Published in final edited form as:
Anaerobe. 2013 August ; 22: 126–129. doi:10.1016/j.anaerobe.2013.04.012.

Transposon mutagenesis of *Bacteroides fragilis* using a *Mariner* transposon vector

Yaligara Veeranagouda[1,2], **Fasahath Husain**[1], and **Hannah M. Wexler**[1,2]
[1]GLAVAHCS, Los Angeles, California
[2]UCLA School of Medicine, Los Angeles, California

Abstract

The *mariner* transposon vector pYV07 was tested for use in the mutagenesis of *Bacteroides fragilis 638R*. The transposon vector efficiently generated mutants in *B. fragilis 638R*. The transposon disrupted genes were scattered throughout the genome of *B. fragilis* 638R. This method serves as a powerful tool to study *B. fragilis*.

Keywords

Bacteroides fragilis; transposon mutagenesis; mutant library; mariner transposon; semi random PCR; rescue cloning

Bacteroides fragilis is an obligatory anaerobic bacterium that usually exists as a commensal in the gut of humans and animals. Stool culture studies performed in the 1970's suggested that the species *B. fragilis* comprises a small proportion of the gut *Bacteroides* and that has been the classical assumption for decades [1–3]. Recent metagenomic studies generally support that assumption [4] although the speciation, data collection and analyses have obviously become much more complex and the genomic methods used to determine abundance of species are completely different than those used by the earlier studies. *B. fragilis* is still considered the primary cause of infections involving *Bacteroides* spp [5–7] although, again, definitive results may be influenced by recent changes in technique and speciation of isolates [7]. It is clear, however, that when *B fragilis* moves out of its niche in the gut, it becomes an opportunistic pathogen and can cause serious infections. It is the anaerobe most frequently isolated from patients with intra-abdominal sepsis, necrotizing skin, perforated and gangrenous appendicitis and soft tissue infections. Enterotoxigenic strains of *B. fragilis* are associated with human diarrheal disease including traveler's diarrhea [8–9].

In many cases, treatment of *B. fragilis* infection is problematic due to its high level of resistance to multiple classes of antibiotics. Many *B. fragilis* clinical isolates are resistant to aminoglycosides, β-lactams, macrolide, and metronidazole [10]. Recent studies have demonstrated an increase in *B. fragilis* isolates that harbor multiple conjugation elements that may confer resistance to multiple antibiotics [11]. These strains further increase the risk

Corresponding author: Yaligara Veeranagouda, GLAVAHCS, Bldg. 115 Room 312, 11301 Wilshire Blvd., LA CA 90073, veereshagy@gmail.com, yvgoudar@ucla.edu, Phone: 310 2683404.

of transfer of multiple drug resistance to bacteria in the neighboring environment. Development of genetic tools to probe the gene functions in *B. fragilis* is necessary to better understand both the development of antimicrobial resistance and pathogenic potential of *B. fragilis*.

Recently we described a transposon mutagenesis method for *B. fragilis* using the EZ-Tn5 transposome [12]. The method involves transposome construction using transposon DNA and the commercially available EZ::Tn5 transposase. Although this transposon mutagenesis method is simple and efficient, it is not cost-effective. Therefore, we sought to develop a simple, efficient and cost effective transposon vector for transposon mutagenesis of *B. fragilis*. *Mariner*-family transposable elements are active in a wide variety of organisms and generate stable random insertions in the recipient genome and are considered a promising mutagenic tool, especially for studying a species which lacks sophisticated genetic tools [13]. Therefore, we reasoned that a *mariner* transposon mutagenesis would be an effective method to construct a random mutant library in *B. fragilis*. Goodman *et al.* [14] developed the *mariner* transposon vector pSAM-Bt which has a hyperactive *mariner* transposase (Himar1C9) [15–16] that inserts the transposon DNA (i.e., the region within the inverted repeat) into the genome of recipient strain at a “TA” site. This transposon vector has been successfully exploited for transposon mutagenesis for *B. thetaiotaomicron* and *Porphyromonas gingivalis* [14]. In the present study, we examined the efficacy of a modified pSAM-Bt in the construction of a transposon mutant library of *B. fragilis 638R*.

The vector pSAM-Bt was a kind gift from Andrew L. Goodman [14]. With this vector, transposon disrupted gene can be easily identified by semi random priming (SRP) PCR. SRP-PCR usually gives short (~50–200 base pairs) sequence which is sufficient to identify a mutated gene if the genome sequence is known. However, if the genome sequence is not known, as in the case of a clinical isolate, rescue cloning of the interrupted gene will yield a much longer sequence for mutated gene identification. Therefore, we added a kanamycin cassette to facilitate the easy retrieval of the mutated gene from both ends of the transposon. The Km (kanamycin) cassette was cloned downstream of the e*rmG* (erythromycin) cassette in pSAM-Bt. The Km cassette along with its promoter was PCR amplified from a pET-27b+ template using KmFXbaI (CTGCAGTCTAGAGTGGAACGAAAACTCACGTTAAGG) and KmRXbaI (CTGCAGTCTAGACATTCAAATATGTATCCGCTC) as primers. The amplified PCR product was digested with XbaI and ligated to XbaI-digested pSAM-Bt. The resulting ligation mix was used to transform *E. coli* S-17 λ pir [17] competent cells and transformants were selected on a Luria Bertani (LB)-Km (40 μg/ml) agar plate. The transformants were isolated and the resultant plasmid is named pYV07 (Fig. 1).

The vector pYV07 was used for mutagenesis of *B. fragilis* 638R as follows. *E. coli* S-17 λ pir-pYV07 and *B. fragilis* 638R were grown overnight in LB/Km and brain heart infusion (BHI) broths, respectively. Overnight cultures were subcultured in antibiotic-free medium and grown to an OD_{600} of 0.15. One ml of *E. coli* S-17 λ pir -pYV07 was then mixed with 10 ml of *B. fragilis 638R*. The mixed cultures were collected by centrifugation, resuspended in 100 μl BHI broth and plated on a single BHI plate. The resulting plates were incubated aerobically for 3h and then anaerobically for overnight at 37°C and the cells harvested by scraping them off the plate. They were then suspended in 1ml BHI broth, centrifuged and then resuspended in 1 ml of BHI broth containing 10% glycerol. The resulting mutant library can be used directly or stored at −80°C for future use. The transposon mutants were isolated by plating the mutant library (20 μl) on BHI/Gentamycin (25 μg/ml)/erythromycin (10 μg/ml)/ plate. The early log phase cells of *B. fragilis* (OD_{600} 0.06 to 0.15) yields a higher number of transposon mutants (208±15 mutants/20 μl of mating mix) than a mid-log phase (0.3 to 0.4 OD_{600}) (65±16 mutants/20 μl of mating mix).

The presence of the *ermG* cassette in 50 randomly selected mutants was confirmed by PCR amplification. All of the mutants yielded a 750 bp PCR product indicating that *ermG* *(* (present in the transposon) was carried by all mutants. It has been previously reported that transposon vectors can also integrate into the genome of the recipient strain by illegitimate recombination. Therefore, we looked for the potential vector backbone integration in fifty transposon mutants by performing PCR to detect the presence of the ampicillin resistant (Amp) gene which resides on the plasmid backbone. None of the 50 mutants yielded a PCR product, indicating that mutants are due to transposon insertion and not due to vector integration.

The quality of random insertion is one of key aspects of a transposon vector. To determine whether pYV07 inserts randomly and not into genetic “hot spots”, we identified the transposon insertion positions in 100 mutants by SRP-PCR [12]. We used this method to retrieve the sequence of the mutated gene next to the IRL (inverted repeat left) but the same method can be used to retrieve the sequence of the gene next to the IRR using the appropriate transposon specific primers. The first round of PCR was performed using OneTaqTM Hot Start 2X master mix (New England Biolabs MA USA) with the SRP1 (GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT) and the transposon-specific primer SAMseq1 (ACGTACTCATGGTTCATCCCGATA) and mutant colony as template DNA. The first round PCR conditions were 10 min at 95°C; 6 cycles of 30s at 95°C, 30s at 30°C, and 1.5 min at 68°C with 5s increments per cycle; 30 cycles of 30s at 95°C, 30s at 45°C, and 2 min at 68°C with 5s increments per cycle; and 5 min at 68°C. One μl of the first round PCR product was used as the template in the second round PCR with SRP2 (GGCCACGCGTCGACTAGTAC) and the transposon-specific primer SAMseq2 (GCGTATCGGTCTGTATATCAGCAA). The second round PCR conditions were 10 min at 95°C; 35 cycles of 45 s at 95°C, 45 s at 55°C, and 1.5 min at 68°C with 5 s increments per cycle; and 10 min at 68°C. The second round PCR product was column purified and sequenced with the transposon specific primer, SAMseq3 (TCTATTCTCATCTTTCTGAGTCCAC). The mutant DNA sequences which contained the IRL (ACAGGTTGGATGATAAGTCCCCGGTCTT) were considered *bona fide* transposon-disrupted genes. The transposon-disrupted gene was identified by comparing the DNA sequence next to the IRL to the genome of *B. fragilis* 638R.

As expected, the *mariner* transposase inserted the transposon into the genome at a “TA” site in all the 100 mutants tested. This insertion preference does not materially affect the quality of the randomness of the mutant library; *B. fragilis* has 347,859 “TA” sites on the positive strand of the genome which theoretically corresponds to one TA site/~ 15 bases (*B. fragilis* has 5,373,121 nucleotides in its genome). As shown in Fig. 2, the transposon insertion sites are evenly distributed across the genome of *B. fragilis 638R*. The selected transposon mutants showed disruptions in the genes involved in polysaccharide (PS) gene clusters (PSA (Cpy31), PSD (Imi2), PSE (Cpy46) and PSJ (Ety15)), RND efflux pumps (Bme8B (MYV52) and Bme1B (Cpy35)), transcriptional regulators (Bme5R (Cpy36)), sigma factors (sigma54 modulation protein (Cef16), ECF-type sigma factor (Cpy4)), transporters (ABC transporter (MYV30)), and RecA (Cpy25). These results indicated that pYV07 is an effective and efficient tool for *B. fragilis* random mutant library generation.

The transposon vector described in this study also facilitates the identification of the transposon-mutated gene by rescue cloning. In this case, the genomic DNA of the transposon mutants would be digested with BglII (or any enzyme which does not cut the transposon DNA), cloned into BglII-digested pUC19 and transformed into *E. coli.* Transformants can be selected on a kanamycin LB/agar plate. Plasmids from kanamycin-resistant transformants contain the kanamycin cassette and flanking genomic sequence. Sequencing of the rescued plasmid with the outwardly directed SAMSeq3 and SAMseqR

(GCCAGGCATCAAATTAAGCAG) primers will yield the transposon junction sequence. Compared to SRP-PCR which usually yields small PCR products, rescue cloning will yield a longer stretch of junction DNA. This feature of the transposon is particularly useful to retrieve the mutated gene sequence if the genome sequence of organism of interest is not available.

Random transposon mutagenesis is a powerful tool for understanding specific gene function in bacteria. Improvement in next generation sequencing (NGS) technology has facilitated the *en masse* identification of a large number of transposon mutants [18]. With the help of NGS technology, transposon vectors have been successfully exploited for identification of essential/fitness genes in many pathogenic bacteria such as *Mycobacterium tuberculosis* [19], *Pseudomonas aeruginosa* [20], *B. thetaiotaomicron* [14] and *P. gingivalis* [16]. These high throughput identification methods are helpful for large scale linking of genotype to phenotypes, identification of gene function and unraveling complex pathways in bacteria. The transposon vector described in the present study is a powerful tool for the characterization of *B. fragilis* genes. We are currently determining the essential genes in *B. fragilis* 638R and our preliminary results indicate that transposon insertions are not detected in likely essential genes. With the help of this transposon vector and NGS technology, we are currently investigating the genes required for *B. fragilis* fitness under a variety of stress conditions.

Acknowledgments

We would like to thank Dr. Andrew Goodman (Yale University School of Medicine) for providing the pSAM-Bt transposon vector. We would also like to thank Dr. Elizabeth Tenorio (Tufts Medical Center, Boston) for advice on the technical aspects of semi-random PCR. 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.

Reference List

1. Moore W, Holdeman L. Human fecal flora: the normal flora of 20 Japanese-Hawaiians. Appl. Microbiol. 1974; 75:961–979. [PubMed: 4598229]
2. Polk BF, Kasper DL. *Bacteroides fragilis* subspecies in clinical isolates. Ann. Intern. Med. 1977; 86:569–571. [PubMed: 322563]
3. Salyers AA. *Bacteroides* of the human lower intestinal tract. Annu. Rev. Microbiol. 1984; 38:293–313. [PubMed: 6388494]
4. Qin J, Li R, Raes J, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010; 464:59–65. [PubMed: 20203603]
5. Brook I. The role of anaerobic bacteria in bacteremia. Anaerobe. 2010; 16:183–189. [PubMed: 20025984]
6. Wexler HM. *Bacteroides*--The Good, the Bad, and the Nitty-Gritty. Clin. Microbiol. Rev. 2007; 20:593–621. [PubMed: 17934076]
7. Tong J, Liu C, Summanen P, et al. Application of quantitative real-time PCR for rapid identification of Bacteroides fragilis group and related organisms in human wound samples. Anaerobe. 2011; 17:64–68. [PubMed: 21439390]
8. Wick EC, Sears CL. *Bacteroides* spp. and diarrhea. Curr. Opin. Infect. Dis. 2010; 23:470–474. [PubMed: 20697287]
9. Jiang ZD, DuPont HL, Brown EL, et al. Microbial etiology of travelers' diarrhea in Mexico, Guatemala, and India: importance of enterotoxigenic Bacteroides fragilis and Arcobacter species. J. Clin. Microbiol. 2010; 48:1417–1419. [PubMed: 20107088]
10. Vedantam G. Antimicrobial resistance in Bacteroides spp.: occurrence and dissemination. Future. Microbiol. 2009; 4:413–423. [PubMed: 19416011]

11. Nguyen M, Vedantam G. Mobile genetic elements in the genus Bacteroides, and their mechanism(s) of dissemination. Mob. Genet. Elements. 2011; 1:187–196. [PubMed: 22479685]
12. Veeranagouda Y, Husain F, Wexler HM. Transposon mutagenesis of the anaerobic commensal, *Bacteroides fragilis*, using the EZ::TN5 transposome. FEMS Microbiol. Lett. 2012; 333:94–100. [PubMed: 22639975]
13. Hayes F. Transposon-based strategies for microbial functional genomics and proteomics. Annu. Rev. Genet. 2003; 37:3–29. [PubMed: 14616054]
14. Goodman A, McNulty N, Zhao Y, et al. Identifying genetic determinants needed to establish a human gut symbiont in its habitat. Cell host. & microbe. 2009; 6:279–289. [PubMed: 19748469]
15. Lampe DJ, Akerley BJ, Rubin EJ, et al. Hyperactive transposase mutants of the Himar1 mariner transposon. Proc. Natl. Acad. Sci. U.S.A. 1999; 96:11428–11433. [PubMed: 10500193]
16. Klein BA, Tenorio EL, Lazinski DW, et al. Identification of essential genes of the periodontal pathogen Porphyromonas gingivalis. BMC Genomics. 2012; 13:578-. [PubMed: 23114059]
17. Simon R, Priefer U, Pühler A. A broad host range mobilization system for In Vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Nature Biotechnology. 1983; 1:784–791.
18. van OT, Bodi KL, Camilli A. Tn-seq: high-throughput parallel sequencing for fitness and genetic interaction studies in microorganisms. Nat. Methods. 2009; 6:767–772. [PubMed: 19767758]
19. Griffin JE, Gawronski JD, Dejesus MA, et al. High-resolution phenotypic profiling defines genes essential for mycobacterial growth and cholesterol catabolism. PLoS. Pathog. 2011; 7:e1002251. [PubMed: 21980284]
20. Gallagher LA, Shendure J, Manoil C. Genome-scale identification of resistance functions in *Pseudomonas aeruginosa* using Tn-seq. M Bio. 2011; 2:e00315-10.

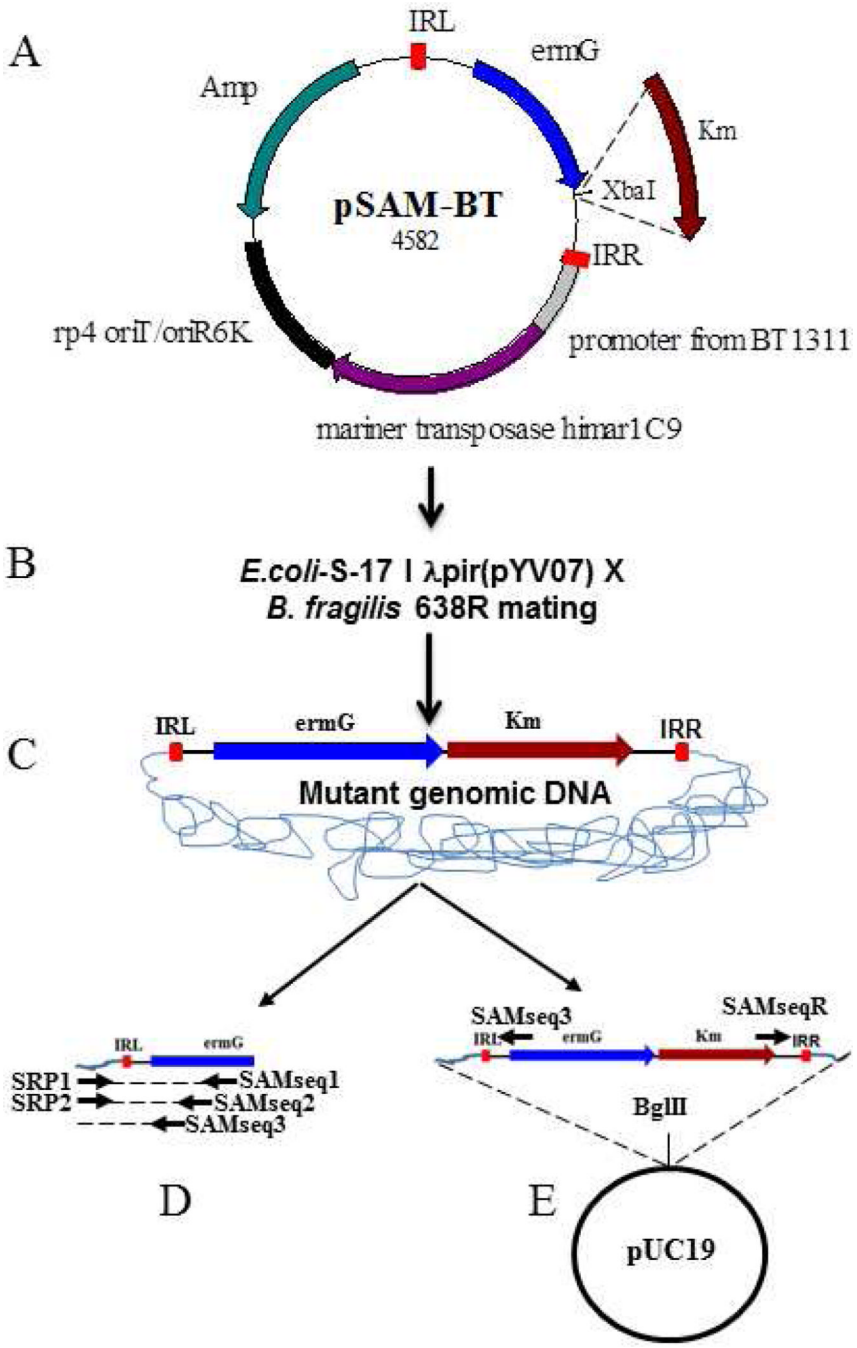

Fig. 1.

Transposon mutagenesis of *B. fragilis* 638R. A) The *mariner* transposon vector pSAM-Bt. IRL, Inverted repeat left; *ermG*-provides erythromycin resistance in *B. fragilis*; rp4 oriT/ oriR6K-conditional origin of replication facilitates plasmid replication only in *E. coli*; Amp-ampicillin resistance gene for *E. coli*. Plasmid pYV07 was created by cloning Km-kanamycin resistance gene at the XbaI site of pSAM-Bt. B) Transposon mutants are generated by mating *E. coli* S-17 λ pir-pYV07 with *B. fragilis* 638R. C) When pYV07 enters *B. fragilis* 638R, the *mariner* transposase, whose expression is driven by *B. thetaiotaomicron* promoter (BT1331), inserts the transposon DNA (ie. IRL, *ermG*, Km and IRR) into the genome. The transposon mutants become resistant to erythromycin. D)

Transposon disrupted gene identification by SRP-PCR and E) Transposon disrupted gene identification by rescue cloning. Mutant genomic DNA can be cut with BglII and cloned into BglII-digested pUC19. Sequencing with SAMseq3 and SAMseqR primer yields transposon junction DNA.

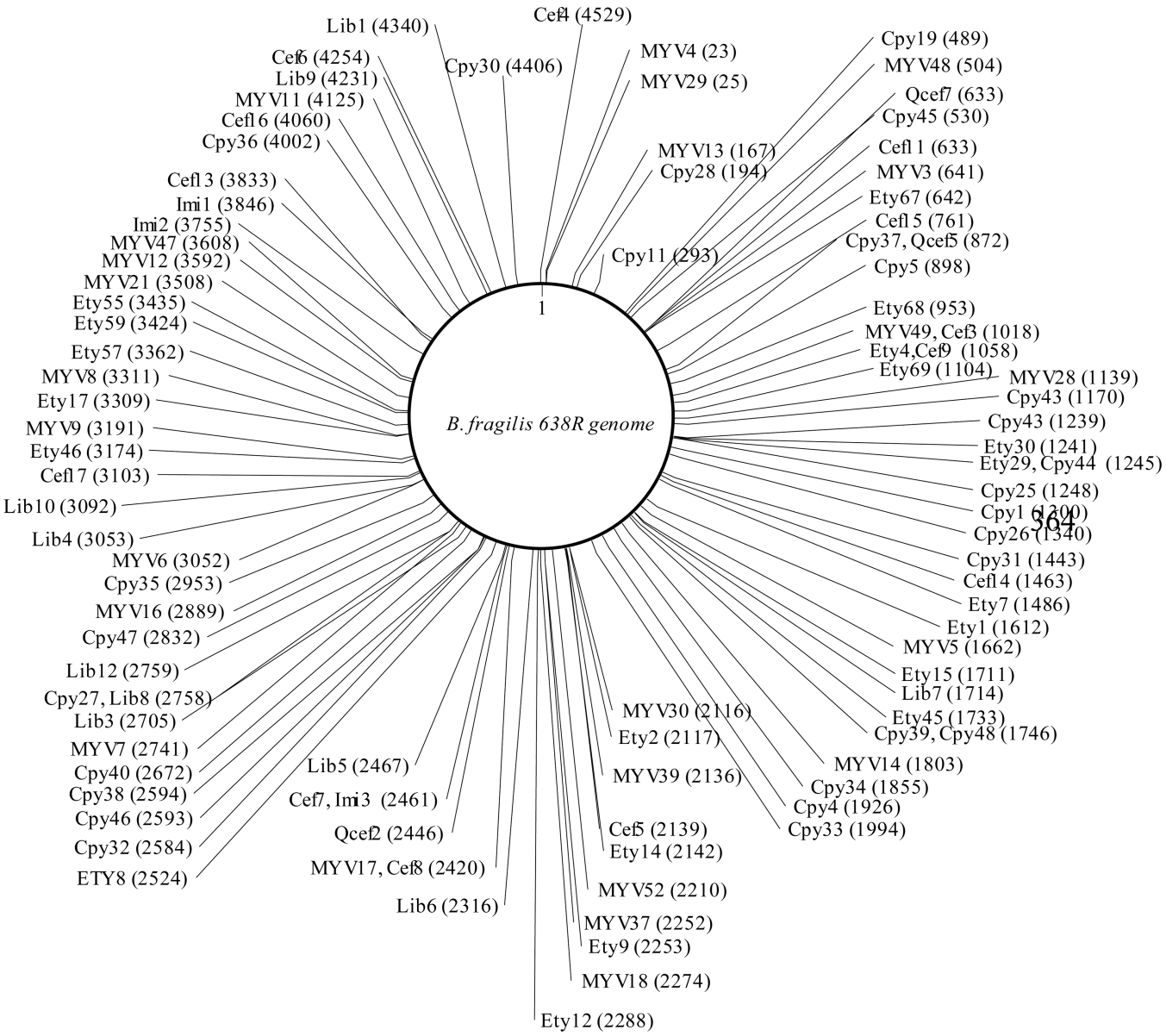

Fig. 2.

Schematic representation of *B. fragilis* 638R transposon mutants. *B. fragilis* 638R has 4,416 genes in its genome. *B. fragilis* transposon mutants are generated using pYV07 and identified by SRP-PCR. The mutant name and the transposon insertion position (locus tag number) are indicated in brackets.