Mycobacteria: Bugs and Bugbears (Two steps forward and one step back)

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Abstract

The use of molecular techniques to study the mycobacteria has advanced greatly since the first genomic libraries of *Mycobacterium tuberculosis* and *M. leprae* were constructed in 1985. However, there are still pitfalls for the unwary. Most of the problems associated with the use of molecular techniques to study mycobacteria can be related to one of the following problems: slow growth rate causing problems with contamination; the formation of macroscopic clumps when grown in culture; resistance to standard chemical lysis procedures; the requirement for containment facilities for pathogenic species; the lack of suitable genetic vectors; and the problems of spontaneous antibiotic resistance. Despite these problems, considerable progress has been made and standard techniques have been developed for the preparation of protein, nucleic acids (DNA and RNA) and cell wall components, chemical and transposon mutagenesis and gene replacement methods, the use of reporter genes and expression vectors, and improved detection and drug sensitivity testing.

Index Entries: *Mycobacterium tuberculosis*; *Mycobacterium smegmatis;* mycobacteria; molecular biology; mycobacteriophages; plasmids; mutagenesis; transposons; recombination; expression.

1. Introduction

Mycobacteria are Gram-positive, rod-shaped bacteria of the Actinomycete family, and are therefore most closely related to the nocardia, corynebacteria, and *Streptomyces*. Their most characteristic feature is their complex cell envelope, containing a high percentage of lipids, which include the mycolic acids. This envelope makes the bacteria resistant to breakage and relatively impermeable to antibiotics, and is responsible for the acid-fast staining property used to identify the organisms. The genomic DNA contains a high guanine plus cytosine (GC) content, ranging from 58 to 69% ***(1)***, which affects the utility of *Escherichia coli* as a surrogate genetic host.

Mycobacteria can infect most species of animals including rodents, birds, and fish. However, their importance lies in the fact that they include major human pathogens. Tuberculosis, caused by *Mycobacterium tuberculosis*, remains the most important infectious cause of mortality in the world, and leprosy, caused by *M. leprae*, still afflicts large numbers of people. Other species can be pathogenic, of which perhaps the most concerning is *M. avium*, which has recently become apparent as a major opportunist in HIV-infected people in the developed world.

Mycobacteria fall naturally and taxonomically into two main groups: slow- and fast-growers. The slow-growers include most of the major human and animal pathogens, whereas the fast-growers include nonpathogenic species. One of these, *M. smegmatis*, is widely used as a convenient, if imperfect, model organism.

2. Molecular Biology: A Brief History

The use of recombinant DNA methods to study the molecular biology of the mycobacteria began

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in 1985, with the generation of genomic DNA libraries from *M. tuberculosis* and *M. leprae* **(2–5)**. These were initially used to identify genes encoding antigenic proteins by screening with serum or monoclonal antibodies in *E. coli*. *E. coli* is a Gram-negative organism with an average GC content of 50%, and it was soon realized that the majority of mycobacterial proteins were not expressed from their own promoters in this cloning host. Greater success was therefore obtained with expression libraries **(6)**.

The use of mycobacteria as hosts for recombinant molecules was made possible by the development of cloning vectors based on mycobacteriophages and mycobacterial plasmids. The identification of suitable antibiotic-resistance markers (initially resistance to kanamycin), and the success of electroporation to introduce such vectors into the cell, has led to the widespread use of nonpathogenic mycobacteria as cloning hosts. The most popular has been *M. smegmatis*, because it is a fast-growing, nonpathogenic organism. Paradoxically, electroporation proved immediately possible with *M. bovis* BCG, whereas it was necessary to isolate an electroporation-competent mutant of *M. smegmatis* [the most widely-used being strain mc^2155 **(7)**]. Other mycobacteria have also been used as cloning hosts, such as *M. vaccae* **(8,9)**. Most cloning vectors are still based on pAL5000, a plasmid isolated from *M. fortuitum* **(10)**, although other replicons and integrating vectors have been developed **(11–19)**.

One of the main problems of the vectors for use in mycobacteria is the low copy number (approx 2–5 copies per cell). This, coupled with the difficulty of lysing mycobacteria, explains why most primary cloning steps are carried out in *E. coli* using shuttle vectors, which carry replication origins for both *E. coli* and mycobacteria.

A set of vectors is available for mycobacteria that can be transferred directly from *E. coli* by conjugation **(20)**. This avoids the use of electroporation, which always carries the risk of creating aerosols by arcing. In addition, the use of phage-based vector systems is gaining popularity because the efficiency of transformation is much higher than can be achieved for plasmid-based vectors **(21)**.

The next obstacle to serious genetic analysis was the production of mutants. Work with chemical mutagenesis has been carried out in several species and efficient transposon mutagenesis has now been achieved in *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis*. The recent success with methods for obtaining gene replacement has enabled the construction of defined mutants **(22–33)**.

3. Molecular Techniques: General Problems

Most of the problems associated with the use of molecular techniques to study mycobacteria can be related to one of the following problems.

3.1. Slow Growth Rate

The slow-growers can take up to 6 wk to form colonies on a plate, and even the fast-growers may take up to 2 wk. This can lead to problems with contamination of cultures, especially with fungi. Cycloheximide can be used to partly overcome the problem of fungal contamination. Most commonly used media have been developed for the isolation of mycobacteria from clinical samples; although they contain partially selective agents, such as malachite green, many require supplementation with nonautoclavable constituents, compounding the problem of contamination. Other media that do not require supplementation have been successfully used for the fast-growers. Media must be selected carefully, depending on the technique. The long incubation time can also lead to problems with plates drying out, and care must be taken to ensure that plates are well-wrapped in Parafilm or clingfilm.

3.2. Clumping

Owing to the nature of the cell wall, mycobacterial cells tend to stick together when grown in liquid, and form macroscopic clumps even when grown with shaking. Some species are more prone to this than others. The effect of aeration on cultures can dramatically alter the amount of clumping and this varies with species. Clumping leads to problems because many standard techniques require dispersed cultures, e.g., optical density

measurement, or ideally, single cells, e.g., for plating, screening for mutants, and infection of tissue culture cells. The problem can be alleviated by the addition of detergents; Tween 80 is the most commonly used. However, this may be only partially effective, and may not be possible where it is important not to alter the outermost layer of the cell wall, or where detergent-sensitive procedures, such as phage infection, are being used. Sonication can be used to break up clumps of mycobacteria, but for pathogenic species this must be carried out with a cuphorn sonicator (inside an appropriate safety cabinet) rather than with a probe, in order to contain the aerosols generated. Passing cell suspensions through a 23-gauge needle several times may also break up clumps, but this is unacceptable for pathogenic species owing to the risk of needle-stick injuries and infection.

3.3. Resistance to Lysis

Mycobacteria are particularly resistant to normal methods of chemical lysis owing to the nature of the cell wall. In addition, the wall contains large quantities of polysaccharides, which can contaminate subsequent preparations of nucleic acids.

3.4. Safety Aspect

One of the most important problems when working with pathogenic mycobacteria is the need for containment. Any procedure that involves the generation of aerosols is potentially dangerous and should be minimized. Thus, the use of non-pathogenic species as model systems for genetic studies is of great importance and convenience. The relative lack of established, disabled host-vector systems means that relatively straightforward recombinant DNA experiments using nonpathogenic hosts will have to be carried out in containment facilities. The recent construction of *recA* mutants of *M. smegmatis* goes some way toward solving the problem ***(34,35)***, but further attenuated strains are required.

3.5. Spontaneous Antibiotic Resistance

As mentioned, mycobacteria can be broadly divided into the slow- and fast-growing species. Most slow-growers possess only one ribosomal RNA (rDNA) operon; this unusual situation means that resistance to agents such as kanamycin can easily be acquired by mutation in the rDNA operon itself ***(36)***, which is not likely to occur when there is more than one operon. Therefore, care must be taken when working with such antibiotics to include appropriate controls when attempting to introduce plasmids, and transformants should always be checked for the presence of the desired construct. Most of the fast-growers contain two rDNA operons, and therefore have a much lower rate of spontaneous resistance to kanamycin.

4. Fractionation

As mentioned, mycobacteria are difficult organisms to lyse, mainly owing to the strength of the cell wall, and methods to isolate subcellular fractions have been developed to cope with this problem. The addition of glycine to growing cells can be used in order to weaken the cell wall before attempting lysis. Intact genomic DNA can be isolated relatively easily, and many methods—chemical, enzymatic, and mechanical—exist to achieve this ***(37)***. RNA isolation poses more problems, because mRNA is very unstable with an extremely short half-life; therefore, lysis must be rapid in order to prevent degradation, and methods, generally mechanical, have been developed for this ***(38,39)***. The successful isolation of intact mRNA from mycobacteria has allowed much more scope with respect to the study of gene regulation and analyses of transcription start sites and transcriptional control of genes are now readily undertaken. Techniques such as differential display, RNA arbitrarily primed polymerase chain reaction (RAP-PCR), and reverse transcription (RT)-PCR are also readily achieved. Such studies are now being used to dissect out genes that are up-regulated in vivo and to examine the effect of phagocytosis on gene expression, both important topics in virulence ***(40,41)***. The ability to prepare good quality mRNA from mycobacteria should also allow the use of ordered DNA microarrays in order to look at genome-wide gene expression under different conditions.

The preparation of protein fractions from mycobacteria is relatively straightforward. Secreted proteins can be recovered simply from the medium

supernatant and subsequently concentrated. For cell wall–associated or cytoplasmic proteins, the cells are generally broken open using mechanical methods and then centrifuged to separate the subcellular fractions. Methods for the isolation and characterization of nonprotein cell wall components such as mycolic acids and lipoarabinomannan are well developed ***(42)***.

5. Genetic Tools

5.1. Vectors

There is a general paucity of mycobacterial genetic tools. As mentioned earlier, the majority of plasmid vectors for use in mycobacteria have all been developed from one plasmid pAL5000 ***(10)***. Replicons derived from other natural plasmids have been used to a lesser extent. An additional problem is that these plasmids do not necessarily function in all mycobacterial species; for example, pAL5000-derived vectors function in most mycobacterial species including *M. smegmatis*, but have been unable to transform *M. intracellulare* ***(28)***.

One advantage of the wide use of the pAL5000 replicon, is that plasmid replication has been studied in some detail and has allowed the development of two different conditionally replicating vector systems: one based on a temperature-sensitive (*ts*) origin and the other based on incompatibility ***(43,44)***.

Plasmid and cosmid isolation have also presented problems, owing to the low copy number and the poor quality of the DNA recovered. Therefore, most plasmid analyses have been conducted after transfer of plasmid DNA isolated from mycobacteria to *E. coli* for analysis. Plasmid and cosmid DNA can be successfully isolated from mycobacteria, but this requires an adaptation to the standard lysis methods and will always be limited by the low copy number ***(45)***. Attempts to increase the copy number of pAL5000 by site-directed mutagenesis of the origin of replication have so far been unsuccessful ***(46)***.

Problems can also be encountered with the stability of certain replicating plasmids; some plasmids undergo spontaneous rearrangements and/or deletions in mycobacteria and recently transposition has been shown to occur into the plasmid itself ***(47,48)***. Therefore the plasmid structure must always be confirmed after transformation and carefully monitored.

5.2. Reporter Genes

Several reporter genes have been successfully used in mycobacteria, including those encoding β-galactosidase ***(49–51)***, chloramphenicol acetyl transferase ***(52,53)***, catechol-2,3-dioxygenase ***(54)***, luciferase ***(55,56)***, and green fluorescent protein ***(57)***. These genes have been used in several ways: as marker genes to monitor plasmid stability or presence; as reporter genes in order to assay promoter activity and provide information about gene regulation and relative promoter strengths, or as markers of cell viability.

5.3. Expression Systems

Mycobacteria promoters do not function well in *E. coli* and many do not possess the standard consensus sequences. The situation seems much more similar to *Streptomyces*, where several different classes of promoter occur ***(58)***. Despite progress in the identification of promoter consensus sequence for mycobacteria ***(59,60)***, there is still a lack of well-characterized promoters for the expression of heterologous proteins. The most widely used promoter for expression in mycobacteria is the *M. bovis* BCG *hsp60* promoter, which is constitutively expressed to a high level and can be further induced by heat shock ***(61)***. Targeting signals, such as the α antigen leader-peptide or lipoprotein-attachment signal can be used to direct the protein towards secretion or the cell wall, respectively ***(61)***. The use of inducible promoters, such as that of the acetamidase of *M. smegmatis* has allowed the controlled expression mycobacterial genes, but so far has only been shown to function in the fast-grower *M. smegmatis* ***(62)***. The lack of good regulatable promoters is a problem, as high levels of constitutive transcription are stressful to the cell and can lead to selection of strains in which transcription is weaker. Again, a problem with vector stability and variability in expression levels can occur,

especially when using strong promoters—in such cases expression of heterologous genes may be reduced or absent.

5.4. Mutagenesis

Production of mutants is essential for understanding gene function. Some progress has been made in this field, but the tools available are still limited. Chemical mutagenesis has been used for the isolation of mutants of several mycobacterial species **(63–67)** and has the advantage that specialized genetic vectors are not required, and that mutations, with effects ranging from total gene inactivation to subtle alterations of phenotype, may be isolated. However, it has several disadvantages, including the use of dangerous chemicals, the inability to select cells carrying mutations, the creation of undefined mutants, the possibility of multiple mutations, and the difficulty in locating the defective gene.

Transposon mutagenesis has been developed for use in *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis* using different insertion sequences and delivery systems **(33,68,69)**. The applicability of a particular transposon to a mycobacterial species depends on an effective delivery system, relatively random insertion, and the absence of the insertion sequence from the host strain. In *M. smegmatis*, a *ts* delivery system has been used in order to overcome the low transposition frequency found with nonreplicating vectors. However, the transposon used, Tn*611*, is not ideal because the delivery plasmid is integrated into the genome. As a functioning pAL5000 origin is lethal in the chromosome **(70)**, this makes the mutants unstable below 39°C. This delivery system has also been successfully used (with a different transposon) to create transposon mutant libraries in *M. tuberculosis* **(33)**.

A BCG transposon delivery system has been described using a partially deficient plasmid origin, and a transposon (Tn*5367*) based on the *M. smegmatis* insertion sequence IS*1096*. Another problem is the low frequency of transposition, this has been overcome by using an alternative delivery system based on a conditionally replicating phage. This delivery system is much more efficient and again has resulted in the production of a transposon mutant library for *M. tuberculosis* **(21)**. This transposon cannot be used in *M. smegmatis* because it is present in the chromosome in multiple copies and it can integrate by homologous recombination **(69)**. However, a mini-transposon with less homology may be usable.

5.5. Recombination

The creation of defined gene knockouts using homologous recombination has not proved to be straightforward in mycobacteria. Although it has been achieved with ease in the fast-grower *M. smegmatis*, initial attempts in the slow-grower BCG were unsuccessful. This was partly owing to a reported high frequency of illegitimate recombination that resulted after transformation with a nonreplicating plasmid. This situation was unexpected and is more similar to mammalian systems, where the frequency of illegitimate recombination is much higher than that of homologous recombination. Recently homologous recombination has been more successful, with both single crossovers and targeted gene replacement being achieved **(22–32)**. Several different delivery systems have been used, including the use of negative selection markers, e.g., *rpsL*–/–$^{+}$ (streptomycin sensitivity) **(71)**, *sacB* (sucrose sensitivity) **(23)**, the use of a *ts* delivery vector (in conjunction with *sacB*) **(24)**, and the use of pretreated suicide vector DNA **(22)**. So, after a long wait, it seems that the ability to generate defined *M. tuberculosis* mutants "at will" is now with us.

5.6. Genome Analysis

The study of mycobacterial genes will benefit greatly from projects to sequence the complete genome of *M. tuberculosis*, *M. leprae*, and *M. avium*. The sequence of *M. tuberculosis* H37Rv has been competed **(72)** and is available in sequence databases (accession number AL123456) or from the Sanger Centre (http://www.sanger.ac.uk), who are also completing the *M. leprae* genome sequence. The Institute for Genome Research (http://www.tigr.org/) is sequencing a second strain of *M. tuberculosis* (CSU93) and *M. avium*. The amount of information arising from these

projects is immense. The ready availability of sequence data for all *M. tuberculosis* genes is an exciting prospect and opens up many avenues for future study. The availability of the complete genome sequence opens up new avenues of research, including the possibility of using DNA microarrays to explore genome-wide gene expression ***(73)*** and the ability to identify the complete protein expression pattern (proteomics). The challenge is now to store, present, and analyze the information relating to the pattern of gene expression at the mRNA level (transcriptomics) and protein expression (proteomics) under different conditions.

6. Detection and Diagnosis

The slow growth rate of mycobacteria has meant that traditional techniques for diagnosing infection take a long time. Obviously, culture is a lengthy process, and subsequent drug susceptibility testing is even more time consuming, the advance of molecular techniques has allowed the development of alternative technologies that have the advantages of sensitivity, specificity, and speed. Many PCR tests have been developed and evaluated using clinical samples; not only is PCR rapid, but it can be designed to identify mycobacteria at the species level ***(74,75)***. Other DNA-based tests have also been used.

Epidemiological studies have been greatly aided by the development of restriction fragment length polymorphism typing ***(76)***, most commonly using the insertion element IS*6110* as a target, although other polymorphic elements have also been described. Spoligotyping provides another rapid means of not only identifying the species, but also typing the strain as well ***(77)***. The addition of such techniques such as rDNA sequencing adds to the versatility of speciation techniques ***(78)***. Biochemical techniques can be used in conjunction with molecular techniques to provide a high degree of confidence in assigning species. Drug susceptibility testing has also been improved with the application of such techniques as polymerase chain reaction–single-strand conformation polymorphism (PCR-SSCP) to detect mutations on the genes encoding for drug targets ***(79,80)*** and the use of phage-based systems ***(81,82)***.

7. Conclusions

The basic tools required for molecular analysis of mycobacteria are now available. They are still limited, but at least they provide the necessary foundations for future progress. The completion of the genome sequences of *M. tuberculosis* and *M. leprae* barely 12 yr after the first gene libraries were constructed is an extraordinary feat. The challenge over the next decade will be to improve and utilize the tools available in order to make the best use of the information now in our hands.

In conclusion, mycobacteriologists are both "two steps forward"—with the sequencing of several mycobacterial genomes completed or in progress—and "one step back"—with the limitations imposed by the availability and stability of our vector systems.

Note Added in Proof

Two recent papers show the speed at which new techniques are being employed and improved. Gene replacement techniques are now being used to generate defined mutants more easily and in greater numbers than before (*83*); and the first use of DNA microarrays to investigate the effect of drug treatment on gene expression has been published *(84)*.

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