MOLECULAR TECHNIQUES IN MYCOBACTERIOLOGY

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MOLECULAR TECHNIQUES IN MYCOBACTERIOLOGY

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STELLINGEN

1. Zainuddin *et al.* leveren, door het achterwege laten van goede controle-experimenten, onvoldoende bewijs voor de transformatie van *Mycobacterium smegmatis* met *Escherichia coli* plasmiden.

   Zainuddin, Z.F., Kunze, Z.M., and Dale, J.W. (1989) *Molec Microbiol* 3: 29-34.

2. De beschrijving van het voorkomen van een lineair plasmide in *Streptomyces rimosus* sluit de mogelijkheid van een artefact veroorzaakt door de ongebruikelijke isolatieprocedure niet uit.

   Chardon-Loriaux, I., Charpentier, M., and Percheron, F. (1986) *FEMS Microbiol Lett* 35: 151-155.

3. De isolatie van DNA uit fossiele bladeren van meer dan 100 miljoen jaar oud overschrijdt, ondanks alle voorzorgsmaatregelen ter voorkoming van contaminatie met 'modern DNA', de grens van het geloofwaardige.

   Poinar, H.N., Cano, R.J., and Poinar, G.O. jr. (1993) *Nature* 363: 677.

4. De conclusie dat vanwege het optreden van hybridisatie met rRNA, het uit *Mycobacterium* sp. geïsoleerde DNA geschikt is voor recombinatie met ander DNA, is onterecht.

   Katoch, V.M., and Cox, R.A. (1986) *Int J of Leprosy* 54: 409-415.

5. Bakker *et al.* houden bij hun onderzoek naar de biogeografie van mariene algen geen rekening met mogelijke vermenging van populaties als gevolg van stromingen of door hechting aan schepen.

   Bakker F.T., Olsen J.L., Stam W.T., and van den Hoek, C. (1992) *J Phycol* 28: 839-845.

6. De discrepanties tussen beide gepubliceerde DNA sequenties van het plasmide pAL5000 tonen eens te meer aan dat sequencen van mycobacterieel DNA geen sinecure is.

   Rauzier, J., Moniz-Pereira, J., and Gicquel-Sanzey, B. (1988) *Gene* 71: 315-321;
   Labidi, A., Mardis, E., Roe, B.A., and Wallace jr, R.J. (1992) *Plasmid* 27: 130-140.

7. Het zoeken naar surrogaat gastheerstammen voor onderzoek naar de pathogenese en behandeling van tuberculose en lepra verdient een hogere prioriteit - dit proefschrift.

8. Het Human Genome Project zou meer geld moeten besteden aan het ontwikkelen van betere sequencing technieken.

9. Erger nog dan genetisch gemanipuleerde sojabonen zijn ethisch gemanipuleerde consumenten.

10. De slogan 'Geniet, maar drink met mate' impliceert door het gebruik van het woord 'maar' dat het ene eigenlijk niet zonder het andere kan.

Stellingen behorende bij het Proefschrift 'Molecular Techniques in Mycobacteriology'.

J.Hermans - 13 januari 1997.

Voor Pascalle,

Lieke, Anke en Eefke

Aan mijn ouders

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Chapter 1

GENERAL INTRODUCTION

Bacterial mono- and dioxygenases in the degradation of hydrocarbons

Many bacterial species are able to utilize aliphatic or aromatic hydrocarbons as source for cell carbon and energy. Under aerobic conditions, degradation of these hydrocarbons is initiated by oxygenase type enzymes. Because of their importance in bacterial metabolism and their possible biotechnological applications, several of such enzymes have been studied in detail, both at the protein and at the genetic level. In Table 1 some of the bacterial oxygenases are listed. A general overview of oxygenases involved in the degradation of hydrocarbons is given in Harayama *et al.* (1992).

With the exception of styrene mono- and dioxygenase and alkene monooxygenase, all of the enzymes mentioned in Table 1 have been studied extensively. Enzymes have been purified and characterized, and in many cases the encoding genes have been localized and sequenced.

Because of their possible use in biotechnological processes, mono- and dioxygenases are important enzymes. They may be used in the production of fine chemicals such as epoxides and *cis*-dihydrodiols. They also may catalize the initial reaction in the complete degradation of toxic waste compounds (Hartmans *et al.*, 1988).

Table 1. Bacterial oxygenases involved in the degradation of hydrocarbons.

Enzyme	Organism	Enzyme purified	Genes localized	References
Methane monooxygenase	methanothrophs	+	+	Murrell, 1992
Alkane hydroxylase	*Pseudomonas oleovorans*	+	+	van Beilen *et al.*, 1994
Benzene dioxygenase	*Pseudomonas putida*	+	+	Tan *et al.*, 1993
Toluene monooxygenase	*Pseudomonas mendocina*	+	+	Whited & Gibson, 1991
Toluene dioxygenase	*Pseudomonas putida*	+	+	Zylstra & Gibson, 1989
Toluene monooxygenase	*TOL* plasmid	+	+	Harayama *et al.*, 1989
Xylene monooxygenase	*Pseudomonas putida*	+	+	Wubbolts *et al.*, 1994
Styrene monooxygenase	*Pseudomonas putida*	-	-	Hartmans *et al.*, 1990
	Pseudomonas fluorescens	-	+	Marconi *et al.*, 1996
Styrene dioxygenase	*Rhodococcus rhodochrous*	-	-	Warhurst *et al.*, 1994
Alkene monooxygenase	*Mycobacterium* sp.	+	-	Weber *et al.*, 1992
	Nocardia corallina	+	-	Miura & Dalton, 1995
	Xanthobacter sp.	-	+	Leak *et al.*, 1996

Production of optically pure epoxides

Because of their reactivity, epoxides are important building blocks in organic synthetic reactions, such as in the production of pharmaceuticals. Epoxides generally occur in two or four enantiomeric forms (stereoisomers). As a result, products derived from racemic mixtures of epoxides also consist of different stereoisomers. Only one of these is biologically active in pharmaceuticals. The other enantiomer at best is an inert contaminant in pharmaceuticals, but may also cause undesired side effects. In the production of pharmaceuticals, it is therefore important to use optically pure epoxides.

To date there are several biological ways to produce optically pure epoxides, as summarized in Fig. 1.

Advantages of the direct epoxidation method are the possibility to obtain epoxides of high enantiomeric excess (up to 100%) at 100% yield from cheap alkene substrates. Disadvantages, however, are the low specific activity of the enzymes and the toxicity of the epoxides towards the organism. Furthermore, because of the need for regeneration of the cofactor NADH, direct epoxidation of alkenes is restricted to whole cells.

Epoxidation of alkenes

Methanotrophic bacteria have been isolated from many different freshwater or marine sources, and they are ubiquitous in nature. They are obligately methylotrophic, converting methane via methanol, formaldehyde and formate to carbon dioxyde (Murrell, 1992). These organisms all produce racemic mixtures of epoxides from alkenes (Weijers *et al.*, 1988).

Styrene monooxygenase (SMO) activity is present in pseudomonads isolated with styrene as substrate (Hartmans et al., 1990; Marconi *et al.*, 1996). This FAD-containing enzyme converts styrene to styrene oxide, an epoxide which is further degraded via phenylacetaldehyde and phenylacetic acid. The aromatic substituent on the double bond appears to be required for activity, as the enzyme is incapable of epoxidation of aliphatic alkenes.

Alkene monooxygenase (AMO), a NADH or NADPH-requiring enzyme, is present in ethene-utilizing strains of *Mycobacterium* sp. that were isolated from soil samples with ethene as substrate. The enzyme converts lower aliphatic terminal and subterminal alkenes to the

corresponding epoxides (de Bont & Harder, 1979). Such an enzyme is also present in *Xanthobacter* sp. (van Ginkel *et al.*, 1986) and possibly in *Nocardia corallina* (Furuhashi,1992).

AMO from *Mycobacterium* sp. as well as from *Xanthobacter* sp. produces epoxides with high enantiomeric excess. Therefore, these strains may find application in the biological synthesis of chiral epoxides, using the technique of direct epoxidation. In Table 2 a few examples are given of optical purities of epoxides obtained using direct epoxidation of alkenes.

Degradation of toxic compounds

A second possible application of bacteria harbouring monooxygenases is the removal of toxic substances from industrial waste streams. Aromatic and aliphatic hydrocarbons are widely used by the chemical industry, either as monomers for the production of synthetic polymers or as solvents. As a consequence, they are present in many industrial effluents, that will eventually contaminate the environment.

In view of their environmental persistence and possible carcinogenicity, reduction of the hydrocarbon content of waste streams is of great importance. Treatment of contaminated wastewater streams is already an established method, and the bulk of contaminants is removed by biological techniques. Also the biological treatment of groundwater and soil is becoming increasingly important.

For waste-gas treatment several physico-chemical techniques exist, but the benefits of biological methods for removal of toxic compounds from waste-gases are increasingly recognised (Weber, 1995). In the case of chloro-substituted lower alkenes, biological removal of these compounds from gaseous emissions might be achieved in biofiltration devices using pure cultures of bacteria that harbour the appropriate degrading enzymes. Application of this technique was studied for the removal of trichloro ethylene by *Xanthobacter* Py2 (Reij *et al.*, 1995) and vinyl chloride by *Mycobacterium* L1 (Hartmans *et al.*, 1992). Many other bacteria containing monooxygenases have been considered for removing chlorinated alkenes (Ensley, 1991).

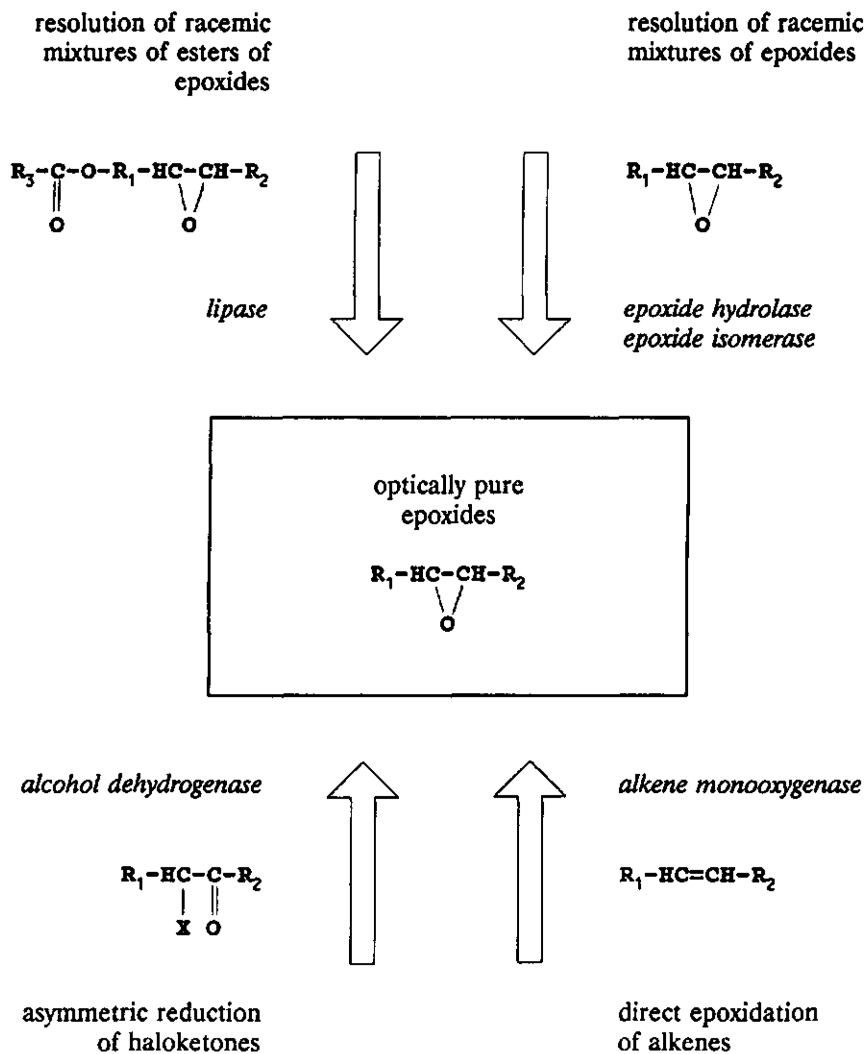

Figure 1. Techniques for the bioproduction of optically pure epoxides.
For details refer to: Weijers *et al.*, 1988; ; Weijers *et al.*, 1992; Leak *et al.*, 1992; de Bont, 1993; Besse & Veschambre, 1994.

Protein characterization of monooxygenases

Methane monooxygenase

Methane monooxygenase from methane-oxidizing bacteria has been studied thoroughly. In *Methylosinus trichsporium* and *Methylococcus capsulatus*, it occurs in two forms, depending on growth conditions: one particulate and one soluble. The soluble methane monooxygenase, on which most research is focused, consists of three proteins: a NADH-reductase component, a hydroxylase component and a regulatory component, all of which have been studied extensively (Murrell, 1992).

Alkane hydroxylase

The protein structure of alkane hydroxylase from *Pseudomonas oleovorans* is also very well studied. This three component enzyme system consists of a rubredoxin component, a rubredoxin-reductase component and a membrane-bound hydroxylase component (van Beilen *et al.*, 1994). The latter is a 42 kD polypeptide, which contains 1 atom of iron and about 20 phospholipid molecules per polypeptide chain.

Xylene monooxygenase

Xylene monooxygenase from *Pseudomonas putida* is a two component enzyme: a membrane-bound monooxygenase component and a cytoplasmic NADH reductase component. The primary structure of the monooxygenase component is homologous to that of the membrane bound *P.oleovorans* alkane hydroxylase, which is not surprising because both enzymes catalyse a similar reaction: the hydroxylation of a methyl group (Wubbolts, 1994).

Table 2. Optical purity of epoxides obtained with different monooxygenases.

Enzyme / organism	Purity	Reference
Methane monooxygenase		
Methylococcus capsulatus	10% (R)-1,2-epoxy propane	Hou *et al.*, 1983
Alkane hydroxylase		
Pseudomonas oleovorans	62% (R)-1,2-epoxy decane	de Smet *et al.*, 1983
Xylene monooxygenase		
Pseudomonas putida	93% (S)-styrene oxide	Wubbolts, 1994
Styrene monooxygenase		
Pseudomonas putida	>98% (S)-styrene oxide	Nöthe & Hartmans, 1994
Alkene monooxygenase		
Mycobacterium L1	>99% (R)-1,2-epoxy propane	Weijers *et al.*, 1988
Xanthobacter Py2	97% (R)-1,2-epoxypropane	Habets-Crützen *et al.*, 1985
Nocardia corallina	87% (R)-1,2-epoxy propane	Furuhashi, 1992

Alkene monooxygenase

In view of the potential applications of monooxygenases both in the production of optically pure epoxides and in the biodegradation of xenobiotic compounds, an interest exists in AMO from *Mycobacterium* strain E3. Until now, it has been rather difficult to biochemically access the proteins of the AMO enzyme. The sigmoidal relationship of AMO-activity versus protein concentration indicated that AMO is a multicomponent enzyme. Further purification showed that it consists of at least two components: one oxygenase (epoxidase) and one NADH-reductase component (Hartmans *et al.*, 1991). The latter has been studied in more detail. It appears to be a 56 kDa monomer, with one FAD prosthetic group and one Fe_2S_2-cluster per molecule (Weber *et al.*, 1992). Recently, AMO from propene-utilizing *Nocardia corallina* strain B276 was resolved into three components: an epoxidase component which consists of two subunits (molecular masses 53 kDa and 35 kDa), an NADH-reductase component (molecular mass 40 kDa) and a 14 kDa 'coupling protein', involved in activity-regulation (Miura & Dalton, 1995). Apart from the molecular masses of the components, the *Nocardia* AMO differed significantly from the *Mycobacterium* AMO in its insensitivity to acetylene-inhibition and in the enantiomeric purity of the epoxides produced (Table 2).

Genetics of monooxygenase type enzymes

Methane monooxygenase

Genes responsible for the oxidation of methane to methanol were among the first monooxygenase type enzymes to be isolated. Using degenerate oligonucleotide probes, designed on the basis of amino acid sequences from the N-terminal regions of soluble methane monooxygenase subunits, the gene cluster coding for the soluble methane monooxygenase of *M.capsulatus* has been isolated, cloned and analysed (Mullens & Dalton, 1987; Stainthorpe *et al.*, 1990). It appears that the coding sequences for all enzyme subunits are clustered in the genome, in a way that is characteristic for enzyme systems. The subject has been reviewed by Murrell (1992).

Alkane hydroxylase

Pseudomonas oleovorans strains that are able to grow on C_6 to C_{12} *n*-alkanes harbour the large OCT-plasmid, which contains the genes coding for the alkane hydroxylase enzyme system (the *alk*BFGHJKL operon and *alk*ST region). Extensive studies of this enzyme system, both on the enzyme level and the genetic level, have rendered the alkane utilization system of *P.oleovorans* one of the best characterized degradative enzyme systems to date (van Beilen *et al.*, 1994). The biotechnological production of alkanols from alkanes and optically active epoxides from alkenes using *P.oleovorans* is currently under investigation (Wubbolts *et al.*, 1994).

Xylene oxygenase

In *Pseudomonas putida* the enzyme xylene oxygenase catalyzes the oxidation of the methyl group of xylenes and toluenes, the first step in their degradation. The gene clusters encoding this monooxygenase type enzyme system (*xyl*CMABN and *xyl*XYZLTEGFJQKIH) were localized on the TOL plasmid and studied extensively with the aim of employing these genes in the production of various aliphatic and aromatic hydrocarbons (Wubbolts, 1994). Since Pseudomonads generally degrade a wide range of hydrocarbons, application of these strains for bioconversions was limited because of the degradation of the products formed. Therefore the *xyl*MA genes, encoding the xylene monooxygenase and its NADH-reductase component, were cloned into a suitable vector and transformed to *Escherichia coli*. Expression of the enzyme was realized by inserting the genes downstream from an active *E.coli* promoter (Wubbolts *et al.*, 1994). At present the production of styrene oxide from styrene by the recombinant *E.coli* strain is under study (Wubbolts *et al.*, 1994).

Outline of this thesis

In the previous sections, several oxygenases have been considered. In almost all cases these enzymes are found in Gram-negative bacteria. As can be seen from Table 1, in many instances the enzymes have been purified and the genes encoding the enzymes have been localized. Also it has been possible to express the enzymes in a heterologous host, such as *Escherichia coli*.

The situation in Gram-positive organisms harbouring monooxygenases, as for instance *Mycobacterium* and *Nocardia* strains, is by far more complex. First of all, standard molecular techniques, developed mainly for working with *E.coli*, appeared to be unfit for *Mycobacterium*. Furthermore, suitable vector plasmids for the manipulation of DNA in *Mycobacterium* were not available.

A main disadvantage of mycobacteria is the low specific enzyme activity of the monooxygenases in their cells. Genetic methods to enhance the activity level of these enzymes either in *Mycobacterium* sp. or in another host therefore would be very welcome. In the present work it was investigated if such genetic methods could be developed for these organisms.

In a first approach, a transformation system based on the standard technique of mixing Ca^{2+}-treated cells with transforming DNA was investigated. However, the presence of a massive cell envelope in mycobacteria made this method unfit for these organisms. Subsequently, a method for the isolation of wall-deficient cells (spheroplasts) was developed. Unfortunately, transformation of these spheroplasts was still unsuccessful because of the low regeneration of spheroplasts to intact cells (unpublished results).

With the advent of the technique of electroporation, the attention was shifted to the use of this method in mycobacteria. Electroporation of whole cells appeared to be a better method for transformation, and a detailed protocol for transformation of *M.aurum* is described in chapter 3.

Vectors designed for manipulating DNA in mycobacteria were very scarce. Even some so-called broad-host-range plasmid vectors appeared to limit their host-range to the Gram-negative bacteria, and were not useful for molecular biological work in mycobacteria. One exception was found in the broad-host-range cosmid pJRD215, that was able to transform *M.aurum*, *M.smegmatis*, *M.fortuitum*, *M.parafortuitum*, and *M.phlei* (chapters 4 and 5).

The causative agents of tuberculosis and leprosy, *Mycobacterium tuberculosis* and *Mycobacterium leprae*, have rendered the genus *Mycobacterium* notorious. Although the pathogenic species comprise only a small minority of the genus, gaining knowledge of the mechanisms of infection and pathogenesis is the driving force behind intensive biochemical and molecular biological research on the genus as a whole. The methods and techniques described in this thesis are of use for the whole field of mycobacteriology, pathogenic as well as non-pathogenic.

Our knowledge of the genetics and the molecular biology of mycobacteria lags behind that of thoroughly characterized species such as *E.coli* or *Bacillus subtilis* (Hartmans & de Bont, 1991). The present situation in the techniques available for genetic engineering in mycobacteria is reviewed in chapter 2.

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Chapter 2

TECHNIQUES FOR GENETIC ENGINEERING IN MYCOBACTERIA ALTERNATIVE HOST STRAINS, DNA-TRANSFER SYSTEMS AND VECTORS

J. Hermans and J.A.M. de Bont

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.

Antonie van Leeuwenhoek (1995) **69**: 243-256.

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 Fig. 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 (Horsbaugh, 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).

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).

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 *Mycobacterium* 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 transformation 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^2155 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^2155, 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 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).

Table 1. Vectors for mycobacteria

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.

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)

Table 1. Vectors for Mycobacteria (continued)

Vector	Origin	Marker genes	Transformed species	Reference
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)

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 additives, 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 chromosome of mycobacteriophage TM4, 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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Chapter 3

TRANSFORMATION OF *MYCOBACTERIUM AURUM* BY ELECTROPORATION: THE USE OF GLYCINE, LYSOZYME AND ISONICOTINIC ACID HYDRAZIDE IN ENHANCING TRANSFORMATION EFFICIENCY

J. Hermans, J.G. Boschloo, and J.A.M. de Bont

A transformation procedure for *Mycobacterium aurum* using electroporation was developed and optimized. Effects of glycine and lysozyme treatments were studied, and isonicotinic acid hydrazide was shown to increase transformation efficiency tenfold.

FEMS Microbiology Letters (1990) **72**: 221-224

Introduction.

The advent of the technique of electroporation has greatly facilitated transformation procedures and many microbial species previously considered as untransformable have now become accessible to molecular biological techniques. Among these are many Gram-positive microorganisms which do not readily take up macromolecules from their environment, as do most Gram-negative organisms. Nevertheless, transformation efficiencies of Gram-positive microorganisms using electroporation are much lower than Gram-negative organisms. Powell *et al.* (1988) suggested that the structure of the Gram-positive cell wall might be the limiting barrier in the transformation by electroporation of the Gram-positive *Lactococcus lactis*, and it was shown that partial degradation of the cell wall resulted in increased transformation efficiencies. Subsequently, many different Gram-positive microorganisms have been shown to be more efficiently transformed after treating the recipient cells with cell wall degrading agents (Wolf *et al.*, 1989; Chater *et al.*, 1982; Holo & Nes *et al.*, 1989).

The Gram-positive genus of *Mycobacterium* has gained considerable interest because of its importance in pathogenicity (Rastogi & David, 1988) and also in view of biotechnological applications (Hartmans *et al.*, 1989). Molecular biological studies in this genus mainly focus on the pathogenic, slow-growing species such as *M. tuberculosis* and *M. leprae.* A transformation system for *M. bovis* BCG and *M. smegmatis* based on electroporation was superficially described (Snapper *et al.*, 1988), but no efforts were made to increase transformation efficiency.

Strains of fast growing, non-pathogenic *Mycobacterium* sp., degrading lower alkenes as ethene and propene and the chlorinated alkene vinylchloride have been isolated from soil. These strains have an alkene monooxygenase enzyme catalyzing the oxidation of alkenes to the corresponding epoxides, which may or may not be further degraded. These strains are interesting for biotechnological removal of toxic waste gases or in the production of optically active epoxides (Habets-Crützen *et al.*, 1985; Weijers *et al.*, 1988).

The construction of a *M. fortuitum* derived plasmid vector, pAL8, has recently been published. Gicquel-Sanzey *et al.* (1989) have constructed pAL8 from the cryptic *M. fortuitum* plasmid pAL5000 by inserting the ampicillin resistance gene from pTZ19R and the kanamycin resistance gene from Tn903 into a supposedly non-essential region of pAL5000. This construct was expressed

and stably maintained in several mycobacterial species, among which *M. fortuitum*, *M. tuberculosis* and *M. smegmatis* (B. Gicquel-Sanzey, personal communication).

In previous, unreported work in our laboratory with *M. aurum* strain L1, it was unsuccessfully attempted to develop a transformation procedure for this fast-growing strain. In these studies, pAL8 was used as a model system to test transformation, since no indigenous plasmid is present in *M. aurum* strain L1.

Using electroporation, it has now been possible to express pAL8 in *M. aurum* L1. The details of the transformation procedure for this fast-growing *Mycobacterium* are presented in this paper, as well as the effect of some cell-wall degrading agents on the efficiency of transformation.

Material and methods

Bacterial strain and plasmid

M.aurum strain L1 was taken from the culture collection of our laboratory. Plasmid pAL8 was obtained from dr. B. Gicquel-Sanzey of the Institut Pasteur, Paris, France.

Media

M.aurum L1 was grown in liquid medium containing 0.5% (w/v) glucose and 0.35% (w/v) yeast extract, buffered at pH 7.0 with 50 mM K_2HPO_4/KH_2PO_4.

Agar plates, consisting of growth medium supplemented with 1.5% agar, routinely contained 50 μg/ml cycloheximide to prevent mold infection. Preliminary experiments showed that cycloheximide did not affect mycobacterial growth. For transformant screening, agar plates were supplemented with 20 μg/ml kanamycin sulphate.

Electroporation

100 ml of growth medium were inoculated with 1 ml of a growing culture of *M. aurum* L1, and incubated at 30°C with shaking for 24 hours. If appropriate, isonicotinic acid hydrazide (INH, isoniazid) was added to 4 μg/ml from a filter sterilized stock solution. Glycine was added to 0.2 M from a separately sterilized stock solution. INH or glycine were added in early logarithmic

growth phase and incubation was continued for another 4 hours. The cells were chilled in a refrigerator and harvested by centrifugation in a Sorvall GSA rotor at 8,000 rpm and 4°C, washed with cold electroporation buffer (EPB, 10% sucrose, 7 mM Hepes pH 7.0, 1 mM $MgCl_2$), resuspended in 0.02 culture volume of cold EPB and kept on ice until further treatment. In case of lysozyme treatment, the harvested cell suspension was washed with cold TE-buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) instead of EPB and resuspended in 5 ml TE with 1 mg/ml lysozyme. The suspension was incubated at 37°C for 1 hour with occasional shaking, harvested, washed and resuspended in EPB as above.

The cell suspensions had a density of approximately 1.0×10^9 cfu/ml. In contrast to other researchers' results with other bacterial species (Wolf *et al.*, 1989), repeatedly freezing and thawing of the cell suspension resulted in excessive lysis of the bacteria, even if not treated with wall degrading agents. Electroporation of these suspensions often was impossible due to 'arcing' in the cuvette. Therefore only fresh cell suspensions were used for electroporation.

100 µl of the suspension was mixed with 2 µg of plasmid DNA in a cooled 0.2 cm electroporation cuvette (BioRad) and immediately subjected to a single electric pulse using the BioRad Gene Pulser, set at 25 µF and 2500 V, with the Pulse Controller set at 200 Ω. These settings generally gave pulse time constants of 3.5 to 4.5 msec. Immediately after electroporation the cell suspension was diluted 10 fold with 900 µl growth medium, transferred to a sterile screw cap tube and incubated at 30°C for 16 to 24 hours. During this regeneration period no cell division took place. Appropriate dilutions of the cell suspension were then spread on growth medium agar without antibiotic to determine the viability. In parallel, portions of 100 µl of undiluted cell suspension were plated on growth medium agar containing 20 µg/ml kanamycin, in order to determine the number of transformants. The minimal inhibitory concentration (MIC) of kanamycin for strain L1 is less than 5 µg/ml. Plates were incubated for 7 to 10 days at 30°C, after which period colonies could be counted.

Results

In initial attempts, cells of *M. aurum* strain L1 were transformed with 2 µg of pAL8 plasmid DNA by electroporation, following a procedure adapted from the one published by Snapper

et al. (1988). The system, using the Gene Pulser settings described in the Methods section, gave satisfactory results with a transformation frequency of 4.5×10^{-6} and an efficiency of 2230 transformants per μg of pAL8 DNA. The true nature of these transformants was checked by showing that the transformants were also resistant to 40 μg/ml ampicillin (MIC = 30 μg/ml), which was due to the expression of the ampicillin resistance gene of pAL8 in the transformants. Cell suspensions mixed with 2 μg of pAL8 but not electroporated, showed no kanamycin- or ampicillin-resistant colonies.

Further experiments focussed on enhancing the transformation efficiency by using mycobacterial cells with degraded cell walls as recipients for pAL8. The effects of the commonly used cell wall degrading agents lysozyme, glycine and INH on transformation efficiency were investigated.

Table 1. Transformation of M.aurum L1 with pAL8 after different pretreatments of the recipient cells.

Culture treatment[1]	Survival[2](%)	Efficiency[3](* 10^3)
no addition	10	2.23
glycine	6	3.64
INH	8	23.40
glycine + INH	2	10.80
lysozyme	0	-

[1]Concentrations of the additions were: glycine, 0.2 M; INH, 4 μg/ml; lysozyme, 1 mg/ml. [2]Survival: number of viable cells after electroporation (percent of original suspension). [3]Efficiency: number of transformants per μg DNA per 10^9 cells electroporated.

As can be seen from Table 1, pretreatment of the cells with 0.2 M glycine resulted in an increase of transformation efficiency with a factor 1.6. Transformation efficiency was increased even further by treating the cells with 4 μg/ml INH during logarithmic growth phase. Furthermore, as judged from cell survival counts after electroporation (Table 1), INH treatment is also a very gentle treatment, causing minimal loss of viability. The combination of INH and glycine

resulted in 4.8 times as many transformants per microgram of pAL8. Surprisingly, after pretreatment of the cells with lysozyme no viable cells were found after electroporation.

Discussion

The few transformation procedures for *Mycobacterium* sp. described in literature before the introduction of electroporation, all employ protoplasted cells, obtained by extensive treatment with cell wall degrading agents. These procedures depend not only on the efficiency of the protoplasting procedure, but also on the ability of the protoplasts to regenerate into viable, dividing cells. Besides being very laborious and time consuming, these procedures appear to be very specific for the mycobacterial species for which they were developed, probably due to the great differences in cell wall structure between mycobacterial species. In previous work in our laboratory, using the protoplasting- and regeneration techniques developed by Rastogi *et al.* (1983) on our *M. aurum* strain L1, no transformants were found with pAL8 as a model system to test DNA uptake. Apparently, this is due to the rigid structure of the cell wall of *M. aurum*, resulting in poor protoplasting and regeneration of the cells.

The technique of electroporation finally opened up the possibility of transformation of whole, untreated cells of *M. aurum*, as was readily shown by the expression in this species of the resistance genes carried by pAL8. Treatment of the cells with some commonly used cell wall degrading agents was shown to enhance transformation efficiency, as could be expected from the results of similar treatments of other bacterial species (Powell *et al.*, 1988; Holo & Nes, 1989; Haynes & Britz, 1989).

Bacteria grown in the presence of glycine show an increased susceptibility to the action of cell wall degrading agents, indicating alterations in their cell wall structure (Winder & McNaughton, 1978). Lysozyme (muramidase) is commonly used as a cell wall degrading agent in DNA isolation procedures, and acts on the innermost peptidoglycan layer of the cell wall, attacking the glycosidic bonds between N-acetyl glucosamine and N-acetyl muramic acid (Strominger & Ghuysen, 1967). INH is used as a strong antibiotic against several pathogenic mycobacterial species. It is an inhibitor of mycolic acid synthesis and, when administered in sublethal concentrations, causes the mycobacterial cell to make weaker cell walls (Winder, 1982).

INH has recently been reported to enhance transformation efficiencies in the species *Brevibacterium lactofermentum* and *Corynebacterium glutamicum*, both related to *Mycobacterium* sp. (Haynes & Britz, 1989).

The results from the above experiments clearly indicate that transformation efficiency is greatly enhanced by the action of INH. This beneficial effect is very probably due to the interference of this agent with cell wall synthesis during growth, thus allowing the mycobacterial cell to take up macromolecules from the environment during the period of destabilization of the cell membrane after electroporation.

The action of lysozyme, even in a relatively low concentration of 1 mg/ml, is probably too vigorous. It seems that even partial breakdown of the peptidoglycan layer of the cell wall results in the loss of resistance of the cells to the strong electrical field during electroporation.

In conclusion, a transformation system for *M. aurum* using electroporation has been developed. For optimal results, pretreatment of the cells with 4 μg/ml INH is required. This procedure should open up ways of applying molecular genetic techniques on this biotechnologically important bacterium. Furthermore, the use of INH in enhancing transformation efficiency might be applicable to other mycobacterial species too.

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Chapter 4

TRANSFORMATION OF *MYCOBACTERIUM AURUM* AND *MYCOBACTERIUM SMEGMATIS* WITH THE BROAD-HOST-RANGE GRAM-NEGATIVE COSMID VECTOR PJRD215

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T. Goosen and J.A.M. de Bont

The transformation of *Mycobacterium aurum* and *Mycobacterium smegmatis* with the Gram-negative RSF1010-derived cosmid pJRD215 is described. The plasmid is stably maintained in both species and the antibiotic resistance determinants to kanamycin and streptomycin are expressed. From Southern blot analysis the cosmid is structurally stable in *M. aurum*, but rearrangements are detected in *M. smegmatis*. The use of pJRD215 in mycobacterial cloning systems is discussed.

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Introduction

The paucity of available molecular information concerning Gram-positive microorganisms can, in large part, be attributed to the lack of genetic tools and methods to access these bacteria. Only in some genera of Gram-positive bacteria, like *Bacillus*, *Lactobacillus* and *Streptomyces*, systems and vectors are available that allow reasonable transformation frequencies on a consistent basis. In the genus *Mycobacterium*, molecular biological studies mainly focus on the pathogenic, slow-growing species. At present, two vector systems are available for these species. Shuttle phasmids, consisting of mycobacteriophage DNA recombined with an *Escherichia coli* cosmid, have been described (Jacobs *et al.*, 1987; Snapper *et al.*, 1988). More recently, *M. fortuitum* derived plasmid vectors have become available (Ranes *et al.*, 1990).

Strains of fast-growing, non-pathogenic *Mycobacterium* sp., able to degrade lower alkenes and chlorinated alkenes as ethene, propene and vinyl chloride, have been isolated from soil. These strains have an alkene monooxygenase catalyzing the oxidation of alkenes to the corresponding epoxides, which may or may not be further degraded. This property of these strains could be of interest for removal of toxic waste gases or in the production of optically active epoxides (Hartmans *et al.*, 1989; Weijers *et al.*, 1988).

A transformation procedure for ethene utilizing *Mycobacterium aurum* strain L1, has been developed. By electroporation it was possible to transform this strain with pAL8 (Hermans *et al.*, 1990). pAL8 was constructed from the cryptic *M. fortuitum* plasmid pAL5000 by inserting the ampicillin resistance gene from pTZ19R and the kanamycin resistance gene from Tn903 into a supposedly non-essential region of pAL5000 (Gicquel *et al.*, 1989). However, pAL8 is not a very suitable cloning vehicle because of its size (9.2 kb) and because it lacks unique restriction sites. We have investigated the availability of other vectors that are expressed in *M. aurum* L1.

We describe the transformation by electroporation of the fast-growing *M. aurum* strain L1 with the Gram-negative RSF1010-derived cosmid pJRD215. This is the first report of the expression of pJRD215 in a Gram-positive organism.

The work was extended by studies with the fast-growing *M. smegmatis*. The easy cultivable *M. smegmatis* is a surrogate host for the cloning and expression of genes from pathogenic, slow-growing mycobacteria, such as *M. tuberculosis* and *M. leprae* (Hopwood *et al.*, 1988).

Material and methods

Bacterial strains and plasmids

M. aurum strain L1 and *E. coli* DH5α were from the culture collection of our laboratory. *M. smegmatis* mc^2155 is described in Snapper *et al.* (1990). *E. coli* TG1 and plasmid pAL8 were from the culture collection of the Institut Pasteur. pBS7, pJRD215, pPL608 and pUB110 were obtained from the Phabagen Culture Collection, Utrecht, the Netherlands. pMG24 was a gift from Dr. J. Kok of the State University Groningen, the Netherlands. pIJ702 was a gift from Dr. A. Akkermans of the Agricultural University Wageningen, the Netherlands. pNZ18 was a gift from Prof. Dr. W. de Vos of the Agricultural University Wageningen, the Netherlands. All plasmids except pIJ702 and pNZ18 were harboured by *E. coli* strains.

Table 1. Plasmids used in the transformation experiments with *Mycobacterium aurum* strain L1.

plasmid	origin of replication from	markers[1]	reference
pAL8	*M. fortuitum*	Ap^R, Km^R	Gicquel *et al.*, 1989.
pBS7	pUB110 + pBR322	Km^R	Yansura and Henner, 1984.
pIJ702	*Streptomyces lividans*	Ts^R, Mel	Hopwood *et al.*, 1985.
pMG24	*Streptococcus cremoris*	Km^R	Kok, pers.comm.
pNZ18	*Streptococcus lactis*	Km^R	de Vos, 1987.
pPL608	*Bacillus subtilis*	Km^R	Williams *et al.*, 1981.
pUB110	*Staphylococcus aureus*	Km^R, Pm^R	Keggins *et al.*, 1978.

[1]Abbreviations of markers: Ap^R: ampicillin resistance; Km^R: kanamycin resistance; Pm^R: phleomycin resistance; Sm^R: streptomycin resistance; Ts^R: thiostrepton resistance; Mel: melanin production.

Media

M. aurum strain L1 was grown in liquid medium containing (per litre) 5 g glucose and 3.5 g yeast extract (Oxoid), buffered at pH 7.0 with 50 mM KH_2PO_4/K_2HPO_4 at 30°C. *M. smegmatis* strain mc^2155 was grown in Mycobouillon, which consisted of (per litre) 4.7 g

Middlebrook 7H9 broth (Difco), 5.0 g Nutrient broth (Difco) and 2 ml glycerol 87% at 37°C. After sterilization, 1% (w/v) glucose was added. Agar plates contained 1.5% bactoagar (Oxoid). Mineral agar plates consisted of mineral growth medium, described by Wiegant and de Bont (1980), solidified with 1.5% bactoagar, and were incubated in a desiccator containing 2% ethene. *E. coli* strains DH5α and TG1 were grown on LB agar (Maniatis *et al.*, 1982). Media were, if necessary, supplemented with filter-sterilized antibiotics (20 μg/ml for kanamycin, 50 μg/ml for streptomycin). For abbreviations of antibiotics, see legends to Table 1.

DNA-isolation

Total DNA from *M. aurum* strain L1 was isolated as follows: 100 ml of an early logarithmic phase liquid culture, grown in erlenmeyer flasks at 30°C while shaking at 200 rpm, was supplemented with glycine to a final concentration of 0.2 M. Incubation was continued overnight under the same conditions. The cell suspension was concentrated 150 times in 10 mM Tris/HCl, 1 mM EDTA (pH 8.0) (TE) and 100 μl of it was treated with 0.5 mg lysozyme (Sigma) for 60 minutes at 37°C. Lysis was completed after the addition of SDS to a concentration of 0.7% and incubation at 37°C for 30 minutes. After the addition of 50 μl of 5 M sodium perchlorate, the lysate was phenol/chloroform-extracted and precipitated with 2.5 volumes of cold ethanol. DNA was recovered from the mixture by spooling the precipitated threads out of the liquid with a bent glass rod and dissolved in 50 μl of TE.
Isolation of total DNA from *M. smegmatis* was as described by Ranes *et al.* (1990).

Plasmid DNA was isolated from *M. aurum* strain L1 by alkaline lysis (Birnboim and Doly, 1979) using 1.5 ml of a cell suspension obtained as described above and treated with lysozyme.

Isolation of plasmid DNA from *E. coli* strains was essentially as described by Birnboim and Doly (1979).

Electroporation

All electroporations were done using the BioRad Gene Pulser, coupled to a Pulse Controller Unit.

Electroporation of *M. aurum* was as described previously (Hermans *et al.*, 1990), without any pretreatment of the cells.

Portions of 100 µl of the transformed cell suspension were plated on growth medium agar plates containing 20 µg/ml kanamycin. The minimal inhibitory concentration of *M. aurum* for kanamycin is less than 5 µg/ml. Plates were incubated for 7 to 10 days at 30°C, after which colonies could be counted.

Electroporation of *M. smegmatis* was as described by Ranes *et al.* (1990).

Electroporation of *E. coli* was according to the BioRad Instruction Manual.

Restriction and hybridization experiments

Restriction endonuclease digestions of DNA samples were done as prescribed by the manufacturer (BRL). Blots of total genomic DNA were made using the Southern transfer technique (Maniatis *et al.*, 1982) and Hybond N^+ membranes (Amersham, U.K.). Denatured pJRD215 DNA was labelled with [^{32}P] α-dATP using the Boehringer Random Primed Labelling Kit (Boehringer, F.R.G.). Hybridizations were done according to the Amersham Hybond N^+ instruction manual.

Determination of plasmid stability in transformants

Single transformant colonies (*M. aurum* and *M. smegmatis*) were streaked on kanamycin agar and incubated. Single colonies were picked and inoculated into 5 ml of growth medium without antibiotic. After one day of incubation at the growth temperature, which corresponds to 3 generation times of *M.aurum*, and 4 generation times of *M. smegmatis*, 100 µl of the suspensions was inoculated into 5 ml of fresh growth medium. This was repeated 7 times, whereafter appropriate dilutions of the suspension were plated out on growth medium agar without antibiotic. After growth, 100 colonies from these plates were stabbed onto medium with 20 µg/ml kanamycin, the percentage of kanamycin resistance was determined, and taken as a measure for plasmid stability.

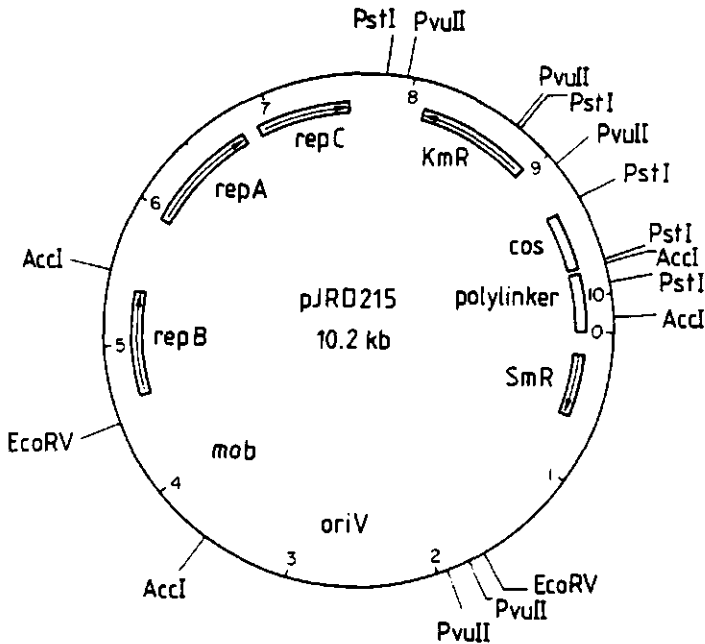

Figure 1. Map of the cosmid pJRD215. Only relevant restriction sites and other structures are shown. Abbreviations: cos: phage Lambda cohesive ends; KmR: kanamycin resistance gene; mob: plasmid mobilization functions; *ori*V: origin of replication; *rep*A, *rep*B, *rep*C: RSF1010 replicons; SmR: streptomycin resistance gene. From Davison *et al.* (1987); Scholz *et al.* (1989).

Results

Transformation of M. aurum strain L1 by electroporation

Cells of *M. aurum* strain L1 were transformed with pJRD215 (Fig.1.), as described in the Experimental Procedures section. Transformants were selected on kanamycin or streptomycin agar after 7 to 10 days of incubation at 30°C, which is slightly longer than the normal colony-forming time for untransformed strain L1 on antibiotic-free medium (7 days). Scoring for kanamycin resistance, strain L1 was transformed at an efficiency of $2 \cdot 10^2$ per microgram, as determined from averaging the results of 3 independent experiments. The minimal inhibitory

concentration (MIC) of *M. aurum* for kanamycin is less than 5 µg/ml. The streptomycin-resistance gene carried by pJRD215 was also expressed in strain L1, where 20 transformants per microgram of pJRD215 DNA were obtained. Also, the kanamycin resistant transformants appeared to be resistant to 50 µg/ml streptomycin. The MIC of strain L1 for streptomycin is less than 10 µg/ml.

In order to determine if other plasmids are capable of transforming *M. aurum*, a series of plasmids originating from Gram-positive organisms was used to transform *M. aurum* under the same conditions as pJRD215 (Table 1). In the case of multiple resistance determinants, transformants were selected on either antibiotic. In several independent experiments, successful transformation was achieved only with pAL8 (Hermans *et al.*, 1990).

Analysis of M. aurum transformants

To distinguish between true transformants and spontaneous kanamycin resistant mutants, we analyzed the tobramycin-resistance of transformants. Tobramycin is a kanamycin-like antibiotic, insensitive to the aminoglycoside phosphotransferase produced by the kanamycin-resistance gene. Spontaneous kanamycin-resistant mutants are likely to be resistant to tobramycin, whereas transformants are not. All kanamycin resistant colonies were sensitive to 10 µg/ml tobramycin.

Determination of the occurrence of pJRD215 in M. aurum strain L1

To establish the state of the vector within the mycobacterial host, DNA was extracted from one transformant colony and characterized in several ways.

In a first approach, total genomic DNA from the transformant was digested to completion with *Pst*I. Blots of genomic DNA were then hybridized with *Pst*I digested radioactively labelled pJRD215 DNA. The resulting autoradiograms clearly showed the presence of all of the pJRD215/*Pst*I bands, indicating that the cosmid is present in the mycobacterial host, without any extensive rearrangements (Fig.2.). The hybridization on the smallest band of 140 basepairs (very faintly visible in lane 4) is too weak to be seen in lane 2 at this exposure time. This band was however seen in autoradiograms that were exposed for a longer period of time. All the other bands expected in this digest are visible. Furthermore, the hybridization of the labelled cosmid on undigested total genomic DNA from the transformant (lane 1) showed that the cosmid is not integrated into the mycobacterial chromosome. From a superficial comparison of the

hybridization intensity of the DNA in lanes 2 and 4, we concluded that the copynumber of the cosmid is less than five copies per genome. The hybridization in lane 3 (untransformed strain L1) is caused by unspecific binding of the probe to the relatively large amount of DNA present.

Secondly, we attempted to extract pJRD215 directly. However, using the plasmid isolation procedure described in the Experimental Procedures section, pJRD215 could not be isolated. This failure was probably due to the low copy number of the cosmid in its new host. Therefore, we tried to isolate cosmid DNA by centrifuging total DNA of the transformant to equilibrium in a caesium chloride/ethidium bromide density gradient. This gradient showed a faint ccc-band, which after isolation appeared to be pJRD215 as demonstrated by gel electrophoresis.

To rule out the possibility of contamination, transformant colonies were tested in the following ways. After microscopic identification of its mycobacterial morphology, strain L1 transformants were shown to be able to grow on mineral agar plates supplemented with 20 μg/ml kanamycin and incubated in a desiccator containing 2% ethene. Furthermore, the banding pattern of total DNA from the original strain, digested with *Pst*I, was virtually identical to the pattern of total DNA isolated from the transformants, cut with the same enzyme (not shown). This is a reliable method of strain identification in several mycobacterial species (Whipple *et al.*, 1987), and hence was taken as additional evidence for the identity of strain L1.

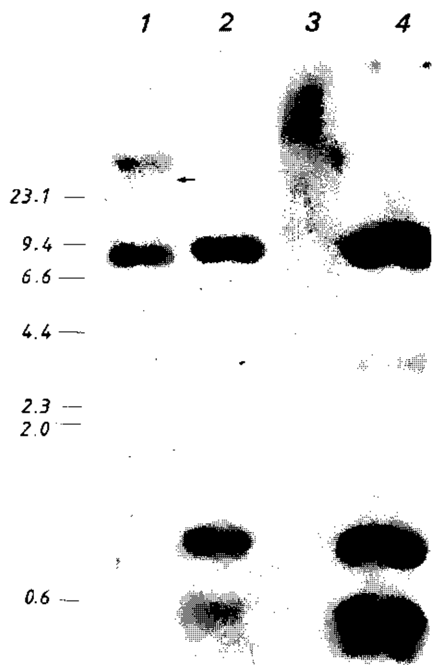

Figure 2. Hybridization pattern of total genomic DNA from *M. aurum* strain L1, probed with *Pst*I digested, ^{32}P-labelled pJRD215. Lanes: 1. 5 μg undigested DNA from transformant; 2. 5 μg *Pst*I digested DNA from transformant; 3. 10 μg *Pst*I digested DNA from untransformed strain L1; 4. 50 ng *Pst*I digested pJRD215. The faint band at about 3 kb in lane 4 is caused by an artifact. The position of Lambda/*Hin*DIII size marker fragments is indicated in the left margin. Fragmentsizes for *Pst*I digest of pJRD215: 8150, 930, 550, 430, and 140 basepairs. Arrow: position of the genomic DNA band in lane 1.

Re-isolation of pJRD215 from E. coli transformants

Total DNA was isolated from a liquid culture derived from one transformant *M. aurum* colony. Cells of *E. coli* DH5α were transformed by electroporation with this DNA and plated on Km-containing agar plates. Plasmid DNA was then isolated from Km-resistant *E. coli* DH5α colonies and identified by restriction enzyme analysis. About 50% of the preparations showed the pattern of fragments expected for pJRD215 after *Pst*I digestion, but the other colonies appeared to harbour a modified plasmid. From the fact that the small *Pst*I-bands were lost from the digest, we concluded that the apparent modification must be a deletion in the region of these fragments. To determine the location of the deletion more precisely, restriction experiments with *Acc*I,

*Eco*RV and *Pvu*II were performed (Fig.3.). In this way, the deletion was mapped between the *Pst*I-site on position 9.4 and the *Acc*I-site on position 10.1.

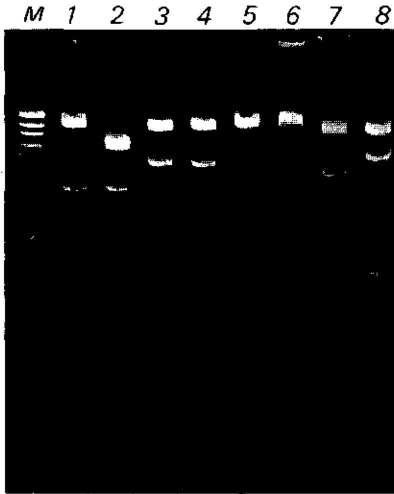

Figure 3. Restriction enzyme digestion patterns of plasmid DNA extracted from 2 representative *E.coli* DH5α colonies transformed with total genomic DNA from *M. aurum* strain L1 transformant. Odd lane numbers: deleted plasmid; even lane numbers: intact plasmid. Lanes 1, 2: *Acc*I; lanes 3, 4: *Eco*RV; lanes 5, 6: *Pst*I; lanes 7, 8: *Pvu*II. M: Lambda/*Hin*DIII size marker.

Transformation of M. smegmatis strain mc²155

Following the successful transformation of *M. aurum* strain L1, we tested the procedure with *M. smegmatis* strain mc²155, a representative for fast-growing mycobacteria. Per microgram of pJRD215 $5 \cdot 10^2$ kanamycin resistant transformants were obtained. The MIC of strain mc²155 for kanamycin is less than 1.5 μg/ml, and the transformants were resistant to 20 μg/ml. The streptomycin resistance gene of pJRD215 was expressed in *M. smegmatis* (resistance to 50 μg/ml streptomycin, the MIC is less than 1.5 μg/ml). Streptomycin-phosphotransferase expression was detected in the transformants (J.Timm, personal communication).

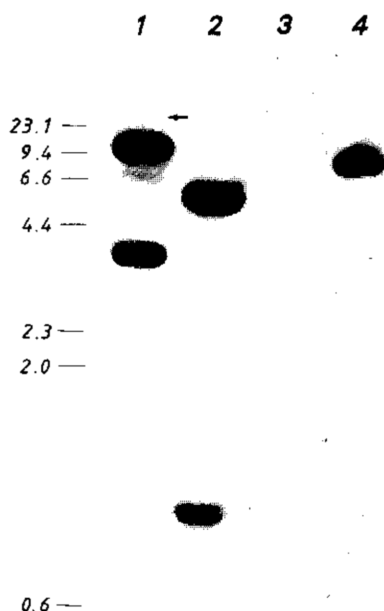

Figure 4. Hybridization pattern of total genomic DNA from *M. smegmatis* strain mc^{2}155, probed with *Pst*I digested, ^{32}P-labelled pJRD215. Lanes: see legend to Fig. 2. The difference of hybridization intensity of the small bands in lane 4 compared with the corresponding bands in Fig. 2 is caused by overexposition of these bands in Fig. 2. The band at about 3 kb in lane 4 is caused by an artifact.

As can be seen from Fig. 4, isolation of total DNA from the transformants and subsequent hybridization with labelled pJRD215 on blots of undigested as well as *Pst*I digested DNA confirmed the presence of pJRD215 DNA in strain mc^{2}155. The absence of three of the five expected bands as well as the fact that the upper band was about 1 kilobase smaller than expected, again indicated that rearrangement of pJRD215 had taken place. This was confirmed by the isolation of genomic DNA from 12 transformant colonies of strain mc^{2}155, and investigation of the hybridization patterns of undigested and *Pst*I digested DNA (not shown). These patterns were all similar to the one shown in Fig. 4, with the exception of one colony, where the small bands were very faintly visible. Retransformation of *E. coli* strain TG1 with total genomic DNA from

this colony showed the rearranged pattern in 6 out of 10 colonies, whereas 4 colonies showed the original *Pst*I pattern of pJRD215 (results not shown).

Determination of the stability of pJRD215 in the transformants

Transformants of *M. aurum* and *M. smegmatis* were grown in antibiotic-free medium for 40 to 50 generations and resistance to kanamycin was determined. In the case of *M. aurum* 94% of the cells had retained resistance to kanamycin, and in the case of *M. smegmatis* 90%.

Discussion

The cosmid pJRD215 is derived from RSF1010, a wide-host-range IncQ plasmid, that replicates in most, if not all, Gram-negative bacteria (Frey and Bagdasarian, 1989). Besides most of the RSF1010 genome, the cosmid contains the phage lambda *cos* site and the Tn5-derived kanamycin resistance gene, while the RSF1010 streptomycin resistance gene was put under the control of the promoter of the pBR322 tetracycline resistance gene (Fig.1.; Davison *et al.*, 1987).

As demonstrated here, pJRD215 is expressed in *M. aurum* and *M. smegmatis*, rendering transformants resistant to kanamycin and streptomycin. It was maintained as a plasmid in both organisms, but after transformation of *E. coli* with total DNA from *M. aurum* and *M. smegmatis* transformants, deletion of part of the cosmid in about half of the transformants was seen. In *M. aurum*, the deleted part was shown to comprise the *cos* site and the polylinker-region. The RSF1010-derived replication functions and the *rep*A-, *rep*B- and *rep*C-genes remain unaffected. These elements are required for replication of RSF1010 in *E. coli* (Scholz *et al.*, 1989). Preliminary experiments with the parental plasmid RSF1010 suggest that it is structurally stable when introduced into *M. smegmatis* (data not shown).

The precise mechanism of the deletion event remains to be investigated, but from the fact that it occurs in a very reproducible manner, it might be concluded that some recombination mechanism is involved.

Both the kanamycin and the streptomycin resistance genes of pJRD215 are expressed in the mycobacterial transformants, a strong indication for functionality of these Gram-negative

promoters in mycobacteria. The finding that the Tn5-derived kanamycin resistance gene is expressed in *Mycobacterium* is in agreement with the results of Snapper *et al.* (1988), who found that the same gene, contained in pIJ666-pAL5000 constructs, was expressed in *M. smegmatis* strain mc^{2}6. The expression of the RSF1010-derived streptomycin resistance gene, under the control of the pBR322 tetracycline promoter, has not been described before. Chater *et al.* (1982), however, describe the expression of the tetracycline resistance gene of pBR322 in *Streptomyces* sp., indicating that its promoter can also be recognized in Gram-positive bacteria.

The efficiency of transformation is too low to permit use of the cosmid as a cloning vehicle for the shuttling of recombinant DNA between *E. coli* and *M. aurum*. In fact, the transformation efficiency of pAL8 in *M. aurum* is a factor 10 higher than pJRD125. However, possibilities exist to enhance the transformation efficiency of pAL8 in *M. aurum* (Hermans *et al.*, 1990), that should be applicable to this system too.

pJRD215 has a low copynumber in *M. aurum*; this seems to be a property of pJRD215, as its copynumber is low in *E. coli* too.

The understanding of the molecular biology of mycobacterial species, both pathogenic and non-pathogenic, could benefit greatly from the development of suitable vector systems that allow direct cloning in *Mycobacterium* sp. and shuttling of DNA between *E. coli* and *Mycobacterium* sp. 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. However, care should be taken with the introduction of the streptomycin-resistance gene into pathogenic mycobacterial species, since streptomycin is one of the first- or second-line drugs used in the treatment of tuberculosis.

The finding that a Gram-negative broad-host-range cosmid can be introduced and maintained in *M. aurum* and *M. smegmatis* might be an important contribution to this field, as it opens up the possibility to shuttle recombinant cosmid DNA with large inserts directly between *E. coli* and *Mycobacterium*. As none of the Gram-positive plasmids tested (Table 1), except the mycobacterial plasmid pAL8, would transform *M. aurum*, this finding is of even greater value, as vectors for these organisms apparently are scarce.

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Chapter 5

TRANSFORMATION OF GRAM-POSITIVE MICROORGANISMS WITH THE GRAM-NEGATIVE BROAD-HOST-RANGE COSMID VECTOR pJRD215

J. Hermans, I.M.L. Suy, and J.A.M. de Bont

A number of Gram-positive bacterial species of biotechnological importance was screened for the expression of the Gram-negative broad-host-range cosmid pJRD215, previously shown to be expressed in *Mycobacterium aurum* and *Mycobacterium smegmatis*. As judged from acquired kanamycin or streptomycin resistance, pJRD215 was expressed in 5 out of 10 species tested: *Bacillus subtilis*, *Brevibacterium* sp., *Corynebacterium glutamicum*, *Lactococcus lactis* subsp. *lactis* and *Mycobacterium parafortuitum*.

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Introduction

Many bacteria of biotechnological importance belong to Gram-positive species, which are relatively difficult to access at a molecular level. The advent of the technique of electroporation has greatly facilitated the introduction of DNA into these species and to date many examples of the transformation of Gram-positive microorganisms with plasmids originating from the same or related species have been published. The development of new vectors to genetically manipulate Gram-positive bacteria is, however, still very desirable in view of the limited number of tools available in the transformation of Gram-positive organisms.

For *Bacillus subtilis*, generally regarded as a model system for the genetic engineering of Gram-positive organisms, and frequently used as a heterologous host because of its secreting capabilities, a few vector plasmids have been developed, mainly with the aim of expressing foreign proteins in *B. subtilis* (Okamoto & Omori, 1986).

Coryneform bacteria like *Brevibacterium lactofermentum* and *Corynebacterium glutamicum* are widely used in the production of amino acids. Although vector systems have been developed for the study and engineering of these organisms, the need for new tools still exists (Yoshihama *et al.*, 1985; Yeh *et al.*, 1986; Cadenas *et al.*, 1991).

The group of lactic streptococci, including *Lactococcus lactis* subsp. *lactis*, has numerous applications in the manufacture of a variety of fermented dairy products. In this group a variety of plasmid vectors has been developed, mainly based on the plasmids which abundantly occur in these species (de Vos, 1987; Xu *et al.*, 1991).

The genus *Mycobacterium* is notorious for its pathogenic members *Mycobacterium leprae* and *M. tuberculosis*. These are subject of extensive studies at the molecular level, with the aims of unravelling the mechanisms of pathogenicity and of developing new and better vaccines against mycobacterial diseases (Rastogi & David, 1988). The non-pathogenic, soil-dwelling group of mycobacteria (e.g. *M. aurum, M. fortuitum*) is of biotechnological importance in the processing of sterol side chains for the production of steroid hormones (Martin, 1984) and for its ability to produce epoxides from lower alkenes in a stereospecific way (Hartmans *et al.*, 1989). At present, a few vector systems are available for these species, based either on mycobacteriophage DNA (Snapper *et al.*, 1988) or on a *M. fortuitum* cryptic plasmid (Ranes *et al.*, 1990). These vectors, however, are very large and difficult to manipulate.

Recently it was shown that the Gram-negative cosmid vector pJRD215 is able to replicate in Gram-positive *Mycobacterium aurum* and *Mycobacterium smegmatis* after transformation (Hermans *et al.*, 1991). This finding has implications for the study of mycobacterial genetics, of both the pathogenic and the non-pathogenic members of the genus. It provides a vector for direct shuttling of DNA between *Escherichia coli* and *Mycobacterium* sp. by which the cloning and engineering of mycobacterial DNA and the subsequent expression in the natural host can be greatly facilitated.

The cosmid pJRD215 was constructed on the basis of the broad-host-range plasmid RSF1010, belonging to the class of IncQ-plasmids, found in many different Gram-negative bacteria (Frey & Bagdasarian, 1989). The Tn5-derived kanamycin-resistance gene was inserted into RSF1010, together with a restriction site bank and the phage Lambda *cos*-site (Davison *et al.*, 1987).

The expression of this Gram-negative cosmid in a Gram-positive bacterium led us to believe that it might be possible to express this cosmid in other Gram-positive species as well. In particular, the study of the genetic systems involved in biotechnological processes might benefit from the use of pJRD215 as a shuttle vector. We therefore decided to screen a number of Gram-positive species for transformation with pJRD215.

Material and methods

Bacterial strains and growth media

The bacterial strains used in this study and their growth media are listed in Table 1.

Identification of microorganisms

Apart from morphological characterization by microscopic appearance, the strains were tested by Gram staining and Ziehl-Neelsen staining (Doetsch, 1981), before and after transformation.

Table 1. Bacterial strains used for transformation with pJRD215.

Organism	Origin[a]	Growth medium[b]
Bacillus subtilis	PBG 1-38	1.
Brevibacterium sp.	DSM 1412	1.
Corynebacterium glutamicum	DSM 20300	2.
Lactobacillus casei	DSM 20011	4.
Lactococcus lactis subsp. *lactis*	DSM 20250	4.
Mycobacterium fortuitum	DSM 43074	3.
Mycobacterium parafortuitum	LC E3	3.
Mycobacterium phlei	DSM 43239	3.
Nocardia sp.	DSM 43191	4.
Rhodococcus erythropolis	DSM 43296	4.
Rhodococcus rhodochrous	DSM 43273	4.

[a]Origin of strains: PBG: Phabagen Collection, Utrecht, the Netherlands; DSM: Deutsche Sammlung von Mikroorganismen, Goettingen, FRG; LC: Laboratory Collection.
[b]Growth media (per liter):
1.: Peptone, 5 g; Meat extract, 3 g; pH 7.0.
2.: Casein-peptone (tryptic digest), 10 g; Yeast extract, 5 g; glucose, 5 g; NaCl 5 g; pH 7.3.
3.: Middlebrook 7H9 broth, 4.7 g; Nutrient broth, 5 g; Glucose, 1 g; glycerol 1 ml; pH 7.0.
4.: Yeast extract, 4 g; Glucose, 4 g; Malt extract, 10.0 g.; pH 7.2.

Electroporation

Electroporation of all strains was done essentially as described previously (Hermans *et al.*, 1990). Logarithmically growing cultures of all strains (250 ml) were harvested by centrifugation at 4°C, washed twice in cold electroporation buffer (EPB, 20% sucrose, 7 mM HEPES, 1 mM $MgCl_2$), and resuspended in 1/100 volume of cold EPB. 100 μl portions of this suspension were mixed with 2 μl of pJRD215 preparation (2 μg) in a cold 2 mm electroporation cuvette and electroporated using the BioRad Gene Pulser connected to a Pulse Controller. Electroporation conditions were as follows: capacitance 25 μF, field strength 12.5 kV/cm, 200 Ω parallel resistance. These settings generally gave retention time constants of 3 to 5 msec. Immediately after

electroporation the suspension was diluted 10-fold with growth medium and incubated at 30°C for 2 to 3 hours. Excepted from this protocol were *B. subtilis* (field strength 3.0 kV/cm) and all mycobacterial strains (incubation time after electroporation 16 hours).

Appropriate dilutions of the cell suspension before and after the pulse were plated onto growth medium agar without antibiotic, for strain identification and to determine initial cell number and survival rate, and onto growth medium agar supplemented with antibiotics in appropriate concentrations (see RESULTS), to determine the number of spontaneous resistant mutants (before the pulse) and transformants (after the pulse).

Results and discussion

Eleven Gram-positive microorganisms (Table 1) were selected to study the possible use of pJRD215 as a shuttle vector. In preliminary experiments, the natural resistance of these bacteria to kanamycin and streptomycin was determined. Log-phase cultures of the strains were streaked onto a series of growth medium agar plates containing antibiotic in a concentration gradient from 10 to 200 μg/ml in steps of 10. Depending on the strain, growth was monitored after 1 to 2 weeks of incubation at 30°C. *L. casei* was resistant to both kanamycin and streptomycin at 200 μg/ml and was not used in further experiments. All other strains appeared to be sensitive to the lowest concentration of streptomycin (10 μg/ml), whereas with kanamycin in *M. fortuitum*, *Nocardia* sp. and *R. rhodochrous* growth was inhibited by 20 μg/ml, and with *R. erythropolis* by 90 μg/ml. Therefore, all selections were done at 30 μg/ml streptomycin or kanamycin, except with *R. erythropolis* (kanamycin 100 μg/ml). No spontaneous antibiotic resistant mutants were found, except with *R. rhodochrous*, where cells developed resistance to streptomycin at a rate of $2*10^{-8}$.

Electroporation of cell suspensions generally resulted in cell survival rates ranging between 25 % and 100 % (Table 2). In the case of *B. subtilis*, a field strength of 3.0 kV/cm was applied, since the standard strength of 12.5 kV/cm gave a survival rate of only 2 %. At 3.0 kV/cm, the survival rate was 25 %, which is in agreement with the findings of Brigidi *et al.* (Brigidi *et al.*, 1990), who could not detect any transformant *B. subtilis* with field strengths higher than 10 kV/cm.

Brevibacterium sp. and *L. lactis* subsp. *lactis* appeared to be unaffected by the electroporation conditions, as can be concluded from their survival rates of 100 %.

As can be seen from Table 2, transformation was successful for 5 of the organisms tested. Electroporated cells from one experiment were screened for transformants on kanamycin and streptomycin separately, rendering greatly varying numbers for transformation frequency and efficiency.

Table 2. Transformation survival, frequencies and efficiencies for electroporation of several bacterial strains with pJRD215.

Organism	Survival[a] (%)	Frequency		Efficiency	
		Km	Sm	Km	Sm
B. subtilis	25.0	0.0	65.0	0.0	37.0
Brevibacterium sp.	100.0	0.0	0.48	0.0	500.0
C. glutamicum	64.0	3.4	0.23	108.0	8.0
L. lactis subsp. *lactis*	100.0	5.4	0.1	1500.0	20.0
M. fortuitum	28.0	0.0	0.0	0.0	0.0
M. parafortuitum	25.0	120.0	52.0	300.0	30.0
M. phlei	68.0	0.0	0.0	0.0	0.0
Nocardia sp.	49.0	0.0	0.0	0.0	0.0
R. erythropolis	42.0	0.0	0.0	0.0	0.0
R. rhodochrous	50.0	0.0	0.0	0.0	0.0

[a]Survival: percentage of viable cells after electroporation; Frequency: number of transformants per number of cells (10^{-8}); Efficiency: number of transformants per microgram of pJRD215-DNA. Frequency and efficiency values shown are corrected for the rate of development of spontaneous resistance to antibiotics.

With *B. subtilis* and *Brevibacterium* sp. transformants were found only on streptomycin containing plates. As the frequency of spontaneous streptomycin-resistance was negligible, these cells were taken to be transformed with pJRD215.

Transformants of *C. glutamicum* were resistant to both kanamycin and streptomycin, whereas the closely related *Brevibacterium* sp. expressed only streptomycin resistance after transformation.

The difference in resistance to the two antibiotics of the transformed strains is probably due to differences in the stability of the gene products from the kanamycin and streptomycin resistance genes.

Transformant colonies, if they appeared, were identified by microscopic examination, Gram stain, and in the case of *Mycobacterium* sp., Ziehl-Neelsen stain. Transformant colonies appearing on plates containing one antibiotic, were restreaked on the same medium and on medium containing the other antibiotic. In all cases this led neither to the loss of the resistance nor to a new resistance emerging.

Transformant colonies were grown in 10 ml of antibiotic-containing growth medium, harvested and lysed by standard procedures. 10 μg of undigested total genomic DNA was separated by electrophoresis on an agarose-gel, blotted and hybridised to radiolabeled pJRD215 DNA by standard procedures (Sambrook *et al.*, 1989). In all cases the presence of pJRD215-sequences in the transformants was conclusively demonstrated after autoradiography (not shown).

From the above results it appears that the Gram-negative cosmid pJRD215 may be used as a shuttle vector not only in *M. aurum* and *M. smegmatis* (Hermans *et al.*, 1991), but also in several other Gram-positive organisms. Surprisingly it was also found that, contrary to mycobacterial species *M. aurum*, *M. parafortuitum* and *M. smegmatis*, two other species *M. fortuitum* and *M. phlei* could not be made accessible for to genetic work by using pJRD215.

So far, no other reports of expression of pJRD215 in Gram-positive bacteria have been published, whereas Gormley and Davies (1991) reported the conjugative transfer of RSF1010 from *Escherichia coli* to *Streptomyces lividans* and *Mycobacterium smegmatis*.

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Chapter 6

GENERAL DISCUSSION - CONCLUDING REMARKS

Members of the genus *Mycobacterium* are the causative agents of two of the worlds most threatening diseases. As outlined in chapter 2, research in mycobacteriology mainly focuses on unravelling the mechanisms of virulence and resistance to antibiotics, with the aim of developing new vaccines. With respect to biotechnological applications, mycobacteria are of growing interest as well, since they are involved in the degradation of recalcitrant organic environmental pollutants, as well as in the production of pharmaceutically interesting compounds.

Despite the importance of this bacterial genus, molecular research in this field still is impeded by the inaccessibility of the species of the genus. Although much progress has been made in recent years, much effort is still devoted to the development of methods and techniques to access the mycobacteria. In this thesis some contributions related to transformation and vector development have been summarized.

Transformation of *Mycobacterium aurum*

Before the upcome of electroporation, very few reliable procedures for transformation of mycobacteria were available. Compared to other bacterial species, transformation rates were low. Procedures for transformation of mycobacteria were generally developed and optimized for one particular species, and involved degradation of the cell wall to render competent cells, spheroplasts or protoplasts. Unfortunately these procedures appeared to be unfit for transformation of other mycobacterial species. The reason for this specificity is not known, but might be found in differences in the architecture of the cell wall between species or different requirements for regeneration of the wall-deficient cells. As the cell wall is a primary characteristic for species determination in *Mycobacterium*, hence differs much between species, this is not an unlikely explanation (Wayne & Kubica, 1986).

Transformation of *M.aurum*, which was the first objective of the work presented here, was unsuccessful using several of these 'classical methods' (Rastogi *et al.*, 1983; Sadhu & Gopinathan, 1982; Udou *et al.*, 1983), even after we developed a procedure to isolate and regenerate spheroplasts from *M.aurum* (Hermans & de Bont, 1987). The plasmid used to monitor the transformation was the mycobacterial plasmid pAL8 (Ranes *et al.*, 1990), that was later successfully transformed and expressed in *M.aurum* using electroporation. Therefore, the failure of transformation of *M.aurum* spheroplasts was attributed to the low regeneration rate of the spheroplasted cells, which was also obvious from cell counts (Hermans & de Bont, 1987).

The application of the technique of electroporation to the transformation of *Mycobacterium* was a big step forward in the molecular study of mycobacteria. It caused the breakthrough that was needed to speed up the development of methods and techniques in this field, and it initiated the construction of many new plasmid-, phasmid- and cosmid vectors (chapter 2.).

Although many mycobacterial species are transformable by electroporation 'as they are', transformation rates can be optimized by pretreatment of the bacterial cells. In chapter 3 the optimization of a transformation procedure employing electroporation for *M.aurum* is described. By treating the cells with cell wall degrading agents, either incorporated in the culture medium or administered to the harvested cells, transformation frequency and efficiency was optimized. This method, applicable to other mycobacterial species as well, was adopted by other researchers to enhance transformation efficiency of mycobacteria (C.Martin, personal communication) as well as other species (Pigac & Schrempf, 1995). The latter authors describe the transformation of several species of *Streptomyces* by electroporation. As in our procedure, transformation efficiency could be greatly enhanced by pretreatment of the cells with a cell wall degrading agent, lysozyme. The fact that lysozyme treatment in our case rendered no living cells after electroporation could be due to the fact that Pigac and Schrempf apply a tenfold lower concentration of lysozyme to the harvested *Streptomyces* sp. However, the concentration of lysozyme that we use was determined empirically to render an optimal spheroplast concentration, while a tenfold lower concentration showed no effect (chapter 3).

Obviously, this work stressed the significance of the cell wall as a barrier to the transport of macromolecules into the cell, and the importance of partially removing it to enhance transformation efficiency, not only in *Mycobacterium*.

Vectors for *Mycobacterium* sp

As described in chapters 2 and 3 of this thesis, the optimization of electroporation as a standard technique for transformation of mycobacteria was possible only after construction of plasmids that could be expressed in *Mycobacterium*. One of these plasmids, pAL8, derived from the *M.fortuitum* endogenous plasmid pAL5000 (Ranes *et al.*, 1990), was used to transform *M.aurum*, *M.bovis* and *M.smegmatis*. It proved its importance by providing the mycobacterial replicon for construction of many vectors (chapter 2) and rendering the tool needed for optimization of electroporation procedures (chapter 3).

The search for other plasmids that were able to transform *M.aurum* concentrated on a range of vectors that originated from other Gram positive bacteria. In chapter 4 the attempted transformation of *M.aurum* with seven plasmids and one cosmid is reported. Of these, only the mycobacterial plasmid pAL8 and, surprisingly, the Gram-negative broad-host-range cosmid pJRD215 (Davison *et al.*, 1987) was successful.

The transformation of *M.aurum* with pJRD215 was the first report of expression of a Gram-negative vector in *Mycobacterium*. pJRD215 or RSF1010, from which pJRD215 is derived, was subsequently transformed to other Gram-positive bacteria as well. Among these are many bacterial species of biotechnological interest: *Bacillus subtilis*, *Brevibacterium* sp., *Corynebacterium glutamicum*, *Lactococcus lactis*, and *Mycobacterium parafortuitum*, all described in chapter 5.

Based on the work described in this thesis, other authors set out to test the feasibility of using these plasmids for cloning in other Gram-positive systems. Recently, the transformation of *Actinomyces* spp. with pJRD215 (Yeung & Kozelsky, 1994) and *Brevibacterium methylicum* with RSF1010 (Nesvera *et al.*, 1994) were reported. Attempts to express pJRD215 in *M.fortuitum* and *M.phlei* however were unsuccessful, as well as the transformation of *Nocardia* sp., *Rhodococcus erythropolis*, and *Rhodococcus rhodochrous* (chapter 5). Clearly, the expression of pJRD215 or RSF1010 depends on species-specific mechanisms that need to be investigated further.

Being a cosmid, pJRD215 is particularly suited for cloning of large inserts. The observed rearrangement of pJRD215 in *M.smegmatis*, described in chapter 4, might hamper its applicability, as *M.smegmatis* is frequently used as alternative host species for the cloning of genes from pathogens. In contrast, no alterations in plasmid structure were detected after transformation

to *M.aurum* (chapter 4) and *Actinomyces* spp. (Yeung & Kozelsky, 1994). Therefore, the use of *M.aurum* as alternative host, in which pJRD215 appears to be stably maintained (chapter 4), might overcome this problem.

From this work it became clear that RSF1010 (IncQ) derived plasmids are important donors of replicons for the construction of new Gram-positive vector plasmids. It initiated research into the applicability of pJRD215 and related plasmids for the construction of hybrid shuttle vectors from Gram-negative and Gram-positive vectors (Gormley & Davies, 1991; Qin *et al.*, 1994). As reviewed in chapter 2, much effort still is spent on development of new heterologous vectors.

Sequencing of Mycobacterial DNA

As outlined in this thesis, the slow growth rate and thick waxy cell wall of mycobacteria have impeded molecular research using standard techniques. The high GC-content of mycobacterial DNA is another burden that mycobacteriologists have to cope with, mainly in DNA-sequencing experiments. Due to the formation of strong secondary structures such as intrastrand hairpin loops in GC-rich regions, the DNA-polymerase enzyme is blocked during the sequencing reactions, causing a band in all four lanes of the sequencing gel. Furthermore, the hairpin loops that form in the single stranded reaction products before or during electrophoresis, cause mobility compressions in the sequencing-gel.

The polymerase chain reaction (PCR) for amplification of DNA, using the heat-stable enzyme *Taq*-polymerase (Saiki *et al.*,1988), has had its offspin to various molecular techniques, among which DNA-sequencing (Murray, 1989). The use of *Taq*-polymerase in a cyclic version of the dideoxy-chain-termination method for sequencing DNA (Sanger *et al.*, 1977), has many advantages, summarized by Rao (1994).

In search of a method for sequencing DNA from *M.aurum*, it was investigated whether this method of cyclic denaturation and elongation might help in sequencing the extremely GC-richt regions we found in *M.aurum* DNA.

From comparison of this method of sequencing with the standard dideoxy-chain termination method with incorporated ^{32}P-dATP (Sambrook *et al.*,1989), it was clear that band resolution improved very much, and GC-rich sequences could be easily read from the gel. In contrast,

band compressions and 'strong stops' hampered the unambiguous determination of the sequence in the standard method.

By optimizing electrophoresis periods, it was possible to read up to 500 bases from one gel, which also showed a more uniform distribution of band-intensity and a lower background of non-specific banding compared to other sequencing methods. Combined with the obvious advantages of short reaction times and employment of standard equipment and techniques, this method might be a valuable tool in mycobacterial genetic studies (Hermans, 1995).

Concluding remarks

Mainly aimed at the pathogenic species *M.tuberculosis* and *M.leprae*, the research in the field of mycobacteriology might have benefits for the biotechnological application of mycobacteria as well. As outlined in the introduction of this thesis, non-pathogenic mycobacteria might find application in the production of chiral epoxides of lower alkenes. But as with many bacterial production systems, manipulation of the genes involved in these processes is a prerequisite to achieve higher production rates, which in turn are needed to make these systems economically interesting.

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SUMMARY

The bacterial genus *Mycobacterium* belongs to the group of Gram-positive bacteria and holds many species, displaying a broad spectrum of properties. About one third is pathogenic to humans or animals, the rest of the mycobacterial species being relatively harmless soil- or water dwellers. Among the pathogenics are notorious species such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*. On an annual basis, 3 million people die of tuberculosis, and 15 million suffer from leprosy. Therefore the pathogenic mycobacteria are subject of intensive study, with as main goals the understanding of the processes of infection and the development of medication and vaccination.

But also the non-pathogenic mycobacteria are subject of intensive study. Some species are able to degrade recalcitrant organic compounds, which makes them interesting for environmental applications. The biotechnological production of valuable intermediates in the synthesis of pharmaceuticals might benefit as well from the application of mycobacteria.

Unfortunately, the genus *Mycobacterium* is not very cooperative. On the one hand there are obvious intrinsic problems in the working with pathogenic microorganisms, on the other hand, mycobacteria are relatively inaccessible to the standard microbiological methods. This is for a great part caused by the presence of a thick cell wall, which impedes the standard methods for the introduction of modified DNA-molecules (transformation) from being applied. Furthermore, the fact that *Mycobacterium* is evolutionarily distant from bacterial species as *Escherichia coli* and *Bacillus* sp., for which most molecular biological techniques are developed, causes the incompatibility of DNA-vectors between *Mycobacterium* and *E.coli*.

Chapter 2 of this thesis gives an overview of recent developments in these areas of research. The development of transformation techniques and DNA-vectors is described in detail. The development of DNA-vectors exhibits a clear tendency from species-specific towards so-called broad-host-range vectors, that can maintain themselves in other bacterial species as well.

Chapter 3 reports on the development of a transformation technique for *Mycobacterium aurum*, based on electroporation of mycobacteria with intact cell walls. Employment of this

technique rendered the solution to the problems encountered when trying to transform *M.aurum* in the 'classical' way, even after partial removal of the cell wall. With these 'spheroplasts' the regeneration of the cell wall appeared to be the barrier to successful transformation.

Transformation of bacteria using the technique of electroporation is based on the submission of a suspension of bacteria to a strong electrical field for a short period of time. In this way the cell membranes become transiently permeable to macromolecules. The plasmid employed, pAL8, originated from a closely related *Mycobacterium* species, enhancing the prospect of successful expression in *M.aurum*. Indeed, pAL8 could maintain and express itself in *M.aurum*, thus enabling the optimization of the transformation technique for *M.aurum*. By treating the cells with wall degrading agents prior to electroporation, transformation yields could still be enhanced.

Unfortunately, pAL8 is not very suitable as a vector for the transport of manipulated genes (cloning). Therefore, further investigations were aimed at finding suitable vectors for the cloning of mycobacterial genes. In a first approach a series of plasmids from related Gram-positive microorganisms were tested for transformation capability of *M.aurum*. Surprisingly, the vector plasmid that was least related to *Mycobacterium* was the only one to be expressed in *M.aurum*. This plasmid, pJRD215, is a construct based on a so-called broad-host-range plasmid, which is found in many Gram-negative bacterial species, none of which is related to *Mycobacterium*. In fact, this was the first report of the transformation of a Gram-positive bacterium with a Gram-negative broad-host-range plasmid.

Many bacterial species involved in biotechnological processes are Gram-positive, and might be transformed with pJRD215 as well. Chapter 5 reports the transformation of five more Gram-positive bacterial species that could be transformed with pJRD215, which widens the range of application of this plasmid.

SAMENVATTING

Het bacteriële geslacht *Mycobacterium* behoort tot de groep van de Gram-positieve bacteriën en omvat vele soorten, die een breed spectrum aan eigenschappen vertonen. Zo is ongeveer een derde van de soorten ziekteverwekkend voor mens of dier, terwijl de resterende soorten relatief onschuldige water- en bodembewoners zijn. Bij de ziekteverwekkers zijn beruchte soorten als *Mycobacterium tuberculosis* en *Mycobacterium leprae*. Per jaar sterven er 3 miljoen mensen aan tuberculose, en 15 miljoen mensen lijden aan lepra. Vandaar dat met name de ziekteverwekkende mycobacteriën onderwerp van intensieve studies zijn, voornamelijk met als doel het begrijpen van de processen die zich bij de infectie afspelen, en het ontwikkelen van geneesmiddelen en vaccinaties.

Maar ook de niet-ziekteverwekkende mycobacteriën zijn het onderwerp van intensieve studie. enkele soorten zijn namelijk in staat om recalcitrante organische verbindingen af te breken, waardoor zij interessant zijn voor milieutechnische toepassingen. Ook bij de biotechnologische productie van waardevolle intermediairen van de synthese van pharmaceutica kunnen mycobacteriën ingezet worden.

Jammer genoeg is het geslacht *Mycobacterium* niet erg coöperatief. Enerzijds kleven er vanzelfsprekend grote practische problemen aan het werken met ziekteverwekkende microorganismen, anderzijds zijn mycobacteriën ontoegankelijk voor de standaard methodieken die gebruikt worden bij microbiologische studies. Dit wordt voor een groot deel veroorzaakt door de dikke celwand, die ervoor zorgt dat het inbrengen van gemodificeerde DNA-moleculen in de bacteriecel ('transformatie') niet via de gebruikelijke techniek kan geschieden. Verder is het feit dat *Mycobacterium* evolutionair gezien ver af staat van bacteriesoorten als *Escherichia coli* en *Bacillus*, waarop de meeste moleculair biologische technieken geëent zijn, er debet aan dat de standaard DNA-vectoren voor het transporteren van gemanipuleerde genen zich niet kunnen handhaven in mycobacteriën.

In hoofdstuk 2 van dit proefschrift wordt een overzicht gegeven van de meest recente ontwikkelingen op deze gebieden. De ontwikkeling van transformatietechnieken en DNA-vectoren wordt uitgebreid belicht. Deze laatste vertonen een duidelijke ontwikkeling van op soortseigen

DNA gebaseerde vectoren naar zogenaamde *broad-host-range* vectoren, die zich ook in andere bacteriesoorten weten te handhaven.

In hoofdstuk 3 wordt melding gemaakt van de ontwikkeling van een techniek voor de transformatie van *Mycobacterium aurum*, gebaseerd op electroporatie van mycobacteriën met intacte celwanden. Deze techniek bood uitkomst toen de 'klassieke' transformatie van *M.aurum* geen resultaat gaf, zelfs niet met bacteriecellen waarvan de celwand grotendeels verwijderd was. Bij deze 'sferoplasten' bleek de regeneratie van de celwand na de transformatie de bottleneck.

Transformatie van bacteriën met behulp van electroporatie berust op het blootstellen van een bacteriesuspensie aan een sterk electrisch veld gedurende zeer korte tijd, waarbij het celmembraan tijdelijk doorlaatbaar wordt voor macromoleculen. Het gebruikte plasmide, pAL8, was afkomstig van een nauw verwante *mycobacterium* soort, waardoor de kans op handhaving en expressie in *M.aurum* groot was. Inderdaad bleek pAL8 zich in *M.aurum* te kunnen handhaven en tot expressie te komen, waardoor de transformatietechniek met behulp van dit plasmide kon worden geoptimaliseerd. Door de cellen eerst te behandelen met stoffen die de celwand verzwakken, kon de opbrengst van de transformatie nog verhoogd worden.

Jammer genoeg is pAL8 niet geschikt als vector voor het transport van gemanipuleerde genen (clonering). Vervolgonderzoek richtte zich daarom op het vinden van geschikte vectoren die gebruikt konden worden voor de clonering van mycobacteriële genen. In eerste instantie werden daarbij plasmiden bekeken die geïsoleerd waren uit verwante Gram-positieve microorganismen, waarvan de kans op expressie groot geacht werd. Vreemd genoeg bleek het plasmide dat het minst verwant was aan *Mycobacterium* als enige te functioneren. Dit plasmide, pJRD215, is een construct gebaseerd op een zogenaamd *broad-host-range* plasmide, dat gevonden wordt in veel Gram-negatieve bacteriesoorten, geen van alle verwant met *Mycobacterium*. Feitelijk was de melding van de transformatie van *M.aurum* met pJRD215 het eerste rapport van de expressie van dit Gram-negatieve *broad-host-range* plasmide in een Gram-positieve bacterie.

Veel bacteriesoorten betrokken bij biotechnologische processen zijn Gram-positief, en wellicht kan pJRD215 ook in deze bacteriesoorten toegepast worden. In hoofdstuk 5 wordt gemeld dat pJRD215 in vijf andere Gram-positieve soorten ook tot expressie komt, hetgeen het toepassingsgebied van dit plasmide verder uitbreidt.

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CURRICULUM VITAE

Jo Hermans werd geboren op 29 oktober 1957 te Kerkrade, waar ook de lagere school en het gymnasium 'Rolduc' werd doorlopen. Na het behalen van het diploma in 1977 werd gestart met de studie Biologie aan de Katholieke Universiteit te Nijmegen. Afstudeervakken waren Genetica (12 maanden), Zoölogie (6 maanden) en Microbiologie (6 maanden). Dit laatste vak werd uitgevoerd aan de toenmalige Landbouwhogeschool te Wageningen. Na het behalen van het doctoraalexamen in april 1983 werd het onderzoek van het afstudeervak Microbiologie voortgezet, hetgeen uiteindelijk resulteerde in het hier beschreven promotieonderzoek. Na afloop hiervan was hij als onderzoeker gedurende drie jaar aangesteld bij het Instituut voor Systematiek en Populatiebiologie van de Universiteit van Amsterdam.
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