Transposon Mutagenesis of *Borrelia burgdorferi*

NOTES

The transposon vectors pMarGent and pGKT (Fig. 1) have been constructed for random mutagenesis of *Borrelia burgdorferi*. The borrelial promoters used to drive expression of the transposase and the antibiotic-resistance markers on the transposons are recognized and functional in *E. coli*. The transposase is therefore active in *E. coli* and allows transposition of the gentamicin marker during growth of the culture for plasmid DNA isolation, maintaining the gentamicin resistance of the cells but resulting in an inactive variant of pMarGent. The plasmid preparations derived from different *E. coli* colonies, therefore, yield a range of plasmid forms and transposase activities. We generally isolate plasmid DNA from 6 different *E. coli* clones and transform each plasmid preparation into a high-passaged *B. burgdorferi* strain (such as B31-A-34) to determine which preps yield the most transposon mutants. To reduce the chances of isolating inactive plasmid preparations from *E. coli*, we constructed the vector pGKT (Fig. 1B) by cloning the kanamycin-resistance marker adjacent to the transposase gene of pMarGent. Growth of *E. coli* colonies harboring pGKT in the presence of both antibiotics requires that both the transposase (linked to the kanamycin marker) and the transposon-portion of the plasmid (inverted terminal repeats, origin of replication, and the gentamicin marker) be retained. Intact plasmid preparations can also be analyzed by diagnostic restriction enzyme digests such as NotI (which cuts twice), or NdeI (cuts three times). We have found that pGKT appears to be more stable than pMarGent resulting in more plasmid preparations with high transposition frequencies.

PREPARATION OF PLASMID DNA

1. Transform pMarGent or pGKT (Fig. 1) into competent *E. coli* cells and plate in the presence of the appropriate antibiotic (i.e. gentamicin for pMarGent or gentamicin **AND** kanamycin for pGKT).
2. Inoculate a starter culture with an *E. coli* colony into growth medium and incubate overnight at 30ºC with appropriate antibiotic selection (30º was used to allow the *E. coli* to reproduce but not remain in stationary phase for a long period).
3. Inoculate a large culture containing growth medium + antibiotic selection with approximately 100-fold dilution of the overnight culture (e.g. a 100 mL culture inoculated with 1 mL of the overnight). Incubate 4-6 hours and isolate plasmid DNA.

TRANSFORMATION AND PLATING OF *B. BURGDORFERI* CELLS

Refer to the protocol on transforming & plating *B. burgdorferi* for more detailed information.

1. Add transposon plasmid DNA (10-20 µg in a 5 µL volume of sterilized H_2O) to 50-100 µL of competent cells.
2. Electroporate cell/DNA mix in a prechilled 0.2 cm-gapped cuvette. Standard transformation settings for the electroporator are: 2.5 kV, 25 µF, and 200 Ω.
3. Immediately after transformation, quickly resuspend cells in a final volume of 5 mL BSK medium and incubate overnight at 35ºC.
4. After overnight recovery, enumerate live *B. burgdorferi* cells using a Petroff-Hausser chamber, it is prudent at this point to prepare a glycerol stock of the transformation outgrowth for future use. Plate in solid BSK medium with appropriate antibiotic selection. Subsurface colonies develop in ~6-10 days, depending on the strain.

IDENTIFICATION OF TRANSPOSON INSERTION SITE

1. Confirm that colonies arising under selective pressure are transposon mutants (and not spontaneously-arising antibiotic-resistant variants) by PCR for the presence of the gentamicin-resistance marker. Stab the *B. burgdorferi* colony with a sterile toothpick, then swirl the toothpick in a tube containing the PCR reaction mix. Primers JK61 and JK62, used for amplifying a fragment of the gentamicin-resistance gene, produce a 520 base pair PCR product.

   JK62 (Gent F): 5' – GGCAGTCGCCCTAAAACAAAGTT

   JK61 (Gent RC): 5' – TCTCGGCTTGAACGAATTGTTAGGT

2. Once confirmed as transposon mutants by PCR, aspirate colonies from the plate using a sterile Pasteur pipet and transfer to liquid BSK medium (5-15 mL) containing appropriate antibiotic(s). Incubate cultures at 35ºC until cells reach high densities (between $5 \times 10^7 - 1 \times 10^8$ cells/mL).

3. Isolate genomic DNA from 5 mL cultures by pelleting the cells, removing the supernatant and resuspending in 500 µL sterile H_20. Lyse cells by the addition of 25 µL of 10% SDS and 5 µL of a 10 mg/mL RNase solution, and incubate at room temperature for 5 min. Phenol:chloroform extractions are repeated (usually 2-3 times) to remove protein and purify genomic DNA. Precipitate DNA with ethanol or isopropanol, wash with 70% ethanol, and resuspend in 50 µL sterile H_20 or TE. Alternatively, genomic DNA can be isolated from a larger 15 mL volume using the Wizard Genomic DNA Purification Kit (Promega), following manufacturer's recommendations for gram-negative bacteria.

4. Digest approximately 500 ng of genomic DNA overnight with the restriction enzyme *Hind*III in a 15 µL volume. This procedure was empirically determined and may be optimized, although it has proven reliable. The restriction enzyme *Hind*III was chosen because the recognition sequence is absent from the transposon but is relatively frequent in *B. burgdorferi* DNA.
5. Remove about 8 µL of digested DNA and ligate in ~10 µL volume at 14ºC for 6 hours (or overnight). Transform entire ligation into chemically competent *E. coli* cells and plate about ½ of the transformed cells on LB-plates containing gentamicin. Incubate plates at 37ºC overnight.
6. Isolate plasmid DNA from *E.coli* colonies.
7. Sequence the *B. burgdorferi* DNA flanking the transposon insertion from the plasmid DNA using primers flg and col:

   col: 5' – CAGCAACGCGGCCTTTTTACG

   flg: 5' – GCTTAAGCTCTTAAGTTCAACC
8. Identify the transposon insertion site by submitting the sequence results to the NCBI nucleotide BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence data using these primers will contain a small amount of vector DNA (including the inverted terminal repeat sequence that serves as a recognition site for the transposase: 5' – ACAGGTTGGCTGATAAGTCCCCGGTCT). Because of the large number of highly related gene families in the *B. burgdorferi* genome, some BLAST results will return multiple genes, requiring more detailed analysis to identify the exact location of the transposon insertion. Also, each rescued transposon should contain a single HindIII site (this can be confirmed through sequence analysis or by digestion and gel

electrophoresis). Multiple HindIII sites would indicate that during the rescue of the transposon several HindIII fragments were ligated together and therefore complicate the identification of the insertion site.

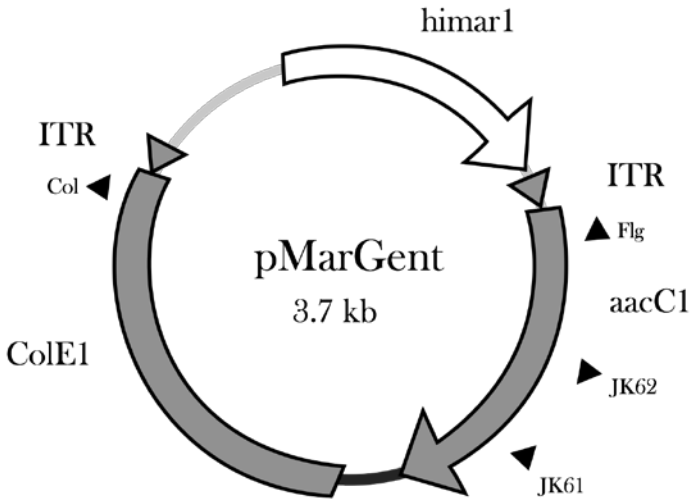
REFERENCES:

Stewart, P. E., J. Hoff, E. Fischer, J. G. Krum, and P. A. Rosa. 2004. Genome-Wide Transposon Mutagenesis of *Borrelia burgdorferi* for Identification of Phenotypic Mutants. *Applied and Environmental Microbiology*. 70:5973-5979.

Stewart, P. E. and P. A. Rosa. 2008. Transposon mutagenesis of the Lyme disease agent *Borrelia burgdorferi*. In *Bacterial Pathogenesis: Methods and Protocols*. Series: *Methods in Molecular Biology*, F. DeLeo and M. Otto (ed.), Humana Press, Totawa, NJ.

Fig. 1

A.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pMarGent
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JK62
JK61

B.

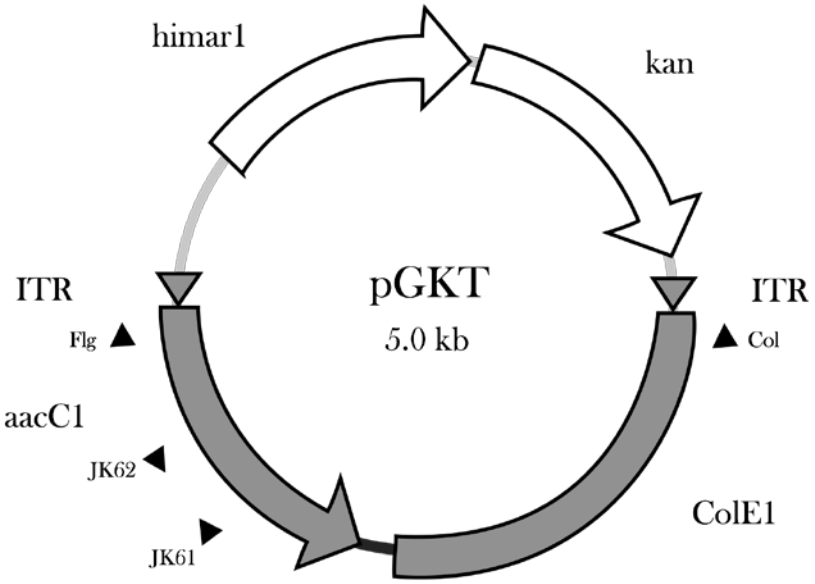
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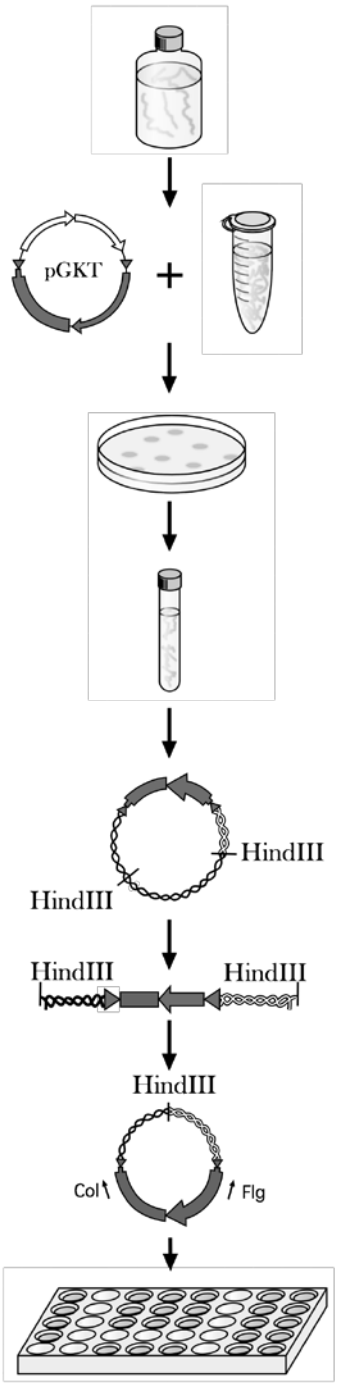
Fig. 2



Growth and competence preparation of B. burgdorferi

Electroporation

Plating

Transfer of individual colonies to liquid culture

Isolation of genomic DNA

Digestion of genomic DNA with HindIII

Ligation of genomic DNA and rescue of transposon and flanking DNA in E. coli

Sequencing with outward-pointing primers (→)