Transposon mutagenesis of the anaerobic commensal, *Bacteroides fragilis*, using the EZ::TN5 transposome

Yaligara Veeranagouda1,2, Fasahath Husain1 & Hannah M. Wexler1,2

1GLAVAHCS, Los Angeles, CA, USA; and 2UCLA School of Medicine, Los Angeles, CA, USA

Correspondence: Yaligara Veeranagouda, GLAVAHCS, Bldg. 115 Room 312, 11301 Wilshire Blvd., Los Angeles, CA 90073, USA. Tel.: +1 310 2683404; fax: +1 310 268 4458; e-mail: veereshagy@gmail.com

Received 2 February 2012; revised 24 April 2012; accepted 23 May 2012. Final version published online 18 June 2012. DOI: 10.1111/j.1574-6968.2012.02602.x

Editor: Robert Gunsalus

Keywords

*Bacteroides fragilis*; transposon mutagenesis; mutant library.

Abstract

Genetic analysis of *Bacteroides fragilis* (BF) is hindered because of the lack of efficient transposon mutagenesis methods. Here, we describe a simple method for transposon mutagenesis using EZ::TN5, a commercially available system that we optimized for use in BF638R. The modified EZ::TN5 transposon contains an *Escherichia coli* conditional origin of replication, a kanamycin resistance gene for *E. coli*, an erythromycin resistance gene for BF, and 19 basepair transposase recognition sequences on either ends. Electroporation of the transposome (transposon–transposase complex) into BF638R yielded $3.2 \pm 0.35 \times 10^3$ CFU g$^{-1}$ of transposon DNA. Modification of the transposon by the BF638R restriction/modification system increased transposition efficiency sixfold. Electroporation of the EZ::TN5 transposome results in a single-copy insertion of the transposon evenly distributed across the genome of BF638R and can be used to construct a BF638R transposon library. The transposon was also effective in mutating a BF clinical isolate and a strain of the related species, *Bacteroides thetaiotaomicron*. The EZ::TN5-based mutagenesis described here is more efficient than other transposon mutagenesis approaches previously reported for BF.

Introduction

*Bacteroides fragilis* is a Gram-negative, anaerobic bacterium associated with the gastrointestinal (GI) tract of animals and humans (Gilmore & Ferretti, 2003) and is the major *Bacteroides* species isolated from human infections (80%) (Bennion et al., 1990; Wexler et al., 1998; Wexler, 2007). As a commensal, it hydrolyzes complex polysaccharides and produces volatile fatty acids used by the host as source of energy (Wexler, 2007). When BF escapes the GI tract, it can cause serious infections (Gilmore & Ferretti, 2003).

Investigation of the BF genetic makeup and its regulatory processes will aid in understanding how BF can evolve from a benign commensal to a multidrug-resistant pathogen. The function of most genes cannot be determined from primary sequence analysis alone (Cerdeno-Tarraga et al., 2005; Patrick et al., 2010), and the creation of mutants (Mazurkiewicz et al., 2006) is a useful tool for deducing gene function. As transposons are known for their random insertion into the genome, they have been widely used for the construction of mutant libraries (Jacobs et al., 2003; Gallagher et al., 2007).

To date, two transposons (Tn4351 and Tn4400) have been used for generation of random mutations in BF. However, each has certain drawbacks. A Tn4351 transposon derivative (used for BF, *Bacteroides thetaiotaomicron* and related bacteria) may integrate into the genomic DNA along with its vector, thereby complicating the molecular characterization of the mutated gene (Shoemaker et al., 1986; Chen et al., 2000a). In addition, mutants generated by Tn4351 can contain multiple Tn4351 insertions, which further hinder characterization of the mutants (Shoemaker et al., 1986). A modified Tn4400 transposon vector pYT646B (Tang & Malamy, 2000) generates mutants by inverse transposition;
Transposon mutagenesis of Bacteroides fragilis

however, this transposon can also incorporate at multiple positions in a single mutant, potentially complicating further analysis (Chen et al., 2000b; Tang & Malamy, 2000).

Ease of identifying the disrupted gene is also an important factor in the utility of these transposons. Tn4400 has a HindIII site within the transposon sequence, so that sequences flanking IS4400R (right inverted repeat) can be identified by self-ligation of HindIII-digested genomic DNA of the mutant and subsequent rescue cloning and sequencing. However, retrieving the gene fragment adjacent to the IS4400L (left inverted repeat) is more difficult because of the lack of appropriate restriction enzymes (Tang & Malamy, 2000).

Owing to the restrictions and drawbacks in the existing systems, we sought to develop an alternative, efficient, and reliable transposon tool for BF (http://www.epibio.com). This system provides an efficient and reliable method of inserting transposon cassette) flanked by inverted repeats. This system provides a HindIII site within the transposon sequence, so that sequences flanking IS4400R (right inverted repeat) can be identified by self-ligation of HindIII-digested genomic DNA into the genome of many different microorganisms

This study reports the development of a simple EZ::TN5-based approach for transposon mutagenesis in BF. Mutants generated by this method contain a single mutation in a single mutant, potentially complicating further analysis (Chen et al., 2000b; Tang & Malamy, 2000). Introduction of the kanamycin resistance gene (km) into pYV01

The kanamycin gene (km) along with its promoter was PCR-amplified with Km F EcoRV and Km R EcoRV primers (Table 1) using the Bacteroides shuttle vector pFD288 as template DNA (Smith et al., 1995) and ligated into pGEM®-T Easy. Escherichia coli Top 10 chemically competent cells were transformed with the ligation mix, and transformants were selected on LB-Amp agar plate, yielding plasmid pT-ermF-4. The ermF was retrieved from pT-ermF-4 by Sac I digestion and ligated into Sac I-digested pMOD-3 < R6Kγori/MCS >. Escherichia coli Top10 competent cells were transformed with the ligation mix, and transformants were selected on LB-Amp agar plate, yielding pYV01.

Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ermF F Sacl</td>
<td>GATATCGAGCTCCCTGTAACACAGTGCC</td>
</tr>
<tr>
<td>ermF R Sacl</td>
<td>GATATCGAGCTCAATTGCCAGCGTATG</td>
</tr>
<tr>
<td>Km F EcoRV</td>
<td>GATATCGAGCTCGTGAAGAACACTACGGAGTCG</td>
</tr>
<tr>
<td>Km R EcoRV</td>
<td>GATATCGAGCTCCATTCAATATGTATCCGCT</td>
</tr>
<tr>
<td>pFKRepAF</td>
<td>AGAGTCGATATCCGTCAGAGGATCC</td>
</tr>
<tr>
<td>pFKRepAR</td>
<td>AGATCTCCCAGGTACAACTACGAGGATCC</td>
</tr>
<tr>
<td>EzTnSeq3R</td>
<td>GCAGCCGATATTCGGACAGCCGAGAA</td>
</tr>
<tr>
<td>EzTnSeqFP</td>
<td>GGGCGAGGCTGACATTAGACCCAGTGAC</td>
</tr>
<tr>
<td>SRP1</td>
<td>GGACCCAGCTCGAGCTGACAGGATCCGAGG</td>
</tr>
<tr>
<td>EnTnSeqN1R</td>
<td>CGATTTTCAGGAAAGTCGAGTTCGA</td>
</tr>
<tr>
<td>SRP2</td>
<td>GGGCGAGCCGAGGTACAGGATCCGAGG</td>
</tr>
<tr>
<td>EzTnSeq2R</td>
<td>GTTCTCCAGGCCAAGGGAGATCCGAGG</td>
</tr>
<tr>
<td>SRP3</td>
<td>TGGATGAGGATCCGAGGATCCGAGG</td>
</tr>
<tr>
<td>ermF- BamHI-F</td>
<td>AACCCAGGGATCCCTAGGAGGATGAGG</td>
</tr>
<tr>
<td>ermF- BamHI-R</td>
<td>AACCCAGGGATCCCTAGGAGGATGAGG</td>
</tr>
</tbody>
</table>

Materials and methods

Strains and culture conditions

All strains were grown as described (Pumbwe et al., 2005). Escherichia coli Top10 (Invitrogen, NY) was used as the host for cloning. Ampicillin (Amp) (100 μg mL⁻¹), erythromycin (Erm) (10 μg mL⁻¹), kanamycin (40 μg mL⁻¹), and gentamycin (40 μg mL⁻¹) were used for selection as indicated.

DNA analysis

DNA preparation, restriction digestions, gel electrophoresis, and analysis were performed as previously described (Pumbwe et al., 2006b). Sequencing was carried out by Laragen (Culver City, CA).

EZ::TNS transposon vector construction

The EZ::Tn5 carrying plasmid pMOD-3 < R6Kγori/MCS> (EPICENTRE® Biotechnologies) was modified for use in BF638R.

Introduction of ermF into pMOD-3 < R6Kγori/MCS>

The erythromycin resistant gene (ermF) along with its promoter was PCR-amplified with ermF F SacI and ermF R SacI primers (Table 1) using the Bacteroides shuttle vector pFD288 as template DNA (Smith et al., 1995) and ligated into pGEM®-T Easy. Escherichia coli Top 10 chemically competent cells were transformed with the ligation mix, and transformants were selected on LB-Amp agar plate, yielding plasmid pT-ermF-4. The ermF was retrieved from pT-ermF-4 by Sac I digestion and ligated into Sac I-digested pMOD-3 < R6Kγori/MCS >. Escherichia coli Top10 competent cells were transformed with the ligation mix, and transformants were selected on LB-Amp agar plate, yielding pYV01.
ers (Table 1) using pET-27B(+) as template DNA. The amplified PCR product (0.95 kb) was purified and ligated into pGEM®-T Easy. *Escherichia coli* Top 10 cells were transformed with the ligation mix, and transformants were selected on LB-Km agar plate, yielding plasmid pT-Km-2. The *km* gene was retrieved from pT-Km-2 by EcoRV digestion and ligated into SmaI-digested pYV01. *Escherichia coli* Top10 competent cells were transformed with the ligation product, and transformants selected on LB-Km agar plate, yielding plasmid pYV02; this plasmid was used for transposome preparation (see below).

**Restriction/modification (R/M) of pYV02 in BF638R**

pYV02 was passed through BF638R, so that the transposon would be properly modified by the host methylation system to avoid subsequent degradation. For this purpose, *repA* (for replication in *Bf*) was PCR-amplified using primers pFKRepAF and pFKRepAR using pFK12 as template DNA (Haggoud *et al.*, 1995). The amplified PCR product (1.68 kb) was purified, digested with SmaI/Eco RV, and ligated into SmaI site of pYV02. BF638R was transformed with the ligation mix by electroporation, and transformants selected on BHI-Km agar plate, yielding plasmid pYV03.

**EZ::TN5 transposome preparation and transposon mutagenesis of BF638R**

Transposomes were prepared according to manufacturers’ protocol with the following modifications. EZ::TN5 transposon DNA was retrieved from either pYV02 or pYV03 (*Bf*-R/M vector) following PvuII digestion. The resulting 2.6-kb fragment was gel-purified and column eluted (Qiaquick Gel Extraction Kit; Qiagen, Inc., Valencia, CA) with TE buffer (10 mM Tris–HCL (pH7.5), 1 mM EDTA). For transposome preparation, 2 μL of EZ::TN5 transposon DNA (100 ng μL⁻¹) was mixed with 4 U (4 μL) of En-Tn5™ transposase (EPICENTRE® Biotechnologies) plus 2 μL of glycerol (100%) and incubated for 1 h at room temperature. The resulting transposon–EZ::TN5 transposase mixture (transposome) was stored at −20 °C and used for mutagenesis of *Bf*.

**BF electrocompetent cell preparation**

A single colony of BF638R grown on BHI was inoculated in 5 mL BHI broth and incubated anaerobically overnight (16 h) at 37 °C. Cultures were diluted (1 : 100) in 100 mL BHI broth and allowed to grow to an OD$_{600nm}$ of 0.3–0.4. Cells were then harvested by centrifugation at 5020 g for 5 min and washed five times with 50 mL of ice-cold 10% glycerol. Cells were finally suspended in 1 mL of 10% glycerol, and 100 μL aliquots were used for electroporation.

**Electroporation conditions**

Transposome (2 μL) was mixed with 100 μL BF638R competent cells in a 0.2 cm electroporation cuvette and incubated on ice for 30 min. Electroporation was performed using a BioRad Gene Pulser™ (200 Ohms, 25 μF and 2.5 kV). Following electroporation, 900 μL of pre-reduced BHI broth was added and the mixture incubated anaerobically for 3 h at 37 °C. The cells were then plated on BHI-Erm agar plate (to select for transposon mutants) and incubated anaerobically for 3 days at 37 °C.

**Southern blot analysis**

The probe, ermF, was PCR-amplified using ermF-BamHI-F and ermF-BamHI-R primers with pFD288 as template DNA. Biotin-16-dUTP (Roche Applied Bioscience, Indianapolis, IN) was incorporated into the probe during PCR amplification. Genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen). Genomic DNA (2 mg) was digested overnight with BglII, electrophoresed (0.8% agarose), and transferred to Nytran SuperCharge Nylon membrane (Whatman, Piscataway, NJ) using the Turboblotter Rapid Downward Transfer Systems (Whatman). DNA was cross-linked to the membrane by baking at 80 °C for 2 h. Hybridization and detection of probe was performed with the biotin Chromogenic kit (Fermentas, Glen Burnie, MD).

**Identification of transposon-disrupted gene by rescue cloning**

Genomic DNA was prepared from transposon mutants and digested with BglII (any enzyme that does not cut within the transposon could be used). Subsequently, the digested DNA was purified, self-ligated with T4 DNA ligase, and introduced into electrocompetent EC100D pir-116 E. coli (EPICENTRE® Biotechnologies) by electroporation. The circularized fragments containing the transposon replicate as plasmids and the transformants were recovered on LB agar plates containing kanamycin (LB-Km). Transposon junction plasmids were isolated from selected transformants and sequenced using transposon-specific outward primers EZTNSeq3R and EZTNSeqFP (Table 1), which anneal to ≤ 100 bp upstream of the mosaic end left (MEL) and the mosaic end right (MER), respectively. Sequences were then compared to the protein sequence database (GenBank) using the Blastx algorithm. For each mutant, the junction between the
Transposon sequence (the Tn5 inverted repeat sequence ending with CTGTCTCTTATACACATCT or AGATGTGTATAAGAGACAG) and the genomic DNA sequence as well as the 9-bp target duplication (a characteristic of Tn5 insertions) were identified.

**Identification of the transposon-disrupted gene by nested PCR using SRP**

The SRP-PCR was developed as described by Chen et al. (2000b). The first round of PCR was performed using OneTaq™ Hot Start 2x master mix (New England Biolabs, MA) with SRP1 and EnTnSeqN1R (transposon specific) primers and template DNA from the mutant. The first-round PCR conditions were 10 min at 95 °C, six cycles of 30 s at 95 °C, 30 s at 30 °C, and 1.5 min at 68 °C with 5 s increments per cycle; 30 cycles of 30 s at 95 °C, 30 s at 45 °C, and 2 min at 68 °C with 5 s increments per cycle and 5 min at 68 °C. One microliter of the first-round PCR product was used as the template in the second-round PCR with primers SRP2 and EzTnSeqN2R. The product of second-round PCR was column purified and sequenced with primer EzTnSeq3R. Sequences that contained the MEL sequence were considered *bona fide* transposon-disrupted genes. SRP3 was used as an alternative to SRP1 in the first-round PCR in cases where SRP1 did not yield the desired PCR product.

**Results**

**Transposon mutagenesis of BF638R using modified EZ::TN5 transposome**

The transposon vector pYV02 (Fig. 1a) was constructed as described in Materials and methods. Digestion of pYV02 with PvuII yielded a transposon that contained the *E. coli* conditional origin of replication (R6K-ori), the kanamycin resistance gene (*Km*), *ermF* (erythromycin resistance gene for selection of transposon insertion in BF), and 19-basepair transposase recognition sequences (mosaic ends, ME) on either ends (Fig. 1b). R6K-ori and *km* enable rescue of the transposon with the surrounding mutated gene sequence in *E. coli*. Transposase was added to the customized EZ::TN5 product forming the transposome which was then introduced into BF638R by electroporation (Fig. 1c). The transformants were selected on BHI/Erm agar plate. About 20 randomly selected transformants were tested for the presence of *ermF*; all potential mutants showed the expected PCR product (1.2 kb band) (data not shown). The efficiency of EZ::TN5 transposon insertion in BF638R was $3.2 \pm 0.35 \times 10^3 \, \mu g^{-1}$ of transposon DNA.

**Passage of the transposon DNA through BF638R increases efficiency of transposon mutagenesis**

The BF genome contains extensive endogenous R/M systems that protect host DNA by recognizing and cleaving foreign DNA (Cerdeno-Tarraga et al., 2005; Patrick et al., 2010). As the transposon DNA was prepared from *E. coli*, the BF638R R/M system might degrade the transposon DNA which would impair transposition efficiency (Salyers et al., 2000). Therefore, pYV02 was electroporated into BF638R, so that it would be restriction modified by the BF638R system to increase transposon efficiency, as described in Materials and methods. The transposomes were then prepared from pYV03 and electroporated to BF638R. The BF638R-modified transposon was nearly six
times more efficient \( (1.9 \pm 0.3 \times 10^4) \) than before modification, confirming that bypassing the host R/M system can increase transposon efficiency.

**Southern hybridization confirmed that EZ::TN5 delivered a single copy of transposon per genome**

Chromosomal DNA was prepared from eight randomly selected mutants and digested with BglII (which has no recognition site within the \( \text{ermF} \) gene). Following Southern hybridization using a biotin-labeled \( \text{ermF} \) probe (Fig. 2), all strains contained only a single hybridizing DNA fragment, demonstrating that each mutant contains only single copy of \( \text{ermF} \). This property of the transposon is very important as it enables the study of the effect of a single-gene disruption in a given mutant. This modified EZ::TN5 system is superior to other transposon systems described for \( BF \) in consistently delivering only a single copy per chromosome.

**Modified EZ::TN transposon inserts properly into the BF638R chromosome**

Externally added DNA may undergo illegitimate recombination with the bacterial chromosome (Desomer *et al.*, 1991; Kalpana *et al.*, 1991; Chua *et al.*, 2000). To confirm that this was not occurring, we rescued the genomic region flanking the EZ::TN transposon from the mutants and looked for a 9-bp target site duplication in the mutant DNA. Analysis of the DNA sequence flanking the EZ::TN transposon at MEL and MER revealed that each insertion was flanked by the 9-bp duplication characteristic of the Tn5 insertion (Table 2) (Berg & Berg, 1983), confirming that the antibiotic-resistant transconjugants arose by transposition of the EZ::TN transposon into the host chromosome.

**Modified EZ::TN can be used to construct a mutant library in BF638R, and the resultant mutants can be rapidly identified by SRP-PCR**

The library was screened for auxotrophic mutants to demonstrate the usefulness of the modified EZ::TN5 transposome in mutant library construction. Five hundred \( BF638R \) transposon mutants were replica plated onto minimal media with or without Casamino acids (0.5% w/v) (Baughn & Malamy, 2002). One of 500 transposon mutants screened failed to grow on minimal medium without Casamino acids, suggesting that a gene in an amino acid biosynthesis pathway was disrupted (Mutant EZY6).

The disrupted gene in the auxotrophic mutant was identified by the SRP-PCR (Fig. 3). The identification of the 19-bp inverted repeat on the amplified PCR products confirmed that isolated auxotrophic mutant was a ‘true’ transposon insertant. We also identified the transposon-disrupted gene using the alternative rescue cloning method described in Materials and methods. Both the methods independently indicated that EZY6 had a mutation in \( \text{argC} \) (acetylglutamyl phosphate reductase, \( BF638R_0529 \)), a gene in the arginine biosynthesis pathway. We found that the SRP-PCR technique was faster and simpler than the rescue cloning method for identifying the disrupted gene.

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**Table 2. Confirmation of transposon insertion in BF638R transposon mutants:** the mutated genes in eight randomly selected mutants were retrieved by rescue cloning and sequenced with forward (EzTnSeqFP) and reverse (EzTnSeq3R) primers that read from MER and MEL, respectively. The nine nucleotides next to the mosaic ends are shown.

<table>
<thead>
<tr>
<th>Mutant name</th>
<th>9 bp duplication</th>
</tr>
</thead>
<tbody>
<tr>
<td>EZY4</td>
<td>MER: ATATAAGAG</td>
</tr>
<tr>
<td></td>
<td>MEL: CTCTTATAT</td>
</tr>
<tr>
<td>EZY5</td>
<td>MER: GGTAATGG</td>
</tr>
<tr>
<td></td>
<td>MEL: CCAATTG</td>
</tr>
<tr>
<td>EZY6</td>
<td>MER: CTCCAGAAC</td>
</tr>
<tr>
<td></td>
<td>MEL: GTTCGAGG</td>
</tr>
<tr>
<td>EZY7</td>
<td>MER: GGTTAAGT</td>
</tr>
<tr>
<td></td>
<td>MEL: ACAAACC</td>
</tr>
<tr>
<td>EZY8</td>
<td>MER: GTACGAGG</td>
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<tr>
<td></td>
<td>MEL: GCCTCCTAC</td>
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<tr>
<td>EZY9</td>
<td>MER: GTGCTACAC</td>
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<td>EZY10</td>
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<tr>
<td>EZY11</td>
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</tr>
<tr>
<td></td>
<td>MEL: ATACGATCC</td>
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</table>

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**Fig. 2.** Transposition of transpososome in BF638R. Southern hybridization: DNA from transposon mutant was digested with BglII and transferred to a nylon membrane. The \( \text{ermF} \) gene present on chromosomal DNA was probed with the biotin Chromogenic kit. Lane 1. M Biotinylated 2 DNA Ladder, Lanes 2–7 are EZY mutants.
Selected mutants that grew slowly on minimal medium were also chosen for further study. The mutated genes were identified by SRP-PCR, and results are presented in Fig. 4. Mutants had transposon insertions in two-component regulators (EZY7), cell division proteins (EZY11), aminotransferase (EZY17), GMP biosynthesis pathway (EZY19), transport-related proteins (EZY21), and various other genes. The disrupted genes were scattered throughout the genome of BF638R (Fig. 4), confirming that the custom EZ::TN5 transposome described here can randomly insert the transposon into the B. fragilis chromosome.

**Modified EZ::TN5 transposome can be used to create mutants in BF clinical isolates as well as in the related species, B. thetaiotaomicron**

The utility of the customized EZ::TN5 transposon for generating mutants in BF 9343 (ATCC 25285), BF clinical isolates, and B. thetaiotaomicron (Pumbwe et al., 2006a) was examined. The transposome was prepared from BF638R-modified pYV03. The efficiencies of the transposition in the clinical strain BF14412 and B. thetaiotaomicron were \(3.6 \pm 0.67 \times 10^3\) and \(6.3 \pm 1.2 \times 10^3\), respectively, indicating that the system may be useful for some clinical strains of BF as well as B. thetaiotaomicron. No mutants were generated in BF 9343 or the clinical isolate BF7320. It is possible that pYV03 DNA modified by the BF638R R/M system was recognized as foreign and cleaved by the resident type I and II R/M system of BF 9343 (Cerdeno-Tarraga et al., 2005). Other plasmids frequently used in BF638R are also difficult or impossible to introduce into BF 9343 (data not shown). In general, more efficient transposon mutagenesis is achieved by prior modification of plasmid carrying the transposon by the host of interest.

**Discussion**

We developed an improved system for transposon mutagenesis in BF using the EZ::TN5 system. Previous attempts to mutagenize BF by transposons have been hindered by either vector integration and/or multiple insertions (Shoemaker et al., 1986; Chen et al., 2000a). Also, those methods often used labor-intensive filter mating techniques to introduce the DNA. The method described here has several advantages: (1) transposons can be introduced into BF by electroporation, (2) all insertion events are independent, (3) no vector delivery system is required and vector cointegration can be completely avoided, and (4) no suicide vector or native inducible promoters to drive transposase expression are needed. We found that the transposon inserts evenly across the chromosome. Also, analysis of the insertion points of the EZ::TN5 transposon indicates that although there is some sequence context preferred of insertion by Tn5, the insertion is sufficiently random for its effective use in construction a library of transposon mutants (Shevchenko et al., 2002).

EZ::TN5 transposon mutagenesis also provides flexibility for subsequent identification of the transposon-disrupted gene. For example, if the genome sequence is not available for the organism of interest, the genes adjacent to the mutated gene can be retrieved and identified by rescue cloning and sequencing. On the other hand, if the genome sequence is available, the mutated gene can be amplified by SRP-PCR and identified by genomic means and large numbers of mutants can be easily
screened. Prior passage of the transposon vector in related strains increases downstream efficiency of transposon mutagenesis. This system provides a useful genetic tool that will facilitate deeper understanding of the pathogenic mechanisms of this important human commensal/pathogen.

Acknowledgement

This research is based upon work supported in part by the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development, Biomedical Laboratory Research and Development and in part by the NIAID (NIH) Grant Number 1R56AI083649-01A2. We would like to thank Drs Elizabeth Tenorio and Yi Wen for their helpful comments and advice regarding mutant identification and Southern Blots, respectively.

Reference


