Random Transposon Mutagenesis of Campylobacter jejuni

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Genetic studies of Campylobacter jejuni have been limited due to the lack of a transposon mutagenesis method. Here, we describe a novel technique for random transposon mutagenesis using a mariner-based transposon into C. jejuni strain 480. Insertions were random, as demonstrated by Southern blot analysis and insertional junction sequencing. We have demonstrated, for the first time, random in vivo transposon mutagenesis of C. jejuni.

Campylobacter jejuni has been identified as the leading cause of acute bacterial diarrhea in the United States, and yet the mechanisms by which this bacterium causes disease in humans are not well understood (11). This paucity of information is partially due to a lack of genetic tools and the relatively recent understanding of Campylobacter spp. as important human pathogens. In particular, the lack of an in vivo transposon mutagenesis method for the efficient generation of random mutants of Campylobacter spp. has restricted molecular genetic studies. To date, the generation of C. jejuni mutant libraries has been limited to transposon shuttle mutagenesis (4) and homologous insertional mutagenesis (2, 16). Unsuccessful attempts have been made to introduce both Gram-positive- and Gram-negative-organism-based transposons into Campylobacter spp. (5). For example, nonrandom site-specific insertion mutants were generated by a TaphoA transposon in C. jejuni (13). Recently, a highly permissive mariner-based transposon known as Himar1 has been utilized for efficient in vivo random transposon mutagenesis in Escherichia coli and mycobacteria (10). We therefore designed and tested an in vivo mariner-based transposon mutagenesis system for the production of random insertional mutants of C. jejuni.

Bacterial strains, media, and growth conditions. C. jejuni 480 is a highly electrocompetent strain isolated during an outbreak of campylobacteriosis (provided by B. A. M. van der Zeijst) (3). Strain 480 was grown routinely on Mueller-Hinton (MH) agar supplemented with 5% sheep’s blood, vancomycin (10 μg/ml), polymyxin B (2.6 U/ml), and trimethoprim (5 μg/ml) at 42°C under microaerobic conditions (5% O2, 10% CO2, 85% N2 gas).

Construction of C. jejuni mini-transposon. We constructed an in vivo mini-transposon system for C. jejuni using the mariner family of mini-transposons previously applied to other bacteria (1, 10). Primers (5'-CCAAACGGTGGGCTGAGGAGGATCTTCTAGATGCTCGGCGGTGTTCCTTTCCAAGGTT3') and (5'-CCAAACGGTGGGCTGAGGAGGATCTTCTAGATGCTCGGCGGTGTTCCTTTCCAAGGTT3') were used to amplify the E. coli/C. jejuni-compatible chloramphenicol resistance (Cm) cassette of pRY111 (provided by P. Guerry) (15). This product was cut with NdeI and subcloned in MluI-digested plasmid pEMCAT (1; A. Camelli, unpublished results), replacing the resident Cm-gene within the mini-transposon to create pEMCAT. To allow Himar1 transposase expression in C. jejuni, a C. jejuni-specific promoter was used to replace the original Himar1 promoter. A C. jejuni promoter, GenBank accession no. AJ002027, was used for this purpose. This promoter was found to be active in C. jejuni and inactive in E. coli, as indicated by β-galactosidase activity (14), and was chosen due to concerns that an overactive transposase might be toxic to E. coli (10). The C. jejuni promoter was constructed using partially complementary primers (P1, 5’-CCATCTAGAAAGCTTACTTATGTAAATTATAATTCTCATTATTTCCTTTTCCTTTCCAGGAGGTT3); P2, 5’-CCAGCATGCCCACCTATAGCCCTCTTTCTTAAATGTGTTTATGCCCTTATAATAGCAGG3'). P1 and P2 were annealed and extended with Taq and Pf (Stratagene) DNA polymerase (10:1) and the product was cut with NdeI. A promoterless Himar1 transposase was amplified from a plasmid containing the C9 hyperactive mutant of the Himar1 transposase (6) by using primers (H1, 5’-CAGCTTCACATAACACATTTGTGTATGGATGACAACTCCCCATCGG3); H2, 5’-CAGCTTCACATAACACATTTGTGTATGGATGACAACTCCCCATCGG3). This product was cut with NdeI and ligated to the NdeI-digested promoter product. Primers P1 and H2 were then used to amplify the C. jejuni-compatible promoter-Himar1 fusion by PCR, and the product was cloned in pCR2.1 (Invitrogen) DNA to generate pCRPH. The C. jejuni-compatible promoter-transposase construct and mini-transposon sequences were moved into pUC19 in separate ligation steps following double digestions of pCRPH and pEMCAT with HindIII-SphI and SphI-BamHI, respectively. This resulted in the construction of pOTHM (Fig. 1), which is incapable of replicating in C. jejuni and thus serves as a suicide delivery vector.

Mini-transposon delivery into C. jejuni. pOTHM was introduced by electroporation into the C. jejuni strain 480 as described by Wassenaar et al. (13). This strain was chosen because of its ability to readily accept exogenous DNA. One microgram of pOTHM was used to electroporate approximately 50 μl of a solution at 5 × 1011 bacteria/ml at 1.25 V, 600 Ω, and 25 μF in a 0.1-cm cuvette. Cells were allowed to recover on Columbia blood agar plates (8) for 4 to 5 h at 37°C under microaerobic conditions. The bacteria were harvested from the plate surface and resuspended in 0.5 ml of MH broth. Transformants were selected at 37°C for 72 h on MH agar supplemented with 5% sheep’s blood and 5 μg of chloramphenicol per ml. This procedure typically resulted in 37 ± 4 (mean ± standard deviation) chloramphenicol-resistant colonies per electroporation (n = 5). Transformants were picked and characterized as described below.

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Analysis of pOTHM insertions. Southern blot analysis was performed on 19 randomly picked transformants obtained from a single electroporation experiment. Genomic DNA was isolated using the G-nome DNA kit (Bio101), digested with HindIII, electrophoretically separated on a 1.8% agarose gel, and transferred to a Hybond N+ membrane (Amersham). The membrane was then probed with DNA containing the 5' and 3' portions of the mini-transposon (Fig. 2B) by using the ECL RPN 3000 detection system (Amersham). Two fragments of various sizes would be expected for each transformant with these probes, provided that a single random transposition event had occurred. C. jejuni 480 wild-type chromosomal DNA was used as a negative control and failed to hybridize with the probes as expected (data not shown). As demonstrated in Fig. 2A, each of the 19 transformants had two uniquely sized bands, suggesting that a single random transposon insertion had occurred in each transformant.

To further confirm the randomness of insertion, the mini-transposon chromosomal junctions were sequenced from 12 mutants obtained from two independent transposition experiments and the sites of insertion were mapped on the complete chromosomal sequence of C. jejuni NCTC 11168 (C. jejuni NCTC 11168 Sequencing Group at the Sanger Centre [http://www.sanger.ac.uk/Projects/C_jejuni/]) (Fig. 3). Inverse PCR of the 12 transformants was performed using HindIII-cut circularized transformant genomic DNA. Primer 1 (5'-CTTCCCAACGTAAATATCGGCAGTAG-3') and primer 2 (5'-TATCGCTCTTGAGGAACATATGTTG3') extended outward from within the Cm' cassette and were used to determine the

![Diagram of pOTHM mini-transposon](https://example.com/diagram.png)


![Southern blot analysis of Campylobacter mini-transposon insertion strains](https://example.com/blot.png)

FIG. 2. Southern blot analysis of Campylobacter mini-transposon insertion strains. (A) Lanes 1 to 19, randomly chosen transformants isolated from one transformation experiment. (B) Plasmid pOTHM was cut with HindIII and Nol, and fragments were separated by agarose gel electrophoresis. DNA fragments containing the 5' and 3' mini-transposon junctional sequences, probes 1 and 2, respectively, were then isolated and used together as a probe. We believe differences in band intensities relate to variations in DNA transfer.
insertion site within the C. jejuni genome. Primer walking-directed sequencing of inverse PCR products (using primer 1) was performed with the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Perkin-Elmer). Cycling of oligonucleotides was performed in the thermal cycler (GeneAmp 9600; Perkin-Elmer) by following the instructions in Perkin-Elmer protocol P/N 402078. Reaction products were run on a 373 DNA Sequencer, Stretcher (Applied Biosystems). The sequence data obtained from each of the 12 junctions demonstrated a high degree of identity to different segments of the C. jejuni NCTC 11168 chromosome. Furthermore, each of the 12 derived sequences is of either known Campylobacter genes or genes encoding putative proteins homologous to other bacterial species and ORFs of unknown function (Table 1). Analysis of the 30-bp sequence flanking each of the 12 insertion sites revealed no consensus sequence other than the invariant TA dinucleotide which the Himar1 mariner transposon recognizes (7, 10), which might indicate insertion site preference. Based on these results, we conclude that the mini-transposon inserts with a high degree of randomness throughout the C. jejuni genome.

Significance. We have been able to demonstrate, for the first time, random in vivo transposon mutagenesis of C. jejuni. Prior attempts to mutagenize C. jejuni by in vivo transposition have been hindered by a combination of incompatibilities with promoter usage resulting in little or no expression of the transposase (12), the absence of species-specific cofactors necessary for transposition, and a lack of natural transposons (7). For these reasons, we chose to test the ability of the highly permissive Himar1 transposase to mediate random insertional mutagenesis in C. jejuni. As described above, this novel method of transposon mutagenesis in C. jejuni has resulted in the generation of highly random insertional mutations. The efficiency of the procedure is high enough that an insertion library of high complexity can be readily generated. This will greatly aid genetic analysis of this intestinal pathogen by screening for putative virulence determinants that have been inactivated by the insertion mutagenesis process.

Infect. Immun.

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REFERENCES


TABLE 1. Mini-transposon insertion into putative C. jejuni NCTC 11168 ORFs

<table>
<thead>
<tr>
<th>Transformant</th>
<th>Homologous ORF* (GenBank accession no.)</th>
<th>E valuea</th>
<th>Organismb</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>unk4 (AAA23030)</td>
<td>0.0</td>
<td>C. jejuni</td>
</tr>
<tr>
<td>2</td>
<td>orfX (CA73267)</td>
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<td>E. coli</td>
</tr>
<tr>
<td>3</td>
<td>hauA (U08349)</td>
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<td>Haemophilus influenzae</td>
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<td>4</td>
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<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>xprB (AAA62787)</td>
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<td>E. coli</td>
</tr>
<tr>
<td>6</td>
<td>Unknown</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>7</td>
<td>radA (AAC33293)</td>
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<td>8</td>
<td>knB (BAA32063)</td>
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<tr>
<td>9</td>
<td>fhaB (AAA22077)</td>
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<td>11</td>
<td>sdaB (L07763)</td>
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<td>E. coli</td>
</tr>
</tbody>
</table>

a DNA sequences of mini-transposon junction sites were used to determine C. jejuni open reading frames (ORFs) using the Sanger Center C. jejuni NCTC 11168 BLAST program. Homologous ORFs and E values were determined using the GenBank database BLAST program.

b Organism in which the homologous ORF was found. NA, not applicable.


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