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Techniques for genetic engineering in mycobacteria

Alternative host strains, DNA-transfer systems and vectors

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Abstract

The study of mycobacterial genetics has experienced quick technical developments in the past ten years, despite a relatively slow start, caused by difficulties in accessing these recalcitrant species. The study of mycobacterial pathogenesis is important in the development of new ways of treating tuberculosis and leprosy, now that the emergence of antibiotic-resistant strains has reduced the effectiveness of current therapies. The tuberculosis vaccine strain *M. bovis* BCG might be used as a vector for multivalent vaccination. Also, non-pathogenic mycobacterial strains have many possible biotechnological applications. After giving a historical overview of methods and techniques, we will discuss recent developments in the search for alternative host strains and DNA transfer systems. Special attention will be given to the development of vectors and techniques for stabilizing foreign DNA in mycobacteria.

Introduction

Mycobacteria are Gram-positive, non-motile, pleomorphic rods, belonging to the family of Actinomycetales. The species in this genus show great diversity in many aspects (Wayne & Kubica, 1986). They are widespread in nature, their habitats ranging from water and soil for the relatively harmless saprophytic species, to the intracellular environment of higher vertebrates for the pathogenic members of the genus. Colony morphology is highly variable, from round and glossy to irregular-shaped and granulous. In some species cell morphology depends on growth rate: long rod-shaped during exponential growth, more coccoid during stationary phase. Some species have specific growth requirements, which in the case of *M. leprae* leads to the impossibility to grow this species outside a living host. An interesting property of some non-pathogenic soil-dwellers is the degradation of recalcitrant organic compounds such as vinyl chloride (Hartmans et al., 1992) or phenanthrene (Waterhouse et al., 1991) and the production of secondary metabolites, such as steroids and optically active oxides of lower alkenes (Martin, 1984; Weijers et al., 1988; Hartmans et al., 1989).

Diverse as they are, the common feature that historically grouped these species together into one genus is the acid-fastness of the cell wall. As a result of the incorporation of waxy materials, the cell wall of *Mycobacterium* is able to retain specific arylmethane dyes even after acidic decolorization (Wayne & Kubica, 1986). This feature among the bacteria is unique to mycobacteria and corynebacteria (Barksdale & Kim, 1977).

Criteria for subdividing the genus are based on growth rate, pigmentation and pathogenicity. The first divides the genus into fast and slow growers, the criterion being the ability to display visible growth from dilute inocula within seven days (Wayne & Kubica, 1986). A correlation between growth rate and the number of ribosomal RNA genes has been postulated, with only one copy of the genes in slow growing mycobacteria, and two in *M. smegmatis* and *M. phlei* (Bercovier et al., 1986). Based on pigmentation,

the group of slow growers is divided into photochromogens, scotochromogens and nonphotochromogens (Runyon, 1970). All pathogenic members of the genus are slow growing species.

Determination of mycobacterial 16s-ribosomal RNA sequences has led to the construction of the phylogenetic tree shown in figure 1., in which all major divisions of the genus, based on growth-rate, pigmentation or pathogenicity, are reflected (Pitulle et al., 1992).

Historically, the genus *Mycobacterium* has gained attention because of its clinical importance. The species forma, *M. leprae*, was described in 1870 as the causative agent of leprosy, and *M. tuberculosis* has been known since 1882. Despite considerable effort to control these diseases since then, leprosy still affects 15 million people, whereas *M. tuberculosis* is responsible for 3 million deaths per year, with one third of the population of the earth infected. Due to the emergence of the AIDS epidemic, infection with *M. tuberculosis* is again on the rise in developed countries (Bloom & Murray, 1992). Infection with bacilli from the *M. avium-M. intracellulare* complex is the most common cause of systemic bacterial infections in HIV-seropositive individuals, with 30% of HIV-deaths caused by *M. avium* infection (Horsburgh, 1991).

The molecular biology of the genus *Mycobacterium* is the subject of extensive research in many laboratories worldwide. The main driving force behind these studies is the need for gaining insight into the mechanisms of pathogenicity (Rastogi & David, 1988). This search focuses on the identification of the pathogenic antigens, elucidation of the mechanisms of drug action and resistance, and the development of multivalent vaccines, employing the vaccine strain *M. bovis* BCG. Also, biotechnological applications of mycobacteria might benefit from knowledge of the genetic systems involved in regulation of replication and gene-expression (Hartmans et al., 1989). The use of recombinant DNA techniques in mycobacterial genetics should be of great help in achieving these goals.

Unlike comparable bacterial systems of pathological or biotechnological interest, the molecular biology of mycobacteria suffers from several great drawbacks. In addition to the above-mentioned impossibility of growing *M. leprae in vitro*, the cultivation of pathogens in general is difficult, requiring special laboratory-accommodations (Jacobs *et al.*, 1991). The search for alternative hosts for the cloning and expression of mycobacterial sequences has therefore great priority. Also, the lack of a good system for the introduction of DNA into mycobacterial cells has hampered the development of mycobacterial genetics. And finally, the search for and construction of vectors for the manipulation of mycobacterial DNA has been an important issue in the last few years. It seemed that many so called wide-host-range vectors, developed for and applicable to non-*Escherichia coli* genetics, do not extend their promiscuity beyond the Gram-negative/positive border (Chater & Hopwood, 1989; Franklin & Spooner, 1989; Hermans et al., 1993).

Starting about ten years ago, the more classical genetic approach to the study of mycobacteriology was gradually superseded by molecular techniques. Mainly through the development of transformation techniques and suitable vectors, recombinant-DNA techniques became applicable to mycobacterial systems. As a result, the study of the molecular mechanisms of pathogenicity and the development of new antigens based on *M. bovis* BCG came within reach.

The genus *Mycobacterium* has been excellently reviewed from a microbiological viewpoint by Hartmans and de Bont (1992; non-medical), Good (1992; medical, mainly *M. tuberculosis*) and Shinnick (1992; *M. leprae*). The topic of transformation of mycobacteria has been reviewed concisely by Hatfull (1993). In the following, we will attempt to summarize recent developments in the search for alternative hosts for cloning mycobacterial DNA, the techniques for transforming mycobacterial cells, and the development of new vectors for mycobacterial genetics.

Alternative hosts for mycobacterial genetic studies

To circumvent the difficulties of growing and manipulating pathogenic bacteria (*in casu M. tuberculosis* or *M. leprae*), alternative or surrogate host-species for mycobacterial genes have attracted much attention. As stated by Hopwood et al. (1988), it will be important to study mycobacterial genes in hosts in which the natural expression signals - especially those governing transcription - have a good chance of being faithfully recognised. This is especially important in *Mycobacterium*, since the high GC-content of its DNA (ranging from 56% to 69%) may imply a use of different transcription and translation signals.

Hopwood et al. (1988) describe the use of *E. coli* as a cloning host. Here, transcription and translation signals from the host have to be present in

recombinant plasmids in order to express mycobacterial genes, which results in fusion-proteins, with N-terminal sequences derived from the *E. coli* vector and C-terminal sequences from the mycobacterial DNA. As these fused proteins are unlikely to show any activity, detection of expression must be done immunologically, with antibodies directed against epitopes from the mycobacterial protein (Thole *et al.*, 1985).

To achieve faithful expression of mycobacterial genes, *Streptomyces lividans* is considered a good candidate, because of its ability to recognise promoters from several other bacterial genera and because of the availability of gene cloning techniques in this organism (Bibb & Cohen, 1982; Hopwood et al., 1985). Results of experiments with *M. bovis* BCG (bacille Calmette Guèrin), *M. tuberculosis* and *M. leprae* indicate that many genes from these species can be expressed in *S. lividans* from their own promoters, making the isolation of genes or gene-sets involved in pathogenicity feasible (Kieser et al., 1986; Lamb & Colston, 1986).

The obvious hosts for manipulating the genes of pathogenic mycobacteria are the non-pathogenic members of the genus. In analogy to other bacterial genera, DNA-transcription and translation are likely to respond to the same signals in all mycobacteria. Therefore the chance of faithfully expressing cloned genes in a homologous organism is very high. In this respect, several species have been investigated, among which *M.bovis* BCG and *M. smegmatis* (Jacobs et al., 1991). One of the main problems encountered when using mycobacterial hosts for mycobacterial genes is the difficulty to grow these organisms in culture. The tuberculosis vaccine strain *M. bovis* BCG, which has been used to vaccinate more individuals than any other live bacterial vaccine, is the sister-species of *M. tuberculosis* (fig.1.) and is therefore a good candidate for the introduction of recombinant mycobacterial genes (Stover et al., 1991). Unfortunately, *M. bovis* is a member of the slow growing group of mycobacteria, and forms colonies on agar plates only after 3 to 6 weeks. Above that, it shows a tendency to grow in clumps, with cells remaining attached to one another after division. Since in most genetic experiments the availability of single, loose cells is a prerequisite, growth media and conditions have been developed to avoid clumping as much as possible (Jacobs et al., 1991).

Also with the fast growing *M. smegmatis*, clumping of cells in culture is a problem, though less than with *M. bovis*. The incorporation of Tween-80 in the growth medium reduces clumping and aids in the preparation of single cell suspensions (Jacobs et al., 1991). Low transformation efficiencies have been overcome by isolating efficient plasmid transformation (*ept*) mutants of *M. smegmatis* (Snapper et al., 1990; see below).

Another candidate for cloning mycobacterial genes is *M. aurum*. Though little recognised, this species has several advantages over other mycobacterial hosts. It is a member of the fast growing group of mycobacteria, which forms glossy, gold-coloured colonies on solid medium after one to two days, requiring only glucose and yeast-extract for its growth. Furthermore, it shows no tendency to form clumps in liquid culture, even in growth medium without Tween-80. Transformation systems for *M. aurum*, based on spheroplast fusion or electroporation have been developed and vectors for this species have been examined (Rastogi *et al.*, 1983; Hermans et al., 1990; Hermans et al., 1991). *M. aurum* is sensitive for most of the drugs used in the treatment of tuberculosis and leprosy (David et al., 1980), so it might be of great help in the study of the genetic basis of drug resistance in *M. tuberculosis* and *M. leprae*. These properties make *M. aurum* an excellent candidate for cloning and expression of genes of other mycobacteria.

Techniques for DNA transfer

One of the main reasons for the slow development of mycobacterial genetics has been the lack of a good laboratory system to introduce DNA into mycobacterial cells. In many papers addressing the problem, this has been attributed to the presence of a massive cell wall, containing glycolipids and other waxy materials or the presence of an efficient *rec*-system (Slosárek et al., 1978; Rastogi et al., 1983). In general, DNA-uptake into bacterial cells can be mediated through conjugation, spheroplast or protoplast fusion, transduction, transfection or transformation, the latter two either direct or by electroporation. Each of these systems has been studied for its suitability for DNA-manipulation in mycobacteria.

Conjugation

The process of conjugation in *Mycobacterium* was extensively reviewed by Greenberg and Woodley (1984). No major contributions to this field have been published since. What appears to be a major mechanism of genetic exchange in *E. coli* and several other bacterial species, was found in the genus *Mycobac-*

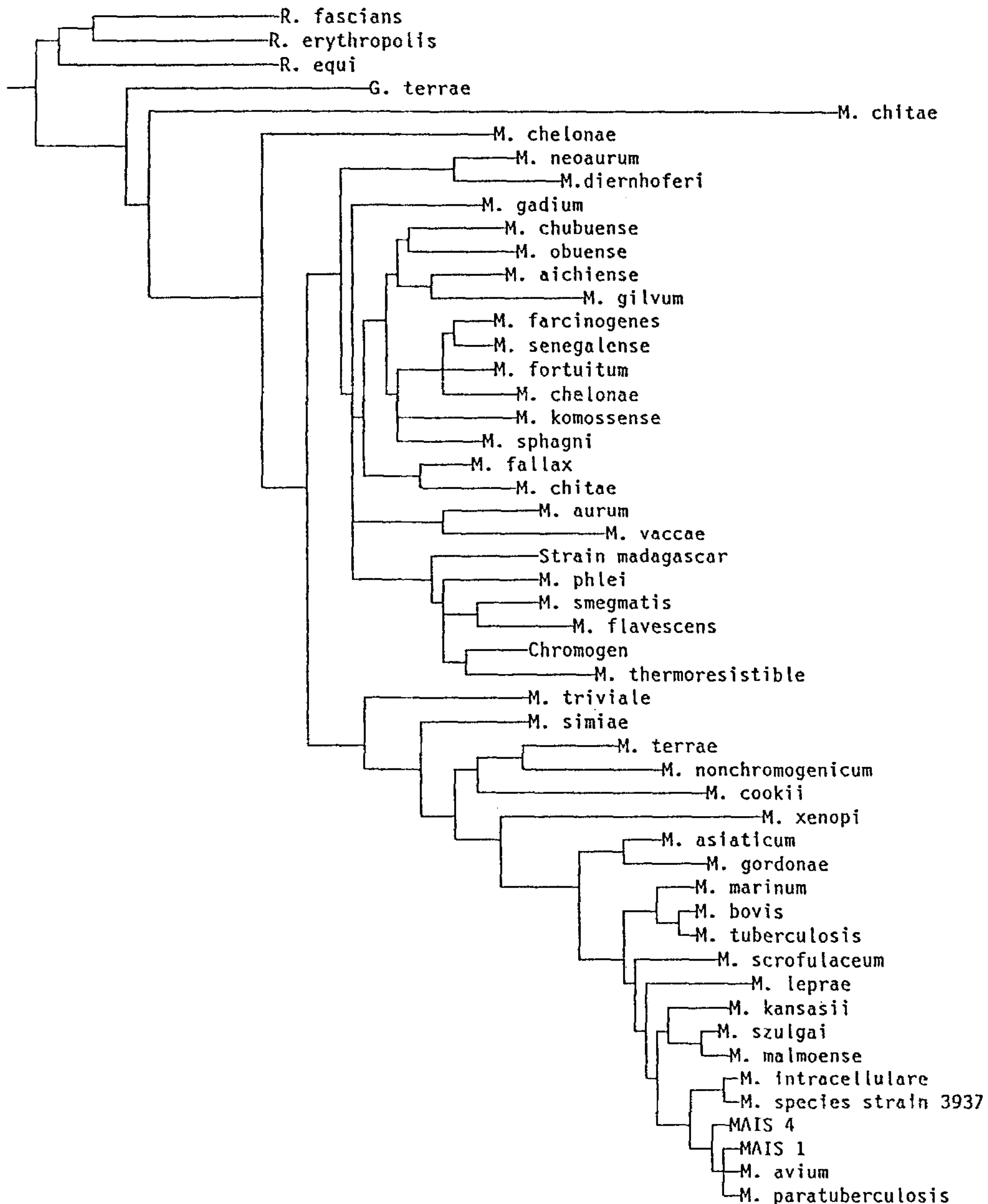

Fig. 1. Phylogenetic tree of the genus *Mycobacterium* based on 16S rRNA sequences. The tree was rooted using three members of the genus *Rhodococcus* and one member of the genus *Gordonae* as outgroups. MAIS: *Mycobacterium avium-intracellulare* group. From Pitulle et al. (1992).

terium only in *M. smegmatis*. The process appeared to be unidirectional between different mating types, but no plasmid-bound sex-factor comparable to the *E. coli* F-plasmid was found.

Recently, two cases of conjugation-driven intergeneric transfer of plasmid DNA between *E. coli* and *M. smegmatis* have been reported. Lazraq et al. (1990) describe the construction of shuttle plasmid pMY10 with replication-origins from pBR322 (*E. coli*) and pAL5000 (*M. fortuitum*) and the origin of transfer from wide-host-range plasmid pRK2. This plasmid can be transferred from *E. coli* to *M. smegmatis* by conjugation. The wide-host-range *IncQ* plasmid RSF1010 (Frey & Bagdasarian, 1989) was transferred by conjugation between *E. coli* and *M. smegmatis* (Gormley & Davies, 1991). Besides opening up possibilities for the

development of transfer systems between these genera, these observations indicate that horizontal gene-transfer may occur on a much wider scale than previously thought.

Spheroplast and protoplast fusion

The fusion of whole cells with degraded cell walls is another way to achieve recombination of genetic material from different strains or species. After bringing these spheroplasts or protoplasts into close contact, the cell membranes can be fused by the addition of polyethylene glycol.

Obviously, the degradation of the cell wall is a crucial step in the procedure. Since the mycobacterial cell wall contains many lipids and polysaccharides (Rastogi et al., 1986), it is resistant to the action of lysozyme. Therefore, its degradation was possible only after sensitization of the cell wall for lysozyme. Besides using lysozyme-sensitive mutants, which for obvious reasons is not very practical, in most reports this is done by incorporating high concentrations of one or more amino acids, mostly glycine, in the growth medium. After harvesting, the administration of lysozyme renders cells with degraded cell walls (Sato et al., 1965; Rastogi & Venkitasubramanian, 1979; Rastogi & David, 1981; Udou et al., 1983). Sadhu and Gopinathan (1982) report that lipase is much more effective than lysozyme in the production of *M. smegmatis* spheroplasts, even with untreated cells. Spheroplast formation of *M. aurum* was optimal using lysozyme on glycine-sensitized cells, and addition of lipase had no effect on spheroplast yield (unpublished observations).

The final critical step in spheroplast fusion is the regeneration of the vulnerable wall-deficient cells. This is usually done on agar plates with iso-osmotic growth medium and takes about 7 days in the case of *M. aurum* (Rastogi et al., 1983). By electron microscopic observation, Udou and co-workers observe complete reversal to the bacillary form of *M. smegmatis* spheroplasts after 64 hours on rich, iso-osmotic growth medium, either on agar plates or in liquid culture (Udou et al., 1982; 1983).

These reports found practical application in recombination studies using spheroplast fusion. The fusion of spheroplasts derived from two carotenoid pigment mutants of *M. aurum* is described by Rastogi et al. (1983). Complemented recombinants appeared at a frequency of 2.5×10^{-3} under optimal conditions, and incidentally even partial diploids occurred.

Transduction

Using bacteriophages as vectors, DNA can be transferred from one bacterium to another in a process called transduction. A temperate bacteriophage can, after excision from the bacterial chromosome, inadvertently take up a portion of the host's genome into its own genome, and transfer it along with its own DNA to a new host. Then, the phage's DNA may be inserted into the chromosome of the bacterium and be expressed.

In *Mycobacterium*, reports of transduction are scarce. Mycobacteriophage I3 has been used in *M. smegmatis* to transduce auxotrophic markers (Sundar Raj and Ramakrishnan, 1970) and resistance to streptomycin and isoniazid (Saroja & Gopinathan, 1973). Jones and co-workers described the occurrence of an R-plasmid in *M. smegmatis* strain 607, coding for an unstable, inducible resistance to streptomycin. This plasmid is transducible by the lytic mycobacteriophage D29 to other *M. smegmatis* strains (Jones & David, 1972) and to *M. tuberculosis* (Jones et al., 1974). Apparently phage D29 is unable to transduce chromosomal markers. In the view of interspecific drug-resistance transfer, these papers are very important, but the observations have neither been confirmed nor evaluated (Greenberg & Woodley, 1984).

Investigations into the process of transduction have not been continued, probably due to the inefficiency of the technique, while other more reliable methods for DNA-transfer became available. Nevertheless, these early investigations have stressed the importance of mycobacteriophages in mycobacterial genetics, and have resulted in the isolation and description of many phages (Mizuguchi, 1984).

Recently, Anes et al. (1992) revive transduction techniques in a paper in which they describe the lysogenization of *M. smegmatis* with a modified derivative of mycobacteriophage Ms6. They were able to insert the *aph*-gene, inferring kanamycin resistance, stably into the genome of *M. smegmatis*. This technique might open up possibilities to construct new vaccines by introducing new antigenic properties into the tuberculosis vaccine strain BCG, and stably maintaining them there.

Transformation and transfection

The introduction of 'naked' DNA into a bacterial cell is termed transformation or, if the DNA is derived from a bacteriophage, transfection. The first reports on trans-

formation of mycobacteria describe the acquisition of streptomycin- and isoniazid resistance of susceptible *M. avium* strains after close contact with total genomic DNA isolated from resistant strains (Katunuma & Nakasato, 1954; Tsukamura et al., 1960). Especially in these early years of transformation, many failures were reported as well (e.g. Bloch et al., 1959). When Tokunaga and Nakamura (1968) had shown that competent mycobacteria readily take up DNA from their environment, the failures were attributed to some later stage of the transformation process, probably the recombination of the transferred genes with the recipient's DNA. Therefore, Norgard and Imaeda (1978) used UV-resistant strains of *M. smegmatis* that were supposed to have a more effective DNA-repair and recombination system. By transforming methionine- and leucine-auxotrophic and streptomycin-sensitive strains of *M. smegmatis* to prototrophy and resistance, they determined requirements of the transformation process, such as the presence of Ca^{2+}-ions and the need for a 'recovery'-period on rich medium after the transformation.

Detection of successful transformation with total genomic DNA depends on the expression of the transformed DNA, so on its integration into the recipient's genome. Successful transfection on the other hand is apparent from the lysis of the infected bacteria, and there is no need for the introduced DNA to integrate into the recipient's genome. This should simplify the study of uptake of DNA considerably. Indeed, Sadashiva Karnik and Gopinathan (1983) use transfection to investigate the effect of several parameters on artificially induced competence for phage I3 DNA in *M. smegmatis*. From their study, the cell wall appears to be the major barrier for the introduction of DNA into the intact mycobacterial cell. Jacobs et al. (1987) use transfection of spheroplasted *M. smegmatis* to construct a shuttle phasmid (see below). Enhancement of transfection efficiencies was studied by Naser et al. (1993), who reported that the efficiency of transfection of *M. smegmatis* spheroplasts improves tenfold when performed at 5°C compared to transfection at room temperature. Recent reports of mycobacterial transformation with plasmid DNA that do not employ the technique of electroporation are very rare.

Developed for the introduction of DNA into mammalian and plant cells (Langridge et al., 1987), electroporation or electrotransformation was soon discovered by microbiologists. It facilitated the introduction of DNA into many different bacterial genera, Gram-negative (Wirth et al., 1989) as well as Gram-positive (Luchansky et al., 1988; Dunny et al., 1991), many of which were previously considered untransformable.

Since transformation was the bottleneck in many studies on mycobacterial genetics, attempts were made to apply the technique of electroporation to these systems as well. Initially, electroporation of *M. smegmatis* with plasmid DNA yielded 1 to 10 transformants per microgram DNA (Snapper et al., 1988), which was equal to the efficiency of classic transformation. By isolating *ept* (efficient plasmid transformation) mutants of *M. smegmatis* wild type strain mc^{2}6, the efficiency of electroporation was enhanced to more than 10^5 transformants per microgram DNA (strain mc^{2}155; Snapper et al., 1990). The authors state that the *ept*-phenotype is probably based on a mutation affecting plasmid replication or maintenance. The mc^{2}155 strain was subsequently used by many researchers in mycobacterial genetics (Ranes et al., 1990; Gormley & Davies, 1991; Hermans et al., 1991).

Having established a plasmid-transformation system for mycobacteria, attempts were made to optimize transformation efficiencies, for *M. smegmatis* mc^{2}155 (Cirillo et al., 1993), as well as for other mycobacterial species such as *M. bovis* BCG (Ranes et al., 1990) and *M. aurum* (Hermans et al., 1990). By treating the cells with wall degrading chemicals like glycine or isoniazid, electroporation efficiency of *M. aurum* was enhanced tenfold ($2.3 \cdot 10^4$ transformants per microgram plasmid DNA; Hermans et al., 1990).

Baulard et al. (1992) developed an electroporation based method for rapid plasmid analysis of mycobacterial transformants, called electroduction. After mixing a colony of transformed *M. bovis* BCG or *M. smegmatis* mc^{2}155 with a cell suspension of *E. coli*, plasmid transfer was effected by submitting the mixture to a single electric pulse, as with electroporation. 10–100 (*M. bovis*) or 10^4 (*M. smegmatis*) *E. coli* transformants were obtained using the pAL5000 derived shuttle plasmid pRR3 (Ranes et al., 1990).

Vectors for mycobacterial genetic studies

Besides a good host-bacterium and an efficient transformation system, an absolute requirement for DNA manipulation is the availability of a vector molecule to shuttle mycobacterial DNA sequences between strains or species. The construction of recombinant DNA molecules involves the joining of extraneous DNA with a vector molecule that is capable of maintaining

itself inside the host of choice; that has the features needed for inserting foreign DNA; and that carries marker genes that allow the selection of recombinant molecules in the host. As mentioned above, *E. coli* or, to a lesser extent, *S. lividans* are often used as alternative hosts. Therefore, the ideal vector should be able to replicate in one of these, or both, for easy construction and manipulation of recombinant molecules, as well as in *Mycobacterium*, for faithful expression of manipulated genes. For ease of cultivation, maintenance of the vector in *Mycobacterium* should be independent of selective pressure. The success of introduction of new antigenic properties into *M. bovis* BCG for the construction of multivalent vaccines depends on their stable maintenance in BCG. Since vaccination with BCG is done with live bacteria, it is important that novel protective antigens are not lost subsequent to vaccination (Stover et al., 1991).

Vectors of mycobacterial origin

In the construction of a suitable vector for mycobacterial genetics, an endogenous plasmid is the obvious point from which to start. Many plasmids have been isolated from mycobacteria. In 9 out of 20 strains from the *M. avium-intracellulare* complex, Crawford and Bates (1979) demonstrated the presence of plasmid DNA. One of the strains contained three plasmids, which were linked to the presence of a restriction-modification (R-M) system in that strain (Crawford et al., 1981). Unfortunately, the R-M system is not suited as a selectable marker for the presence of a plasmid. Therefore, a small plasmid from another strain, pLR7, was used for the insertion of selectable genes from *E. coli* plasmid pBR322, as a first step towards the construction of a vector system (Crawford & Bates, 1984).

A strain from the closely related species *M. scrofulaceum* contained four plasmids, one of which (pVT1, 181 kb.) encodes a mercury-(II)-reductase which determines high levels of mercury resistance (Meissner & Falkinham, 1984). A search for plasmids in *M. fortuitum* and related species revealed six different plasmids, but no growth-requirements, biochemical properties or differences in susceptibility to the 18 drugs tested could be attributed to them (Labidi *et al.*, 1984). None of the above plasmids was used for vector construction.

Observations of plasmids in soil-dwelling mycobacteria associated with degradation of recalcitrant organic compounds might help in the elucidation of biodegradative pathways, and yield tools for biotechnological applications (Guerin & Jones, 1988; Waterhouse et al., 1991).

A *M. fortuitum* plasmid (pAL5000; Labidi et al., 1985) has become the best characterized mycobacterial plasmid to date. Following the publication of its complete nucleotide sequence (4837 bp.; Rauzier et al., 1988; updated by Labidi et al., 1992), its structure was analyzed and used for the construction of *E. coli/Mycobacterium* shuttle vectors (Gicquel-Sanzey *et al.*, 1989; Ranes et al., 1990; Villar & Benitez, 1992). By selectively deleting parts of pAL5000 that are not involved in plasmid replication and inserting the genes for kanamycin resistance (KmR) from transposon Tn903 and ampicillin resistance from *E. coli* plasmid pTZ19R, plasmid pAL8 (9.2 kb) was constructed. The KmR gene from Tn903 was previously shown to be expressed in *M. smegmatis* and *M. bovis* BCG (Snapper et al., 1988). After transformation by electroporation to *M. smegmatis* mc^{2}155 and to *M. bovis* BCG, expression of the KmR gene was demonstrated in both species (Ranes et al., 1990). Nevertheless, pAL8 was not suited for cloning experiments because of its lack of unique restriction sites for the insertion of foreign DNA. More suitable in this respect was pRR3, a 6.6 kb shuttle plasmid carrying a smaller fragment from pAL5000 and the same resistance genes as pAL8 (Ranes et al., 1990).

Hybrid vectors consisting of pAL5000 sequences joined with a plasmid carrying *S. lividans* sequences (pIJ666, Kieser & Melton, 1988) were constructed by Snapper et al. (1988). One of these vectors, designated pYUB12, carries the KmR gene from transposon Tn5 and the chloramphenicol resistance gene from *E. coli* plasmid pACYC184. Both genes are expressed in *M. smegmatis*, thus widening the range of useful selective markers for vector construction.

Other vectors based on pAL5000 were constructed by Lazraq et al. (1991a). Plasmid pMY10 and cosmid pDC100, both carrying a KmR gene, were transformed to *M. smegmatis*, *M. tuberculosis*, *M. aurum*, and *M. flavescens*. Unfortunately, in the absence of kanamycin, both vectors disappeared from cultures of *M. smegmatis* within 140 generations.

A temperature sensitive derivative of pAL5000 was used to construct pCG79, carrying the *E. coli* transposon Tn611 with a KmR gene. By raising the temperature to the non-permissive level (39°C), a large number of random insertional mutations was generated in *M. smegmatis* (Guilhot et al., 1994). Thus, transposon delivery vectors are likely to become sophisticated

tools for gene localization and function studies (Gavigan et al., 1995).

Another endogenous mycobacterial plasmid is pMSC262 from *M. scrofulaceum*. Goto et al. (1991) used this plasmid for the construction of pYT937, a small shuttle vector, which was able to transform *M. smegmatis* to kanamycin resistance.

A different approach for constructing plasmid vectors able to replicate in *E. coli* and *Mycobacterium* was chosen by Radford and Hodgson (1991). Their observation that plasmid pNG2 from *Corynebacterium* replicated also in *E. coli*, apparently under the control of the same origin of replication, led them to the construction of plasmids pEP2 and pEP3. These contain the pNG2 origin of replication and a KmR gene from pUC4K (*E. coli*) or a hygromycin resistance gene respectively. These plasmids were then introduced by electroporation into *M. smegmatis* and *M. bovis* BCG and expressed. The use of hygromycin for the selection of transformants is an important development in the search for selectable marker genes. Since kanamycin is used in the treatment of tuberculosis, use of hygromycin instead of kanamycin avoids the risk of creating kanamycin resistant pathogens. Unfortunately the authors give no data on the stability of pEP2 or pEP3 in their mycobacterial hosts.

Plasmid p16R1 was constructed by Garbe et al. (1994). Besides the pAL5000 origin of replication, this plasmid carries the hygromycin resistance gene from *Streptomyces hygroscopicus*. Using hygromycin resistance instead of kanamycin resistance as selective criterion, the authors were able to transform previously untransformable clinical isolates of *M. tuberculosis*.

In search of the mechanisms of phage infection, a detailed analysis of the DNA sequence of mycobacteriophage L5 revealed the presence of a gene conferring immunity to phage infection on the host (superinfection-immunity; Hatfull & Sarkis, 1993) which might serve as a selectable marker in transformation of species where antibiotic-resistance markers are unwanted (Donnelly-Wu et al., 1993).

Vectors of heterologous origin

Vectors totally lacking mycobacterial sequences were considered unlikely to be expressed in mycobacteria. Zainuddin et al. (1989) report the transformation of *M. smegmatis* spheroplasts with *E. coli/S. lividans* plasmid vector pIJ666. These transformants could only be found when selecting for chloramphenicol resistance. Snapper et al. (1990) also find *M. smegmatis*-pIJ666 transformants screening for acquired kanamycin resistance, but suspect the integration of plasmid sequences into the *M. smegmatis* chromosome.

A series of 6 plasmid vectors derived from species ranging from *Bacillus subtilis* to *S.lividans* did not replicate in *M. aurum* (Hermans et al., 1991). Surprisingly, cosmid vector pJRD215 was able to transform *M. aurum* and *M. smegmatis* mc^{2}155, expressing both the Tn5-derived KmR gene and the RSF1010-derived streptomycin resistance gene. The cosmid pJRD215 was constructed on the basis of RSF1010, a wide-host-range IncQ plasmid, that replicates in most, if not all, Gram-negative bacteria (Frey & Bagdasarian, 1989). Besides most of the RSF1010 genome, the cosmid contains the *cos*-site from phage λ and the Tn5-derived KmR gene, while the RSF1010 streptomycin resistance gene was put under the control of the promoter of the pBR322 tetracycline resistance gene (Davison et al., 1987).

Subsequently, Gormley and Davies (1991) demonstrated the transfer of RSF1010 from *E. coli* to *S.lividans* and *M. smegmatis* by conjugation. Plasmid RSF1010 was shown to be stably inherited as a plasmid in both hosts, without any gross rearrangements in its structure. This is in contrast with the finding of major deletions in pJRD215, comprising the *cos*-site and the polylinker region, but leaving the RSF1010 derived functions unaffected (Hermans et al., 1991).

The finding of heterologous plasmids capable of replication in *Mycobacterium* is important for genetic investigations of the genus, as it overcomes the need to construct plasmids with different replication functions for the different hosts. Table 1. summarizes the vectors constructed for mycobacterial genetics.

Techniques for stably maintaining foreign genes in mycobacteria

The problem of stability of vectors in mycobacterial hosts was tackled after the observation that, unlike endogenous plasmids, many mycobacteriophages were able to propagate and maintain themselves in a wide range of mycobacterial species after lysogenization, that is, after inserting themselves into the host's chromosome. An *E. coli* cosmid carrying the coliphage λ *cos*-site was inserted into mycobacteriophage TM4, replicating in *M. avium*, *M. smegmatis*, *M. bovis* BCG and *M. tuberculosis*. The construct, named phasmid phAE1, could be introduced into *E. coli* after packaging in λ and into *M. smegmatis* and *M. bovis* BCG after

Table 1. Vectors for mycobacteria

Vector	Origin	Marker genes	Transformed species	Reference
pYUB12	pAL5000	KmR, CmR	*Msm*	Snapper et al. (1988)
pAL8	pAL5000	KmR	*Mau, Mbo, Msm*	Ranes et al. (1990); Hermans et al. (1990)
pRR3	pAL5000	KmR	*Mbo, Msm*	Ranes et al. (1990)
pMY10	pAL5000	KmR	*Mau, Mfl, Msm, Mtu*	Lazraq et al. (1991a)
pDC100	pAL5000	KmR	*Mau, Mfl, Msm, Mtu*	Lazraq et al. (1991a)
pCG79	pAL5000	KmR, SmR	*Msm*	Guilhot et al. (1994)
pYT937	pMSC262	KmR	*Msm*	Goto et al. (1991)
pEP2	pNG2	KmR	*Mbo, Msm*	Radford & Hodgson (1991)
pEP3	pNG2	HmR	*Mbo, Msm*	Radford & Hodgson (1991)
pJRD215	RSF1010	KmR, SmR	*Mau, Msm*	Hermans et al. (1991)
p16R1	pAL5000	HmR	*Mbo, Msm, Mtu, Mva*	Garbe et al. (1994)
phAE1	TM4	–	*Mbo, Msm*	Jacobs et al. (1987)
phAE15	L1	–	*Msm*	Snapper et al. (1988)
pMV261	pAL5000	KmR	*Mbo*	Stover et al. (1991)
pMV361	L5	KmR	*Mbo*	Stover et al. (1991)
pEA4	Ms6	KmR	*Msm*	Anes et al. (1992)
pMH5	L5	KmR	*Msm*	Lee et al. (1991)
pMH94	L5	KmR	*Mbo, Msm, Mtu*	Lee et al. (1991)
pRM64	D29	KmR	*Msm*	Lazraq et al. (1991b)
pBL415	D29	KmR	*Msm*	David et al. (1992)
pBL525	D29	KmR	*Msm*	David et al. (1992)
pY6002	–	*pyrF*	*Msm*	Husson et al. (1990)
pUS701	–	KmR	*Msm*	England et al. (1991)
pUS702	–	KmR	*Msm*	England et al. (1991)

Abbreviations: *Mau, M. aurum*; *Mbo, M. bovis* BCG; *Mfl, M. flavescens*; *Msm, M. smegmatis*; *Mtu, M. tuberculosis*; *Mva, M. vaccae*; CmR, chloramphenicol resistance; HmR, hygromycin resistance; KmR, kanamycin resistance; *pyrF*, orotidine monophospate decarboxylase; SmR, streptomycine resistance.

transformation (Jacobs et al., 1987). The temperate shuttle phasmid phAE15 was constructed by inserting an *E. coli* cosmid into the temperate mycobacteriophage L1 (Snapper et al., 1988). Using phAE15 as a vector, the KmR gene from transposon Tn903 was introduced and stably maintained in *M. smegmatis*. The mechanism behind this stable integration of foreign genes is probably a site-specific integration into the mycobacterial chromosome.

Based on the same principle and with the aim of introducing foreign antigens into *M. bovis* BCG for the construction of multivalent vaccines, Stover et al. (1991) constructed two nearly identical vectors. They share a KmR gene, the *E. coli* origin of replication and a stretch of DNA containing insertion sites preceded by the promoter of a universally expressed gene (*hsp60*). The difference between the two vectors is the presence of the pAL5000 origin of replication in the first (pMV261), and the attachment site and integration genes from mycobacteriophage L5 in the second (pMV361). Both vectors can be transformed to *M. bovis* BCG, but only pMV361 is stably maintained, after integration into the host's genome.

Plasmid vector pEA4 consisting of the complete mycobacteriophage Ms6 genome with a KmR gene inserted was able to form stable lysogens expressing kanamycin resistance in *M. smegmatis* (Anes et al., 1992).

By cloning the attachment site (*attP*) and the integrase gene (*int*) from the mycobacteriophage L5 genome into an *E. coli* plasmid, Lee et al. (1991) constructed plasmid vectors, designated pMH5 and pMH94, that were able to integrate site-specifically into the genome of *M. smegmatis*. pMH94 also transformed *M. tuberculosis* and *M. bovis* BCG very efficiently. By eliminating the excisionase gene (*oriM*)

from the plasmids, they were stably inserted into the chromosome.

Similar approaches were followed by two other groups. Both used the wide host range mycobacteriophage D29 origin of replication and the KmR gene of Tn903 or Tn5, inserted into *E. coli* plasmids pUC19 (pRM64, Lazraq et al., 1991b) or pHG165 (pBL415 and pBL525, David et al., 1992). The resulting shuttle plasmids replicate both in *E. coli* and *M. smegmatis* and are stably maintained in *M. smegmatis* without integration into the chromosome or the selective pressure of kanamycin. The observation that the replication origin of mycobacteriophage D29 suffices for stable maintenance of plasmid vectors suggests that some kind of phage-specific replication mechanism, which differs from plasmid-replication, is able to stabilize plasmids in mycobacteria.

Thus it seems that mycobacteriophages have properties that can be used for stabilizing foreign genes after introduction into a mycobacterial host.

Husson et al. (1990) used a totally different concept for the stable introduction of foreign DNA into mycobacteria. The *E. coli-M. smegmatis* integrating shuttle vector pY6002 was constructed, harbouring an *E. coli* origin of replication, an *E. coli* selectable marker gene (the ampicillin-degrading ß-lactamase gene), and the *M. smegmatis pyrF* gene, which is responsible for the synthesis of uracil and allows selection in *M. smegmatis*. The vector, not possessing a mycobacterial origin of replication, can only maintain itself in *M. smegmatis* after integration into the chromosome, which is facilitated by homologous recombination with the chromosomal *pyrF* gene. Insertion of a foreign gene into the vector's copy of *pyrF* results in integration of that gene into the chromosome, thereby inactivating the *pyrF* gene. A similar approach was followed by Aldovini et al. (1993), who inserted a KmR gene into the genome of *M. bovis* BCG by homologous recombination with the *uraA* gene. After transformation with a linear DNA fragment containing the *uraA* gene (orotidine-5'-monophosphate decarboxylase) and the KmR gene, about 20% of the transformants had the KmR gene integrated into the homologous *uraA* locus.

Another way for the stable introduction of foreign genes into a mycobacterial host was described by the group of Dale and McFadden. They report the construction of vector plasmids that carry a KmR gene, flanked by two copies of the insertion sequence IS*900* (England et al., 1991). These 'artificial transposons', pUS701 and pUS702, may have other foreign sequences inserted next to the KmR gene, and can be introduced into *M. smegmatis*, where they integrate into the chromosome in the same way as a natural transposon (Dellagostin et al., 1993).

Future developments and applications

Alternative hosts

As mentioned above, the search for non-pathogenic, easy cultivable bacterial strains is given much attention. Although *E. coli* is unfit for expression of mycobacterial genes, it can be used as a host for recombinant mycobacterial DNA if the objective is to obtain large amounts of DNA, for instance in sequencing projects. Replication of recombinant plasmids depends in that case on the presence of an *E. coli* origin of replication. If on the other hand faithful expression of mycobacterial genes is important, other hosts that are evolutionary closer to *Mycobacterium* must be considered.

One non-mycobacterial candidate has been described. *S.lividans* is known to express foreign genes, and many genetic techniques have been worked out for this organism (Hopwood *et al.*, 1985). The discovery of conjugative transfer of plasmid RSF1010 between *S.lividans*, *E. coli*, and *M. smegmatis* (Gormley & Davies, 1991) opens up possibilities for the construction of derivatives of this IncQ-plasmid, that are more suited for genetic manipulation. The RSF1010-derivative pJRD215 was shown to be expressed in *M. aurum*, *M. smegmatis* and *E. coli* (Hermans et al., 1991), as well as in *S.lividans* (E. Gormley, personal communication), but the non-RSF1010 part of this plasmid seems to be unstable in its new host. Investigations into the precise nature of this instability are necessary to improve the performance of new vectors for these species.

M. bovis BCG and *M. smegmatis* have been proposed as mycobacterial surrogate hosts for the genes of pathogenic mycobacteria, and much effort has gone into the optimization of these systems (Jacobs et al., 1991; Stover et al., 1991). But since both species show the tendency to grow in clumps, and *M. bovis* is a slowly growing species, a better candidate might be found in *M. aurum*. Plasmids of both mycobacterial and broad-host-range origin have been shown to be expressed in *M. aurum* (Hermans et al., 1990; Hermans et al., 1991). No information is available on its phage susceptibility. In order to enhance the transformation efficiency without the need for growth medium addi-

tives, the isolation of *ept*-mutants of *M. aurum* might be a good alternative.

DNA transfer systems

The development of the technique of electroporation has rendered a simple, fast and efficient tool for the transformation of mycobacteria. Methods for improving transformation efficiencies by isolating *ept* mutants or by treating the target cells with wall degrading agents are available.

Vectors

The development of cloning vectors for mycobacterial genetics proceeds in two directions. Firstly, vectors of mycobacterial origin are being developed for expression of recombinant sequences in a mycobacterial host. Since stable maintenance of newly introduced genes is an important issue, much effort is given to the enhancement of stability of these sequences. Secondly, vectors of heterologous origin hold the promise of easy shuttling of recombinant DNA between different host-species. Here, changes in plasmid structure after introduction into the new host have to be investigated. The instability of pAL8-derivatives in *M. bovis* BCG and *M. smegmatis*, studied by Haeseleer (1994), might give some important clues to tackle this problem.

In conclusion, the study of mycobacterial genetics has overcome initial difficulties and through the development of specific vectors and transformation systems, has detached itself from a dependence on *E. coli* techniques. The expression of foreign antigens in *M. bovis* BCG, reviewed by Flynn (1994), is one of the challenging areas of mycobacterial studies that hold the promise of major achievements in the near future. Depending on the vector used, foreign antigens can be expressed in the cytoplasm or on the surface of *M. bovis* BCG, or can be secreted. In this way it was possible to engender strong immunological responses against Lyme disease (Stover et al., 1993) or Leishmania parasites (Connell et al., 1993) in mice.

By inserting the firefly luciferase gene into the derived plasmid mycobacteriophage TM4 phAE40, Jacobs *et al.* (1993) developed a quick technique to monitor drug susceptibilities in *M. tuberculosis*. After infection, the luciferase gene was expressed from the *hsp60* promoter, resulting in light production with ATP supplied by the mycobacterial host.

Mycobacterial research will have to face several challenges: The increasing incidence of antibiotic-resistant strains of *M. tuberculosis*, and development of alternative methods for treatment; the development of multivalent vaccines based on *M. bovis* BCG, with a possible application in vaccination against HIV-infection; the unravelling of the mechanisms of pathogenicity of *M. leprae*; the elucidation of biodegradative and biosynthetic processes. Thanks to major technical advances in the past ten years, the techniques and equipment for solving these problems are becoming available.

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