times 10^{-7}$	76.0 (±0.7)
pIM13	*B. subtilis*	1, 19	pMTL85151	—[d]	—[d]

[a] 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 *lacZ*α multiple cloning site (8).
[b] Conjugation frequencies were calculated as the number of transconjugant colonies per CFU of *E. coli* donor.
[c] Segregational stabilities were calculated as percent stability per generation as described in Materials and Methods.
[d] The pIM13-based plasmid pMTL85151 could not be transferred into *C. difficile* R20291.

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 gene-directed 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 *lacZ*α gene harboring 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 *Clostridium botulinum* replicon pBP1 and the *Clostridium butyricum* replicon pCB102, respectively (Table 3). We were unable to transfer the plasmid based on the *Bacillus subtilis* pIM13 replicon. It was notable that cells of *C. difficile* R20291 harboring either the pBP1- or the pCB102-based plasmid took 48 to 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 for transposon delivery because, even though it 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, we constructed the *mariner* plasmids pMTL-SC0 and pMTL-SC1 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 harbor the antibiotic resistance gene *ermB*, the Gram-negative replicon ColE1, and the conjugal transfer function *traJ* in their backbones. 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 an 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 medium supplemented with cycloserine, cefoxitin, and lincomycin and then subcultured on TY medium as described in Materials and Methods. This was done in an attempt to enhance expression from the *tcdB* promoter, which was driving expression of the *Himar1* C9 transposase in pMTL-SC1 (5). Transconjugant clones were finally subcultured onto BHIS medium under selection for the transposon-based *catP* marker. After 12 to 16 h of incubation thiamphenicol resistant (Tm^r) colonies were visible at a frequency of $4.5 (\pm 0.4) \times 10^{-4}$ (calculated as the ratio of Tm^r CFU to total CFU) for the pMTL-SC1 transconjugant cultures. In contrast, no Tm^r colonies were visible for the pMTL-SC0 (no-transposase control) transconjugant cultures. We postulated that the pMTL-SC1-derived Tm^r colonies were the result of one or more independent transposition event(s). To test this hypothesis 17 randomly selected Tm^r colonies, all derived from the same conjugation, were isolated for further analysis.

PCR analysis with primers catP-F1 and catP-R1 (Table 2) revealed that the transposon-based *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 *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 (harboring the *Himar1* C9 transposase) had subsequently been lost from the cells, thus immobilizing the transposon *in situ*. To ensure that pMTL-SC1 had not integrated into the *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 P*tcdB* sequence which is common with pMTL-SC1. The results revealed that no such integration event had occurred (Fig. 2C),

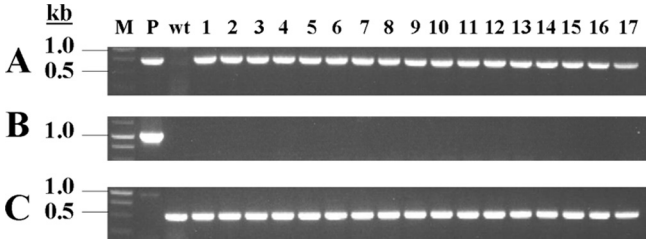

FIG. 2. PCR screens of 17 randomly selected pMTL-SC1-derived Tm^r clones. Genomic DNA prepared from each clone was screened for the transposon-based *catP* gene (A), the plasmid-based *Himar1* C9 transposase gene (B), and an uninterrupted chromosomal *tcdB* promoter sequence (C). Lane M, 1-kb ladder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

providing further evidence that one or more independent transposition events had occurred.

To establish whether each of the 17 pMTL-SC1-derived Tm^r clones was the result of an independent transposition event, Southern blot analysis was carried out. Genomic DNA was isolated from wild-type *C. difficile* R20291 and each of the Tm^r clones, digested with HindIII, resolved on a 0.8% (wt/vol) agarose gel, and transferred to a Hybond H+ nitrocellulose membrane. Probing the membrane for the transposon-based *catP* sequence revealed that the transposon was present on a different size restriction fragment in each of the 17 Tm^r clones and confirmed its absence in wild-type R20291 genomic DNA (Fig. 3). Furthermore, 16 of the 17 clones analyzed had a single transposon insertion while only 1 clone (clone 12) appeared to have a double insertion. These results indicated that each of the 17 Tm^r 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 analyzed by Southern blotting. All of the Tm^r clones sequenced had a single transposon insertion, with the exception

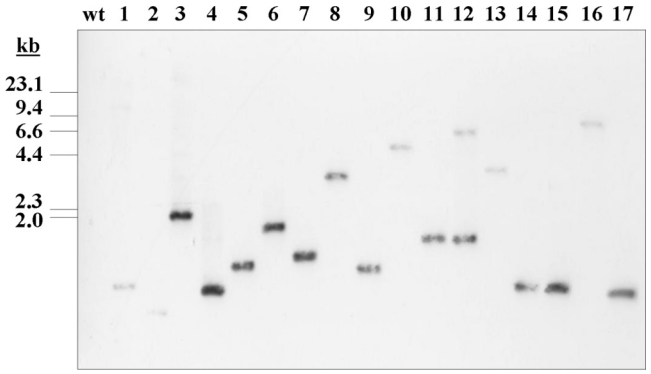

FIG. 3. Southern hybridization analysis of pMTL-SC1-derived Tm^r clones. Genomic DNA samples were digested with HindIII. The membrane was probed for the transposon-based *catP* sequence. Lane wt, wild-type *C. difficile* R20291; lanes 1 to 17, pMTL-SC1-derived Tm^r clones 1 to 17.

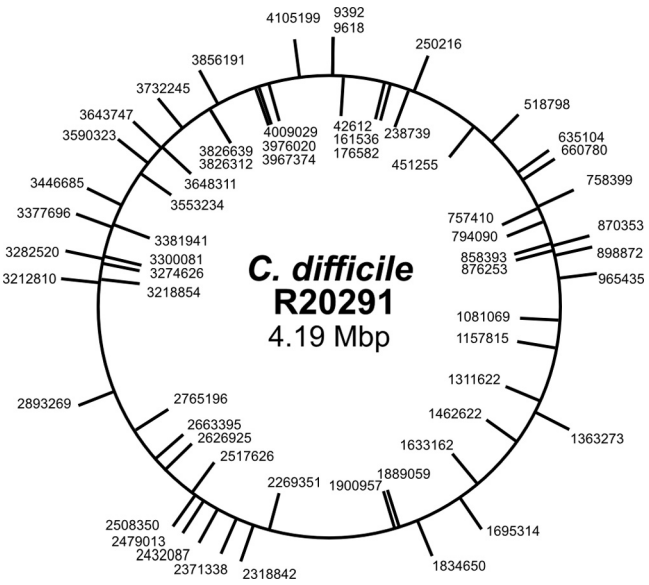

FIG. 4. Genetic map of *mariner* transposon insertions. Sixty independent transposon insertions were sequenced. Insertions in the plus orientation are marked on the circle exterior. Insertions in the minus orientation are marked on the circle interior. Numbers indicate the precise point of insertion according to genome sequence data for *C. difficile* R20291 (Refseq number NC_013316; GenBank accession number FN545816) (28).

of one (clone 12), which had already been found to have a double insertion by Southern blotting. 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 subcultures in the absence of selection (data not shown). Characteristic of *Himar1*-based transposons, all insertions occurred at a TA dinucleotide 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 mutants (spo/ger$^-$) and auxotrophic mutants. We identified one spo/ger$^-$ mutant that failed to grow on BHIS medium supplemented with 0.1% (wt/vol) taurocholate following heat treatment (60°C for 30 min) and one auxotroph that failed to grow on *C. difficile* minimal medium. 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

TABLE 4. Transposon insertion sites in *C. difficile* R20291 mutants

Phenotype	Transposon insertion site[a]	Location in genome (nt)[b]	ORF interrupted[c]	Description
spo/ger$^-$	TGGGAACACGTATGCAAC**TA**—Tn—**TA**TAACTGGTACAGCAGCAG	2517626	*cspBA* (CDR20291_2147)	Putative germination specific protease
Auxotroph	TCCCTTTATAGCTTCTTT**TA**—Tn—**TA**TATAATCTGGCATTTTTA	238739	*pyrB* (CDR20291_0185)	Aspartate carbomoyltransferase catalytic chain

[a] The Tn insertion is indicated by dashes on either side, and the target site duplication is shown in boldface.
[b] nt, nucleotide.
[c] ORF, open reading frame.

aspartate carbomoyltransferase catalytic chain (*pyrB*). A search of the Kyoto Encyclopedia 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 demonstrated 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 Tn*916* and Tn*5397*, 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*.

ACKNOWLEDGMENTS

We thank John Heap for useful discussions throughout this work and Laura Whitehorn for technical assistance.

We acknowledge the financial support of the MRC (G0601176) and the European Union (HEALTH-F3-2008-223585).

REFERENCES

1. **Azeddoug, H., J. Hubert, and G. Reysset.** 1992. Stable inheritance of shuttle vectors based on plasmid pIM13 in a mutant strain of *Clostridium acetobutylicum*. J. Gen. Microbiol. **138:**1371–1378.
2. **Bae, T., A. K. Banger, A. Wallace, E. M. Glass, F. Aslund, O. Schneewind, and D. M. Missiakas.** 2004. *Staphylococcus aureus* virulence genes identified by *bursa aurealis* mutagenesis and nematode killing. Proc. Natl. Acad. Sci. U. S. A. **101:**12312–12317.
3. **Cao, M., A. P. Bitar, and H. Marquis.** 2007. A *mariner*-based transposition system for *Listeria monocytogenes*. Appl. Environ. Microbiol. **73:**2758–2761.
4. **Collins, M. E., J. D. Oultram, and M. Young.** 1985. Identification of restriction fragments from two cryptic *Clostridium butyricum* plasmids that promote the establishment of a replication-defective plasmid in *Bacillus subtilis*. J. Gen. Microbiol. **131:**2097–2105.
5. **Dupuy, B., and A. L. Sonenshein.** 1998. Regulated transcription of *Clostridium difficile* toxin genes. Mol. Microbiol. **27:**107–120.
6. **Gao, L. Y., R. Groger, J. S. Cox, S. M. Beverley, E. H. Lawson, and E. J. Brown.** 2003. Transposon mutagenesis of *Mycobacterium marinum* identifies a locus linking pigmentation and intracellular survival. Infect. Immun. **71:**922–929.
7. **Heap, J. T., O. J. Pennington, S. T. Cartman, G. P. Carter, and N. P. Minton.** 2007. The ClosTron: a universal gene knock-out system for the genus *Clostridium*. J. Microbiol. Methods **70:**452–464.
8. **Heap, J. T., O. J. Pennington, S. T. Cartman, and N. P. Minton.** 2009. A modular system for *Clostridium* shuttle plasmids. J. Microbiol. Methods **78:**79–85.
9. **Hussain, H. A., A. P. Roberts, and P. Mullany.** 2005. Generation of an erythromycin-sensitive derivative of *Clostridium difficile* strain 630 (630Delta*erm*) and demonstration that the conjugative transposon Tn*916*DeltaE enters the genome of this strain at multiple sites. J. Med. Microbiol. **54:**137–141.
10. **Karlsson, S., L. G. Burman, and T. Akerlund.** 1999. Suppression of toxin production in *Clostridium difficile* VPI 10463 by amino acids. Microbiology **145:**1683–1693.
11. **Kuijper, E. J., F. Barbut, J. S. Brazier, N. Kleinkauf, T. Eckmanns, M. L. Lambert, D. Drudy, F. Fitzpatrick, C. Wiuff, D. J. Brown, J. E. Coia, H. Pituch, P. Reichert, J. Even, J. Mossong, A. F. Widmer, K. E. Olsen, F. Allerberger, D. W. Notermans, M. Delmee, B. Coignard, M. Wilcox, B. Patel, R. Frei, E. Nagy, E. Bouza, M. Marin, T. Akerlund, A. Virolainen-Julkunen, O. Lyytikainen, S. Kotila, A. Ingebretsen, B. Smyth, P. Rooney, I. R. Poxton, and D. L. Monnet.** 2008. Update of *Clostridium difficile* infection due to PCR ribotype 027 in Europe, 2008. Euro Surveill. **13:**pii=18942.
12. **Lampe, D. J., B. J. Akerley, E. J. Rubin, J. J. Mekalanos, and H. M. Robertson.** 1999. Hyperactive transposase mutants of the *Himar1* mariner transposon. Proc. Natl. Acad. Sci. U. S. A. **96:**11428–11433.
13. **Lampe, D. J., M. E. Churchill, and H. M. Robertson.** 1996. A purified *mariner* transposase is sufficient to mediate transposition *in vitro*. EMBO J. **15:**5470–5479.
14. **Lampe, D. J., T. E. Grant, and H. M. Robertson.** 1998. Factors affecting transposition of the *Himar1* mariner transposon in vitro. Genetics **149:**179–187.
15. **Lanckriet, A., L. Timbermont, L. J. Happonen, M. I. Pajunen, F. Pasmans, F. Haesebrouck, R. Ducatelle, H. Savilahti, and F. Van Immerseel.** 2009. Generation of single-copy transposon insertions in *Clostridium perfringens* by electroporation of phage mu DNA transposition complexes. Appl. Environ. Microbiol. **75:**2638–2642.
16. **Le Breton, Y., N. P. Mohapatra, and W. G. Haldenwang.** 2006. In vivo random mutagenesis of *Bacillus subtilis* by use of TnYLB-1, a *mariner*-based transposon. Appl. Environ. Microbiol. **72:**327–333.
17. **Maier, T. M., R. Pechous, M. Casey, T. C. Zahrt, and D. W. Frank.** 2006. *In vivo Himar1*-based transposon mutagenesis of *Francisella tularensis*. Appl. Environ. Microbiol. **72:**1878–1885.
18. **Minton, N. P., and J. Morris.** 1981. Isolations and partial characterization of three cryptic plasmids from strains of *Clostridium butyricum*. J. Gen. Microbiol. **127:**325–331.
19. **Monod, M., C. Denoya, and D. Dubnau.** 1986. Sequence and properties of pIM13, a macrolide-lincosamide-streptogramin B resistance plasmid from *Bacillus subtilis*. J. Bacteriol. **167:**138–147.
20. **O'Connor, J. R., S. Johnson, and D. N. Gerding.** 2009. *Clostridium difficile* infection caused by the epidemic BI/NAP1/027 strain. Gastroenterology **136:**1913–1924.
21. **O'Connor, J. R., D. Lyras, K. A. Farrow, V. Adams, D. R. Powell, J. Hinds, J. K. Cheung, and J. I. Rood.** 2006. Construction and analysis of chromosomal *Clostridium difficile* mutants. Mol. Microbiol. **61:**1335–1351.
22. **Paredes-Sabja, D., P. Setlow, and M. R. Sarker.** 2009. The protease CspB is essential for initiation of cortex hydrolysis and dipicolinic acid (DPA) release during germination of spores of *Clostridium perfringens* type A food poisoning isolates. Microbiology **155:**3464–3472.
23. **Purdy, D., T. A. O'Keeffe, M. Elmore, M. Herbert, A. McLeod, M. Bokori-Brown, A. Ostrowski, and N. P. Minton.** 2002. Conjugative transfer of clostridial shuttle vectors from *Escherichia coli* to *Clostridium difficile* through circumvention of the restriction barrier. Mol. Microbiol. **46:**439–452.
24. **Redelings, M. D., F. Sorvillo, and L. Mascola.** 2007. Increase in *Clostridium difficile*-related mortality rates, United States, 1999–2004. Emerg. Infect. Dis. **13:**1417–1419.
25. **Sambrook, J., and W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
26. **Smith, C. J., S. M. Markowitz, and F. L. Macrina.** 1981. Transferable tetracycline resistance in *Clostridium difficile*. Antimicrob. Agents Chemother. **19:**997–1003.
27. **Sorg, J. A., and A. L. Sonenshein.** 2008. Bile salts and glycine as cogerminants for *Clostridium difficile* spores. J. Bacteriol. **190:**2505–2512.
28. **Stabler, R. A., M. He, L. Dawson, M. Martin, E. Valiente, C. Corton, T. D. Lawley, M. Sebaihia, M. A. Quail, G. Rose, D. N. Gerding, M. Gibert, M. R. Popoff, J. Parkhill, G. Dougan, and B. W. Wren.** 2009. Comparative genome and phenotypic analysis of *Clostridium difficile* 027 strains provides insight into the evolution of a hypervirulent bacterium. Genome Biol. **10:**R102. doi:10.1186/gb-2009-10-9-r102.
29. **Vidal, J. E., J. Chen, J. Li, and B. A. McClane.** 2009. Use of an EZ-Tn*5*-

based random mutagenesis system to identify a novel toxin regulatory locus in *Clostridium perfringens* strain 13. PLoS One **4:**e6232.

30. **Wang, H., M. C. Smith, and P. Mullany.** 2006. The conjugative transposon Tn*5397* has a strong preference for integration into its *Clostridium difficile* target site. J. Bacteriol. **188:**4871–4878.
31. **Williams, D. R., D. I. Young, and M. Young.** 1990. Conjugative plasmid transfer from *Escherichia coli* to *Clostridium acetobutylicum*. J. Gen. Microbiol. **136:**819–826.
32. **Wilson, A. C., M. Perego, and J. A. Hoch.** 2007. New transposon delivery plasmids for insertional mutagenesis in *Bacillus anthracis*. J. Microbiol. Methods **71:**332–335.
33. **Zhang, J. K., M. A. Pritchett, D. J. Lampe, H. M. Robertson, and W. W. Metcalf.** 2000. In vivo transposon mutagenesis of the methanogenic archaeon *Methanosarcina acetivorans* C2A using a modified version of the insect mariner-family transposable element *Himar1*. Proc. Natl. Acad. Sci. U. S. A. **97:**9665–9670.