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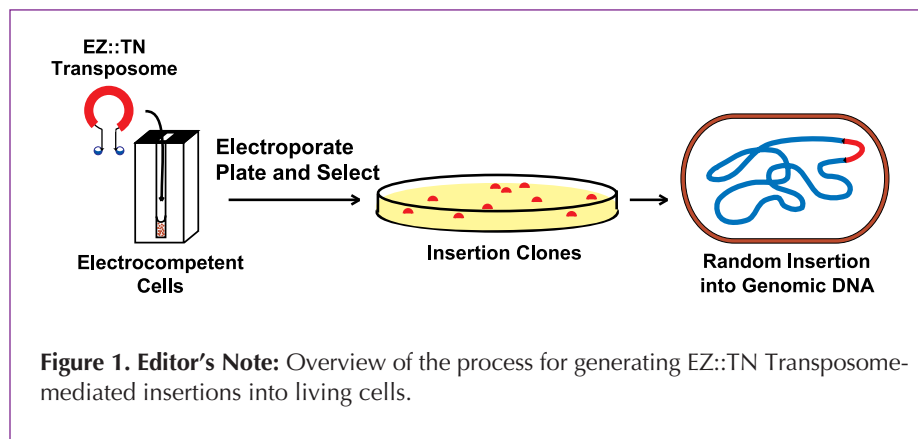
Using the EZ::TN™ Transposome™ for Transposon Mutagenesis in *Mycobacterium smegmatis*

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Mycobacterium smegmatis is the organism of choice for genetic studies of mycobacteria because the strain is fast growing, non-pathogenic, and DNA can be introduced into it by transformation (electroporation), transduction and conjugation.¹⁻³ As such it is used as the genetic testing ground for its more pathogenic cousins *M. tuberculosis* and *M. leprae*. The development of additional genetic tools to facilitate the study of this fascinating group of organisms is important, especially as until recently they have proven to be refractory to genetic analyses. This article describes the application of a Tn5-based transposome system⁴ (Figure 1) to *M. smegmatis* and it is the first description of its use in a gram-positive organism. These results therefore underline the potential of this system as a mutagen for many bacterial species.

Transposons have proven to be one of the most versatile and useful genetic tools for molecular genetic research. They can be used to generate marked gene knockouts, create gene fusions, and act as mobile priming sites for DNA sequence analyses.⁵ More recently, the repertoire of transposon applications has expanded dramatically with the development of *in vitro* transposition systems.⁶⁻¹⁰ These systems have had great impact on the ability to generate insertion mutations and to provide sequencing priming sites by taking advantage of a more efficient biochemical reaction that allows insertion of the transposon to cloned regions of interest.

For many less-characterized organisms, there is still a need for the development of *in vivo* transposon mutagenesis systems to facilitate genetic studies.



continued

This requires both an efficient transposition system for the host organism and a method to deliver the transposon. Most transposon vectors are based on a conditionally replicating phage or plasmid that is used to deliver the transposon into the host under permissive conditions and provide a window of time for transposition to occur. Transposon insertions are then selected with an appropriate antibiotic, while growing the cells under non-permissive conditions eliminates the vector. Unfortunately, for many of these organisms functional transposons, and plasmid or phage vectors have still to be described.

Recently, a simple and elegant *in vivo* transposon delivery and transposition system has been described that is destined to become the transposon mutagenesis method of choice for such organisms.⁴ The only requirement is that DNA can be introduced into the host by electroporation; no vector delivery system is required. Its simplicity relies on the ability to generate a stable EZ::TN Transposome (Tn5 transposon intermediate) that can be electroporated into the bacterial host. Tn5 transposes by a simple insertion mechanism,⁶ and one of the key intermediates in this pathway is the transposome: an excised transposon with the transposase protein bound to the inverted repeats (Mosaic Ends) found at the ends of the transposon. The transposome can be generated *in vitro* using purified transposase protein and a DNA fragment flanked by the inverted repeats. The fact that any gene flanked by the inverted repeats can be used to form this intermediate endows the system with great flexibility, as it can be tailored to the organism of choice. The procedure relies on the fact that transposomes are extraordinarily stable DNA complexes^{11,12} and, in particular, the EZ::TN (Tn5) complex has been shown to maintain its integrity throughout the electroporation process.

Methods

One microliter of the preformed EZ::TN <Kan-1>Tnp Transposome (EPICENTRE) was introduced into *M. smegmatis* strains, mc²155¹³ and mc²874,¹⁴ by electroporation. Electrocompetent cells were prepared and used according to previously described methods.³ Immediately after pulsing, 1 ml of trypticase-soy broth (TS) containing 0.05% Tween 80 was added, and the cell suspension was incubated for 4-6 hrs before plating on TS media containing kanamycin at 10 µg/ml. Plates were incubated for 4-5 days at 37°C to allow colony formation. No transformants were detected in mock electroporations. The auxotrophic screen was performed by growing Kan^r transformants in microtitre plates for 3 days at 37°C before spotting 5 µl onto either TS media or Middlebrook 7H10 media plus ADC³ containing kanamycin.

Chromosomal DNA for Southern analysis was obtained from strains of *M. smegmatis* using established procedures.¹⁵ The Kan^r gene was labeled with [α-³²P] dATP by random priming, and used as a probe of Southern blots of EcoRI- and PstI- digested chromosomal DNA. There are no EcoRI or PstI sites in the EZ::TN <KAN-1> Transposon.

Results and Discussion

Electroporation of the EZ::TN <KAN-1>Tnp Transposome into both strains of *M. smegmatis* generated between 1-5 x 10² Kan^r colonies. This is in contrast to plasmid electroporation, which yields approximately 10⁵ transformants/µg of plasmid DNA. To confirm that these were *bone fide* insertions, chromosomal DNA was isolated from 16 transformants and subject to Southern hybridization analysis. Two different enzymes were used to digest the DNA, and a segment of the Kan^r gene was used as a probe (Figure 2). The analysis shows that none of the hybridizing bands co-migrate in both of the restriction analyses. This demonstrates unequivocally that the insertions are on different DNA fragments and are

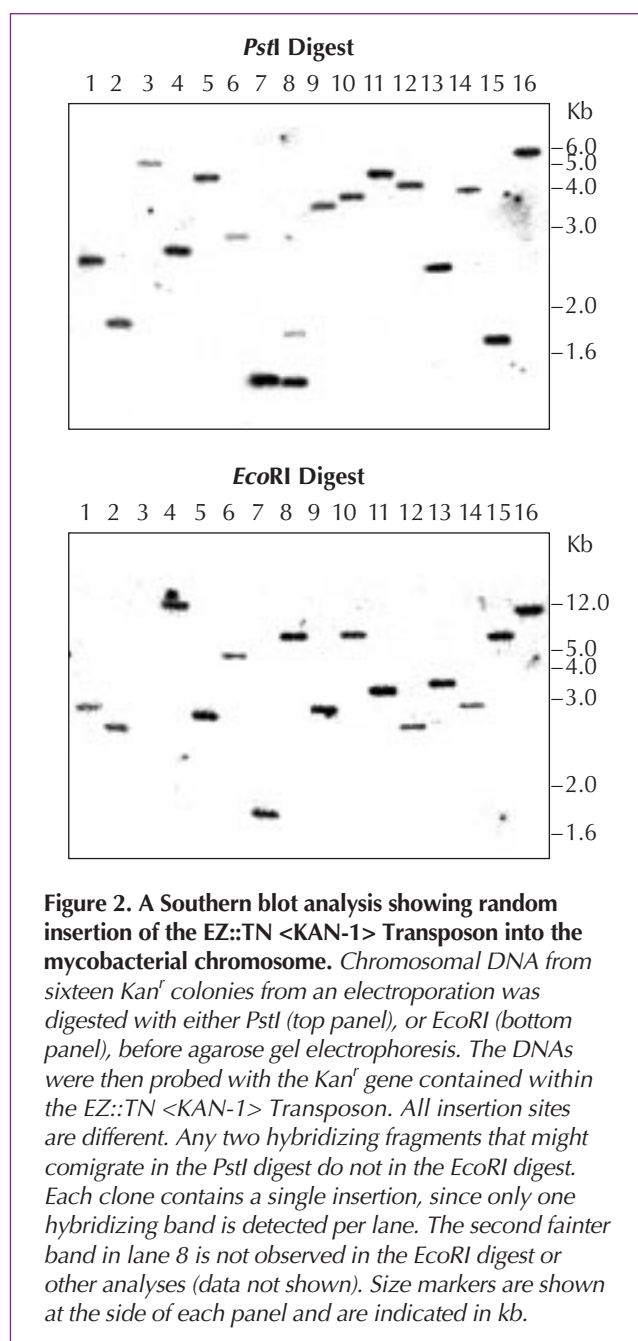


Figure 2. A Southern blot analysis showing random insertion of the EZ::TN <KAN-1> Transposon into the mycobacterial chromosome. Chromosomal DNA from sixteen Kan^r colonies from an electroporation was digested with either PstI (top panel), or EcoRI (bottom panel), before agarose gel electrophoresis. The DNAs were then probed with the Kan^r gene contained within the EZ::TN <KAN-1> Transposon. All insertion sites are different. Any two hybridizing fragments that might comigrate in the PstI digest do not in the EcoRI digest. Each clone contains a single insertion, since only one hybridizing band is detected per lane. The second fainter band in lane 8 is not observed in the EcoRI digest or other analyses (data not shown). Size markers are shown at the side of each panel and are indicated in kb.

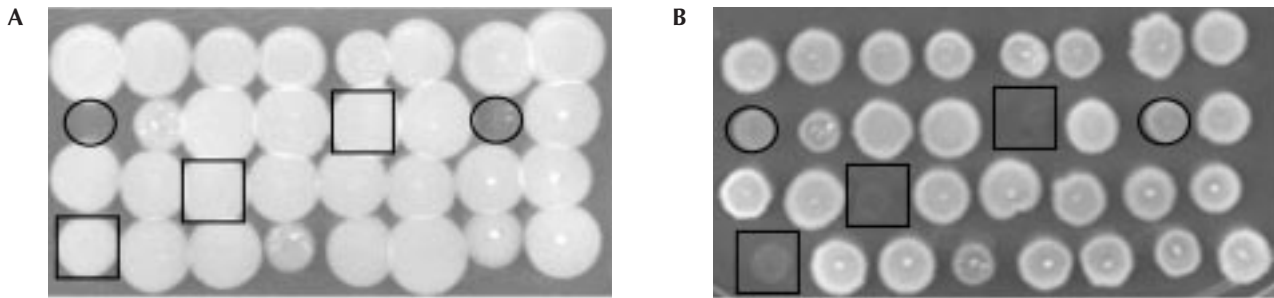


Figure 3. A representative sample of EZ::TN <KAN-1> Transposon insertion mutants used to screen for growth deficiencies. Aliquots of broth cultures were spotted onto either rich media (A), or minimal media (B). Mutants unable to grow on minimal media are highlighted in a box, mutants defective for growth on rich media are circled.

therefore independent. Furthermore, it indicates that the insertion sites are randomly distributed around the genome. None of the isolates contained more than one hybridizing DNA fragment, demonstrating that each Kan^r colony contains a single insertion. This is important as it allows any phenotype to be attributable directly to a single insertion.

To demonstrate that the transposon insertions can be used to isolate mutants, a screen for auxotrophic mutants was performed. Small aliquots of broth cultures were screened for their ability to grow on rich or minimal medium (Figure 3). Out of 400 insertion mutants screened seven failed to grow on minimal media suggesting a deficiency in amino acid biosynthesis. In addition, two mutants could not grow on rich media. This latter class of mutants was unexpected and interesting, and may reflect an inability to grow at the faster growth rate on rich media. For example, *polA* mutants of *Escherichia coli* are unable to grow on rich medium.¹⁶ Importantly, they highlight the unexpected benefits of a random mutagenesis.

This work describes the application of a new transposon system to mycobacteria. There are several features of this system that make it extremely attractive to the mycobacteriologist and also to those working on other bacterial systems. The first is simplicity: insertions are directly selected after electroporation and all insertion events are independent. The system does not require the initial establishment of conditionally replicating vectors. Two temperature-sensitive transposon delivery vectors have been described for mycobacteria that are derived from the phage, TM4¹⁷ and the plasmid, pAL500.¹⁸ Unfortunately, a distinct disadvantage with the plasmid system is that many of the insertions are siblings (Takacs and Derbyshire, unpublished results). This is presumably because of the extended period of time required to establish the transposon delivery plasmid in the host (5-6 days for colony formation on plates at 30°C). Any cell in which transposition takes place at an early stage of growth will continue to divide and form siblings.

The second advantage of the EZ::TN Transposome system is that the transposon is not native to mycobacteria. This allows for easy detection, by Southern analysis or PCR, and ensures that the insertion is stable, as it will not be activated by trans-acting transposases. The two transposon systems most commonly used in mycobacteria are native elements, IS1096 and Tn611, although more recently the eukaryotic transposon *Himar 1* has been used in *M. smegmatis*.¹⁹ IS1096 is found in many copies in most of the common laboratory strains of *M. smegmatis*. Thus it is possible for transposon insertions to be trans-activated. However, in our experience a bigger problem is that integration of the marked IS1096 can occur by both transposition and homologous recombination into existing elements.²⁰ Clearly, these latter recombinants will not result in insertion mutants. Tn611 transposes by a replicative mechanism and thus insertion results in integration of both the transposon and vector into the target genome.¹⁸ This is undesirable as it complicates analyses and leads to unstable insertions.

When using the EZ::TN Transposome system in *M. smegmatis* only a limited number of transformants were obtained in each experiment ($1-5 \times 10^2$), which is mainly due to the low efficiency of electroporation of mycobacteria. Thus it would be necessary to carry out multiple electroporations to ensure saturation of a genome. However, given the simplicity of the experiment this need not be construed as a disadvantage, and in fact ensures multiple independent mutant populations.

Note: The EZ::TN <KAN-1>Tnp Transposome used in this study has been replaced by the smaller, but functionally equivalent EZ::TN <KAN-2>Tnp Transposome.

Acknowledgements

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References

1. Hatfull, G.F. (1993) *Trends Microbiol.* **1**, 310.
2. Hatfull, G.F. and Jacobs, W.R.J. (1994) *Mycobacteriophages: Cornerstones of Mycobacterial Research*, American Society for Microbiology, Washington, D.C., 165.

continued

3. Parsons, L.M. *et al.* (1998) *Mol. Microbiol.* **28**, 571.
4. Goryshin, I.Y. *et al.* (2000) *Nature Biotechnol.* **18**, 97.
5. Berg, C.E. and Berg, D.E. (1996) in: *Escherichia coli and Salmonella: Cellular and Molecular Biology*, eds. Neidhardt, F.C. *et al.*, American Society for Microbiology, Washington, D.C., 2588.
6. Goryshin, I.Y. and Reznikoff, W.S. (1998) *J. Biol. Chem.* **273**, 7367.
7. Akerley, B.J. *et al.* (1998) *Proc. Natl. Acad. Sci. USA* **95**, 8927.
8. Griffin, T.J. *et al.* (1999) *Nucl. Acids Res.* **27**, 3859.
9. Gwinn, M.L. *et al.* (1997) *J. Bacteriol.* **179**, 7315.
10. Haapa, S. *et al.* (1999) *Nucl. Acids Res.* **27**, 2777.
11. Haniford, D.B. *et al.* (1991) *Cell* **64**, 171.
12. Chaconas, G. *et al.* (1996) *Curr. Biol.* **6**, 817.
13. Snapper, S.B. *et al.* (1990) *Mol. Microbiol.* **4**, 1911.
14. Pavelka, M.S. and Jacobs, W.R. (1996) *J. Bacteriol.* **178**, 6496.
15. Jacobs, W.R., Jr. *et al.* (1991) *Methods Enzymol.* **204**, 537.
16. Joyce, C.M. and Grindley, N.D.F. (1984) *J. Bacteriol.* **158**, 636.
17. Bardarov, S. *et al.* (1997) *Proc. Natl. Acad. Sci. USA* **94**, 10961.
18. Guilhot, C. *et al.* (1994) *J. Bacteriol.* **176**, 535.
19. Rubin, E.J. *et al.* (1999) *Proc. Natl. Acad. Sci. USA* **96**, 1645.
20. Machowski, E.E. *et al.* (2000), submitted to *Gene*.

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Figure. Sequencing through a GC-rich trinucleotide repeat. Supercoiled plasmid template containing (CGG)₂₃ was sequenced using the SequiTherm EXCEL II isothermal sequencing protocol.

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