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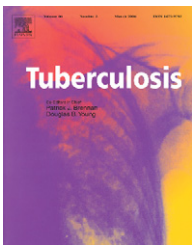

# Rapid recombination screening to test gene essentiality demonstrates that *pyrH* is essential in *Mycobacterium tuberculosis*

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**Summary**
The availability of the complete genome of *Mycobacterium tuberculosis* affords the possibility of screening genes for essentiality under defined conditions. We tested a rapid recombination method for screening and confirmation of gene essentiality which would be more amenable to higher throughput applications. Non-replicating vectors carrying the internal portion of a gene were used as recombination substrates. Such vectors would lead to inactivation of the target gene in a single recombination step. For non-essential genes, recombinants can be obtained; for essential genes, no recombinants can be obtained, thus providing a rapid screening method to determine essentiality in a targeted manner. The incorporation of a promoter in the vector allowed us to establish the essentiality of a single gene in an operon. We confirmed this method worked with several essential (*proC*, *glnE*, *mtrB*, *trpD*) and one non-essential (*tlyA*) gene. In addition, we used the method to demonstrate that the *pyrH* gene is essential.


## Introduction

*Mycobacterium tuberculosis*, the causative agent of tuberculosis, is responsible for almost 2 million deaths every year. Increasing numbers of co-infections with HIV and multi-drug resistant strains have resulted in an urgent need to develop new therapeutics to combat the disease. An increased understanding of the biology of the bacteria is required in order to develop new anti-mycobacterial agents or vaccines.

The availability of the complete genome sequence of several members of the *M. tuberculosis* complex[1–4] affords the possibility of predicting genes which may be of relevance to pathogenesis or may be novel drug targets. In addition, the use of comparative genomics allows us the opportunity to identify a large number of candidate drug targets. One method of validating drug targets is to demonstrate that the gene is essential for growth or infection.

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Current methods for generating large numbers of mutant strains of Mtb are dependent on random approaches (transposon mutagenesis),[5–7] whilst directed methods (gene knock-outs using homologous recombination) are only appropriate for constructing small numbers of mutant strains.[8–10] Using transposon mutagenesis, libraries of mutants can be generated, but if one wants to investigate a particular group of genes, there is no guarantee that the mutants required will be found amongst those generated. In addition, although the absence of a particular mutant from a library raises the possibility that a gene is essential, more formal proof is still required, as a percentage of genes identified are likely to be "false positives".

Construction of defined mutants is now a routine task, but due to the slow growth rate of *M. tuberculosis*, it can take several months and again failure to generate mutants must be followed up by a formal proof of essentiality.[11–13] The classical method for proving that a gene is essential is to demonstrate that the chromosomal copy can only be inactivated or deleted when a functional copy of the gene is provided elsewhere, for example on a replicating[14] or integrating plasmid.[12] We have used this method to demonstrate that both *glnE* and *aroK* are essential in *M. tuberculosis*, but this approach takes 6–9 months.[12,13]

Our study aimed to develop a more rapid method for screening and confirmation of gene essentiality which would be more amenable to higher throughput applications.

## Materials and methods

### Construction of vectors

The hygromycin resistance gene (*hyg*) from pAGAN40[15] was cloned as a *Pst*I–*Not*I fragment into p2NIL[9] to generate p4NIL. The Ag85a promoter from pAPA3 was cloned as an *Eco*RI fragment into p4NIL to generate p5NIL. The primers indicated in Table 1 were used to amplify either the internal segment of a gene or the full gene or operon. Primers incorporated *Pac*I sites and all products were cloned into p4NIL or p5NIL as *Pac*I fragments (Table 1). All inserts were fully sequenced to confirm no mutations had been introduced by PCR.

### Transformation of *M. tuberculosis*

*M. tuberculosis* was cultured using Middlebrook 7H9 liquid medium plus 10% (v/v) OADC (oleic–albumin–dextrose–catalase) supplement (Becton Dickinson) and 0.05% (w/v) Tween 80 or on Middlebrook 7H10 solid medium plus 10% (v/v) OADC. Electroporations were conducted using 1–5 μg of UV-treated DNA[15] and transformants were selected on solid medium using 100 μg/mL hygromycin and 20 μg/mL kanamycin.

### Analysis of recombinants

Transformants were subcultured using solid or liquid medium for DNA preparation. Cells were disrupted in 750 μL of TE buffer (Sigma) in Lysing Matrix B tubes (QBiogene) using the FastPrep machine (QBiogene) on speed 4 for 20 s. Cell debris was removed by centrifugation and the supernatant was filtered through a 0.2 μm filter to remove any intact, viable cells. The supernatant was treated with 0.2 vol of 5% (w/v) sodium deoxycholate at 56 °C for at least 90 min. Proteins were removed by phenol:chloroform extraction and the DNA was precipitated with 3 vol Na acetate pH 4.6, 2.5 vol EtOH at −70 °C for 1 h. Southern transfer was carried out using a vacuum blotter. Hybridization probes were labelled and detected using the AlkPhos Direct system (Amersham).

## Results and discussion

### Essentiality testing

We wanted to develop a method which would allow the construction of larger numbers of defined mutants than currently possible, quickly and at the same time identify those that are essential. Such a increased throughput approach would allow us to quickly determine which of the approximately 4000 genes of *M. tuberculosis* are worthy of further study as drug targets.

Our rationale was to use a homologous recombination method which would allow us to determine in a single step whether a gene was essential or not, based on the number of transformants (recombinants) obtained after electroporation. The method relies on homologous recombination between an introduced plasmid carrying only the internal region of the gene and the chromosomal copy (Fig. 1A). A single recombination event will lead to a single cross-over strain in which the whole plasmid is integrated and flanked by two partial copies of the gene (Fig. 1A). Neither of these copies represents a functional gene and so the strain generated will be a mutant. For essential genes, no transformants will be obtained.

We tested this approach with two genes which are essential under normal culture conditions (in the absence of supplementation), *proC* and *glnE*.[12,16] Non-replicating (suicide) vectors carrying the internal portion of either gene were generated (Table 1). Two resistance genes (*hyg* and *kan*) were included to overcome the problem of spontaneous antibiotic resistance, which occurs at a high enough frequency to obscure homologous recombination when using a single marker.[16] Suicide vectors were used to transform *M. tuberculosis*. No transformants were obtained for *proC* (pWHOT14) in multiple transformations (Table 2). Two transformants were obtained for *glnE* (pWHOT16).

The genotype of the two transformants was analysed using Southern hybridization (Fig. 2C). If the vector had integrated via homologous recombination, two bands should be seen; a 4.3 kb band derived wholly from the vector and a 3.6 kb band derived from integration into the *glnE* locus (Fig. 2A). Both transformants had different banding patterns (Fig. 2C), neither of which was correct for homologous recombination, confirming that the vector had not integrated into the *glnE* locus, but had presumably undergone non-homologous or illegitimate recombination as previously reported in the *M. tuberculosis* complex.[17,18] Since no homologous recombinants were obtained for either gene, the prediction is that these genes are essential. In order to confirm this, we determined if recombination could be obtained using control vectors.