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Scaling down: A PCR-based method to efficiently screen for desired knockouts in a high density *Mycobacterium tuberculosis* picked mutant library

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Summary Transposon mutagenesis produces random mycobacterial mutants at high frequency. Because they are random, however, it is difficult to isolate mutations in particular target genes. Here we describe the use of an arrayed library of *Mycobacterium tuberculosis* together with a PCR screening strategy to rapidly identify strains with defined insertion mutations. This method is useful for many genetic applications.
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Introduction

The ability to perform allelic replacement in *Mycobacterium tuberculosis* has been the single most important technical breakthrough in mycobacterial genetics. This has opened up an entire field of study, rapidly leading to a much more complete understanding of the biology of this important pathogen. Either of two general techniques (plasmid-mediated recombination or phage transduction) has been used by a variety of investigators to create knockout strains.[1–3] Allelic replacement is clearly the gold standard for performing genetic studies in *M. tuberculosis*.

However, this method had drawbacks. Some genes have essential functions and attempts at allelic replacement are difficult or impossible. While other genes can be knocked out, specific constructs can have unexpected effects such as creating toxic fusions or affecting downstream transcription. Most importantly, because of the slow growth of *M. tuberculosis*, it takes quite a long time to obtain any specific mutant.

Transposon mutagenesis is an efficient method for producing mutations in mycobacteria. Using a hyperactive transposase mutant and delivering DNA by phage transduction results in very large libraries of random insertion mutants.[4] Because

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the transposon insertion tags the mutated gene, it is fairly simple to isolate and identify each insertion site. These can be amplified and sequenced using a variety of methods.[5–7] However, sequencing a very large library is expensive and quite labor intensive. In particular, it is difficult to grow mycobacteria in large enough volumes and prepare an amount of DNA appropriate for subsequent steps. Because the chances of finding a mutant of interest increase with the number of mutants sequenced, it may require enormous amounts of sequencing to find specific insertions.

Here we describe an alternative strategy for identifying insertion mutations in targeted genes. We use a pooling and amplification strategy that does not require large volumes and can easily be performed rapidly and reproducibly.

Materials and methods

Strains and media

M. tuberculosis strain H37Rv was grown in Middlebrook 7H9 broth supplemented with 0.05% Tween 80 and Middlebrook OADC or Difco Middlebrook 7H10 agar. All procedures prior to heat inactivation of bacteria were performed in a BSL-3 laboratory.

Construction of arrayed *Mycobacterium tuberculosis* H37Rv transposon library

Using previously described methods by Sassetti et al.[4] a high density *M. tuberculosis* transposon library was utilized. The library was plated on 7H10 agar containing 20 μg/ml kanamycin. The library was plated at a low density. Single colonies of varying size were picked into 96 deep well plates containing 1.2 ml of 7H9 containing kanamycin and cycloheximide. Sixty 96 deep well plates were picked to allow for over two-fold coverage of the *M. tuberculosis* genome. Plates were incubated standing undisturbed at 37 °C for 6 weeks. At 6 weeks the deep well plates were removed from incubation, replicated, and 50 μl aliquots were added to 96 shallow well plates for heat inactivation. Original cultured plates were stored at −80 °C. Replicated plates were incubated standing at 37 °C indefinitely. Fifty microliters of aliquots were heat inactivated by baking plates in a dry oven at 80 °C for 2 h and removed from the BSL3 facility.

DNA amplification

PCR was performed using one primer specific for the gene of interest and a second primer (5′-gctctacgtgggagtcggacaatgttg-3′) that hybridized to the transposon. One microliter of template was used in a total volume of 20 μl with Taq polymerase (TaKaRa) using the buffer provided by the manufacturer. For amplification we used the following conditions: 95 °C for 5 min followed by 30 cycles of 95 °C × 30 s, 65 °C × 30 s, 70 °C for 2 min; and 70 °C for 10 min. Ten microliters of each sample was analyzed by agarose gel electrophoresis.

Results

Library preparation

We used a Himar1-derived minitransposon[8] that can be introduced by transduction using the temperature-sensitive phage ϕAE87.[9] After transduction, we froze the transduced bacteria at −80 °C, then plated aliquots of the frozen cells to determine the titer of transposon mutants. The library was found to contain ~100,000 independent mutants. We used this value to determine the amount of material to plate to obtain well-separated colonies and, thereby, to minimize the amount of contamination at the subsequent picking step.

Colony picking and inactivation

Well-isolated colonies were picked using sterile toothpicks into 96 deep well plates. At the end of a 6-week incubation, a visible pellet could be seen at the bottom of almost every well. For simplicity of analysis, all wells were included whether or not there was detectable growth.

To remove bacteria from the BSL-3 laboratory, cultures were heat inactivated. We determined that this heating step was adequate to kill a high titer culture. This process results in cell death and some lysis, producing relatively viscous samples. Subsequent steps were performed by pipetting this suspension using the indicated volumes.

Construction of pools

Pools for PCR screening were constructed as indicated in Fig. 1. Each well was represented three times—in a whole plate pool and pools consisting of each well in each row and each column. Pools were maintained in 96 well plates for ease of further manipulation.

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