Construction of Mycobacterial Expression Vectors

By

Dawit Kidane

A Thesis presented to the School of Graduate Studies of the Addis Ababa University in partial fulfillment of the requirements of the degree of Master of Science in Biology

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Dr. Tobias Rinke De Wit, Examiner

Dr. Abera Geyid, Examiner

Prof. Endashaw Bekele, Advisor

Dr. Brigitte Wieles, Advisor

Dr. Masresha Fetene, Chairman

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List of abbreviations

Bp = base pairs
kD = kilodalton
PCR = Polymerase chain reaction
pDK = plasmid Dawit Kidane
Trx = Thioredoxin
TR = Thioredoxin reductase
SDS-PAGE = Sodium dodecyl sulfate poly acrylamide gel electrophoresis

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ABSTRACT

Molecular biology studies on *M. leprae* and *M. tuberculosis* involve cloning of genes and high level of expression to provide large amounts of protein. These proteins are used to study their usefulness in sub-unit vaccines or as antigens in diagnostic kits. Most of the work that has been done to obtain protein antigens directly from the pathogens has drawbacks. For example the tedious purification methods and the inability to culture *M.leprae* in vitro. Most of these obstacles can be circumvented by over-expressing proteins in *E.coli* but that has its drawbacks as well. The post-translational modification in *E.coli* is absent or at least different from that in mycobacteria. Furthermore, some mycobacterial proteins can not be produced in *E.coli*. We have initiated studies to develop a mycobacterial expression vector that might sidestep some of the above named difficulties. This study describes the construction of a vector that allows the over-expression of mycobacterial proteins in a non-pathogenic, fast growing mycobacterial host. Firstly, we have cloned a number of model proteins in an *E.coli* expression system. These vectors allow the over-expression of *M.leprae* and *M.tuberculosis* recombinant proteins (45kD, Esat-6 and Trx) in *E. coli.* Secondly, a mycobacterial expression vector (pDK1) was constructed. This vector has a histidine-tag that can be used for affinity purification of proteins. In this way we circumvent tedious biochemical purification systems. It also contains a multiple cloning site for convenient cloning of genes of interest. Thirdly, this pDK1 expression vector was used to introduce the same test proteins as for the *E.coli* expression vector. These test proteins were over-expressed in *E.coli* as well as in a mycobacterial host. Both protein sets can be used to determine whether there is any difference in recombinant proteins obtained from *E.coli* and the semi-autologous system using *M.smegmatis* as a recombinant host.

1. INTRODUCTION

The recent increases in mycobacterial disease in both developing and industrialized countries, together with the emergence of drug resistant strains and the synergy with the human immuno-deficiency virus (HIV) pandemic have led to raise public concerns and to highlight the need for radical improvement in control strategies (Bloom and Murray, 1992). The development, improvement and use of genetic tools to combat mycobacterial diseases lie at the center of current research programs (Young and Cole, 1993). Rational design of new vaccines and drug treatment for any infectious diseases requires the basic molecular genetics to determine the basis of pathogenesis and drug resistance (Jacobs and Bloom, 1994). Identification and immunological characterization of individual components of mycobacteria has been a major focus for research in mycobacterial disease. Most of the studies were initiated to understand the molecular mechanisms involved in the immune response to mycobacteria, and for the potential practical benefits of identifying candidate molecules for use as sub-unit vaccines and as reagents for immuno-diagnostic tests.

1.1 Mycobacteria

Mycobacteria are distinctive from other bacteria due to several unique physical characteristics. One of these characteristics is a thick, mycolic-acid rich cell wall, which differs substantially from those of gram-negative and gram-positive bacteria (Brennan *et al.*, 1990). *Mycobacterium leprae* and

Mycobacterium tuberculosis were categorized on the basis of their similar cell wall structure. The principal cell wall carbohydrate is a co-polymer of arabinose and galactose termed aribinogalactan, which is covalently bound by phosphodiester bonds to the acetyl or glycolyl moieties of murine. Esterified to arabinogalactan are distinctive lipids termed mycolic acids. The mycolic acid is known to reduce the permeability of the cell wall and is largely responsible for the staining property of acid-fastness (Bisshop and Neuman, 1970; Jarlier and Nikaido, 1994). Approximately 60 bacterial species with a similar cell wall make up are known to belong to the genes Mycobacterium (Stewart-Tull, 1982; Grange, 1988).

The second striking feature is an extremely low growth rate. Mycobacteria can be classified broadly into two groups based on growth rate; fast growing species such as *M. smegmatis* have a doubling time of 3 to 4h. The slow growing species such as *M. tuberculosis* have a doubling time of 24h (Wheeler and Ratledge, 1994). *M. leprae* has an even slower doubling time, determined to be 13 days in mice (Levy, 1970,1976). The vast majority of pathogenic strains are slow growing, while the fast growing strains are mostly non-pathogenic saprophytic organisms. Even the fast growing mycobacterium species have doubling times, which are six folds slower than that of the well-characterized bacterium, *E. coli* under similar conditions.

1.2 Leprosy and Tuberculosis.

Leprosy is a chronic infectious disease caused by the organism *M. leprae.* This bacterium is an obligate intracellular, acid fast bacillus which divides by binary fission (Meyres, 1992). *M.leprae* primarily affects the cooler areas of the body, the skin, upper respiratory tract, anterior segment of the eye, superficial portion of peripheral nerves and testes.

M. leprae was the first bacillus to be associated with human infectious disease. Although described as such by Hansen, in 1874, more than a century later it is still impossible to culture the bacteria in vitro. Many of the approaches adopted for studying the basic biology and biochemistry of other bacteria are not applicable to it. To some extent this has been circumvented by the use of experimental animals. The limitations of working in vivo and in particular the uniquely long generation time of *M. leprae* even growing optimally in the tissue of experimental animals, have proven to be major obstacles (Colston, 1993). By far the most widely used experimental animal in leprosy research is the mouse. The infection in mice was first described by Shepard (1960) who used mice for screening and evaluating compounds for activity against *M. leprae.*

At present leprosy is restricted to tropical and subtropical areas such as Africa, South America, India, South Asia, the Philippines, and some south pacific islands. The prevalence of leprosy ranges between 5 and 10 million

cases worldwide (Noordeen, 1994). In tropical and subtropical areas most individuals are resistant and have an effective immune response to the bacillus (Godal *et al.*, 1972). In the areas of elevated endemicity like Ethiopia, the exposure rates measured by antigen-specific lymphocytes transformation tests are high and variable (WHO, 1988).

During the first half of this century, tuberculosis was one of the most intensively researched infectious diseases. Tuberculosis (TB) is caused by 3 closely related Mycobacterium strains within the so-called TB-complex. *M. tuberculosis* and *M. africanum* are both human pathogens. *M. bovis* is an important pathogen that can infect a range of hosts including cattle and humans (Pollock and Anderson, 1997a). It is thought that the progenitor of the *M. tuberculosis* complex, comprising *M. tuberculosis, M. bovis, M. bovis* BCG, *M. africanum*, and *M. microti,* arose from a soil bacterium. The human bacillus may have been derived from the bovine following the domestication of cattle. The complex lacks inter-strain genetic diversity and nucleotide changes are very rare (Sreevatson *et al.*, 1997).

The characteristics feature of the tubercle bacillus include its slow growth, dormancy, complex cell envelop, intracellular pathogens and genetic homogeneity (Wheeler and Ratledge, 1994). *M. tuberculosis* is generally considered to be strictly aerobic, but is believed to be able to remain dormant for several years in a relatively anaerobic environment in the host.

With the introduction of streptomycin in 1945 and Isoniazed (INH) in 1952 the imperative for tuberculosis research decreased (Young and Cole, 1993). For many years the incidence of tuberculosis dropped steadily in developed countries, but it continued to exalt a high mortality in the developing world (Murray *et al.*, 1990).

Tuberculosis remains one of the world's most series health treats with approximately 2 billion people infected world wide and an estimated 2.9 million deaths per year (Kochi, 1991). Among those over 5-years of age tuberculosis kills more people than AIDS, malaria, diarrhea, leprosy and other tropical disease combined. The World Health Organization has calculated that, unless urgent action is taken the annual number of deaths could rise to 4 million by the year 2004 (WHO, 1994).

Infection with the human immune deficiency virus is associated with increased susceptibility to tuberculosis (Barnes *et al.*, 1991). A co-infection with the HIV virus and *M. tuberculosis* renders a person much more likely to develop overt tuberculosis and disease progression is considerably accelerated. At present about 8-10% of the tuberculosis cases world wide are related to HIV infections but the association is much more common in many African countries often 20% or more (Raviglion and Nunn, 1997). Furthermore, globally about 10% of the tuberculosis cases are caused by

strains resistant to one or more drugs. Primary multi-drug resistance is uncommon, about 0.2% (WHO, 1997). Resistance derives from mutations in chromosomal genes leading to overproduction, alteration or loss of the drug target (Cole, 1994). The recent increase in the incidence of tuberculosis, and the appearance of multi-drug resistant strains underscores the need for an effective vaccine against this disease (Jacobs, 1994).

1.3. The immune response to mycobacteria.

Mycobacteria are made up of a vast number of complex components, many of which may have an influence on immunological responses (Brennan, 1986; Closs *et al.*, 1980; Klatser *et al.*, 1984). The encounter between a microbial pathogen and a potential hosts involves a complex series of interactions with the outcome: resistance, infection or disease being mainly dependent on the host's immune response.

From an immunological point of view, resistance to mycobacterial diseases requires a concrete cell mediated immune response in which phagocytes, Th1 type T- cells, Natural killer cells, cytotoxic T- cells and perhaps T suppressor cells are necessary to control the disease.

1.3.1. Immunity of Leprosy

Ridely and Jopling (1966) established a leprosy classification system dividing various forms of the disease in 5 distinct groups. Figure 1 shows

that T-cell dependent immunity to *M. leprae* is high in tuberculoid leprosy patients (TT) with localized disease, but is strikingly absent in lepromatous leprosy patients (LL) who have a high bacillary load and widely disseminated lesions. This high cell mediated immunity is also seen in healthy individuals which indicates that T-cell dependent immunity protects against dissemination of bacteria and of the disease (Ottenhoff, 1994).

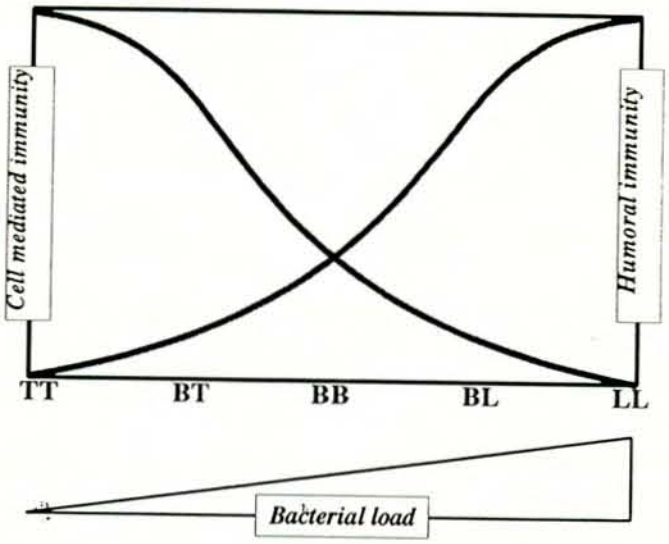


Fig.1. The five different clinical forms of leprosy spectrum: tuberculoid (TT), borderline tuberculoid (BT), borderline (BB), borderline lepromatous (BL), lepromatous(LL) leprosy.

In the classical view, cellular immunity was seen as a process in which macrophages take up bacteria and present them to T-cells which then start to produce interferon- gamma (IFN-γ) which helps the macrophage eliminate the intracellular parasite. However, the cellular immune response is a two edged sword: it protects by limiting bacillary growth, yet it also can harm if turned on improperly by inducing severe pathology like in tuberculoid leprosy and leprosy reactions (Britton, 1993).

1.3.2. Immunity of tuberculosis

There is evidence that the immune response in those who develop tuberculosis is not weak but dis-regulated. It is now known that helper T-lymphocytes (Th) mature along two pathways resulting in so called Th1 and Th2 cells that are distinguishable by chemical messengers called cytokines (Romagnani,1994). A large number of studies in murine models and in the human system have suggested that intracellular pathogens preferentially induce either Th1 or Th2 subsets (Locksley *et al.,* 1992). These two T-cells subset may thus regulate the outcome of intracellular infections, leading to either resistance (Th1) or susceptibility (Th2) to invading micro-organisms. Besides Th cells other cells might play a role as well. Among these cells are cytotoxic T-cells (CTL) which are effector cells and suppressor T-cells (Ts) which are regulatory T cells. CTLs may contribute to anti-mycobacterial protection by lysing mycobacterial infected macrophages and thereby

promoting the uptake of released bacilli by activated macrophages (Ottenhoff and Mutis, 1995).

The protective immune response in tuberculosis is mediated by Th1 cells. Th2 or a mixed Th1-Th2 response renders cells very sensitive to killing by the cytokine tumor necrosis factor (TNFα), This cytokine induces the gross tissue destruction characteristic of progressive tuberculosis (Hernandez-Pando and Rook, 1994).

Although the regulatory role of T-cell subsets in mycobacterial infections still raises a number of questions, the protective role of certain cytokines in mycobacterial disease are now well established. Interferon gamma (IFN-γ) for example is a major macrophage activating factor. IFN-γ is produced by T–cells and natural killer cells and it enables these cells to kill or inhibit the growth of a large range of intracellular parasites (Gallin *et al.*,1995)

1.4. Genetics of mycobacteria

All of the information, which determines the structure of mycobacteria, is obviously available within the bacterial genome. The genome is a total complement set of genes within the cell. Mycobacterial genomes are made up of distinct small regions of DNA conserved between the genomes of mycobacterial species, these conserved regions are flanked by long

segments of dissimilar DNA (Philipp *et al.,* 1996). This basic arrangement shows the pressure to conserve small segments of DNA, which encode essential functions. There is high tolerance for varying arrangements of other coding regions. In those bacteria that have been studied in detail the genome has been found to consist of a single length of DNA in the form of a closed loop.

Molecular weight estimates for mycobacterial genomes ranges from 2.2-4.5 $x10^9$ bp (Clark-Curtiss, 1990). According to a study by Baess and Mansa (1978) , the G + C content in 19 mycobacterial strains ranges from 66.1 to 71.4% of the total base content. Furthermore, modified bases such as base 6- methyl amino purine (Dunn and Smith, 1958) and 5 methyl cytosine (Johnson and Coghill, 1952) have been detected in mycobacterial DNA. The modification of DNA plays an important role in permitting the cell to distinguish between its own and foreign DNA.

The genome of *M. leprae* is represented by four contigs of overlapping clones, which together, accounts for nearly 2.8Mbp of DNA (Eiglmeier *et al.,* 1993). All of the cloned *M. leprae* genes have been positioned on the contig maps together with the dispersed repetitive element. A species-specific repetitive element has also been identified in *M. leprae* (Clark-Curtiss and Docherty, 1989). The *M. leprae* repeat is present in approximately 29 copies in the genome and several of the copies have been

analyzed at the level of nucleotide sequence, identifying genes encoding relevant antigens and enzymes (Woods and Cole, 1990).

Genome sequencing projects are being actively pursued and a large number of cosmids from *M. leprae* have already been completely sequenced (Siegele and Kolter, 1992). In addition to providing information about chromosomal organization, information from the sequencing project will provide valuable support to characterize the biochemical and immunological features of mycobacteria.

The *M. tuberculosis* genome sequence was completed recently (Cole *et al.*, 1998). It differs from other bacteria in that a very high portion of its coding capacity is devoted to the production of enzymes involved in lipogenesis and lipolysis. Two new families of glycine rich proteins with a repetitive structure that may represent a source of antigenic variation were identified as well. Furthermore, the genome is rich in repetitive DNA, insertion elements and new multi gene families and duplicating housekeeping genes. 3924 open reading frames were identified in genome accounting for ~91% of the potential coding capacity. The genome sequence showed that *M.tuberculosis* has the potential to produce several proteins that are normally associated with an anaerobic metabolism (Desmet, 1997).

From the genome sequence, *M. tuberculosis* has the potential to synthesize all essential amino acid, vitamins and enzyme co-factors, although some of

the pathways involved may differ from those found in other bacteria. *M. tuberculosis* is naturally resistant to many antibiotics, making treatment difficult. This resistance is due mainly to the highly hydrophobic cell envelope acting as a permeability barrier but many potential resistance determinants are also encoded in the genome (Brennan and Drapper, 1994). Repetitive DNA sequences, corresponding to mobile genetic elements or insertion sequences, have been particularly useful as taxonomic aids in identifying different strains of *M. tuberculosis* (Hermans *et al.*, 1990).

1.4.1. Vectors

The bacterial genome is often referred to as the chromosome to distinguish it from other genetic elements within the cell termed plasmids. Plasmids are circular, extra-chromosomal, self replicating pieces of DNA. They frequently contain genes that provide a selective advantage to the host such as drug resistance and serve as vectors for gene transfer in bacteria. Naturally, plasmids are transferred from one bacteria to another by conjugation; a specific contact mediated phenomena (Mazodier and Davies, 1991). In vitro, however, plasmid can be taken up from the medium as naked DNA by transfection a process that requires the stringent conditions of electroporation in mycobacteria (Jacobs *et al.*, 1991). Plasmids replicate autonomously from the principal genome and may not be transmitted to all daughter cells during bacterial replication (Grange, 1982). Plasmids have

never been convincingly demonstrated in *M. tuberculosis* although reports of their existence have appeared (Rauzier *et al.*, 1988).

Initial studies in the area of mycobacterial vectors as a vaccine delivery system focused on developing shuttle vectors which could be used to transfer DNA from *E. coli* in to mycobacteria (*M. smegmaties* and BCG) (Jacobs *et al.*, 1987; Snapper *et al.*, 1988). *E.coli*-mycobacterial shuttle vectors have been developed that are able to replicate in *E. coli* and mycobacterial species. The genes encoding the 65kD proteins (Husson *et al.*, 1990; Thole *et al.*, 1990) and 70kD protein (Aldovini and Young, 1991) were cloned and shown to be expressed in BCG using these first shuttle vectors. These initial vectors were large, lacked useful sites for incorporating foreign DNA and were unstable in mycobacteria because they lacked appropriate selection markers. Later more sophisticated smaller vectors were developed which appear to overcome these deficiencies.

The most extensive evaluated mycobacterial plasmid is pAL5000 from *M. fortuitum* (Ranes *et al.*, 1990). The *M. fortuitum* plasmid derived origin of DNA replication operates in other mycobacteria sustaining autonomous replication of plasmid in the *M. tuberculosis* complex and in *M. smegmatis* (Hinshelwood and Stoker, 1992). Some of the later vectors such as pMV261 and pMV361, use the promoter and 5' coding sequence of the BCG heat shock protein gene Hsp60. (Stover *et al.*, 1991). These vectors contain

signals capable of deriving high levels of expression with regulatory elements recognized by mycobacterial RNA polymerase. These latter vectors can be considered as mycobacterial expression vectors.

1.4.2. Cloning and expression of Mycobacterial Genes.

One of the major areas of impact of molecular biology on the study of mycobacteria is cloning of the genes of interest and high level of expression to provide large amounts of protein. This has been widely applied for the production of recombinant proteins. *E. coli* is a good host for cloning and expression of mycobacterial proteins. The first genes to be cloned from mycobacteria were those encoding proteins recognized by monoclonal antibodies and T- lymphocytes involved in the immune response to infection (Young *et al.*, 1985a). More than 50 such antigen encoding genes have now been characterized, and in several cases sequences analysis has allowed the identification and elucidation of the functional role of the encoded protein (Young *et al.*, 1990, 1992). The cloning of small fragments of randomly sheared *M. leprae* DNA into the *E. coli* phage λgt 11 creating an *M.leprae* expression library has been proven very useful (Young *et al.*, 1985b). In this phage, the cloned DNA is inserted into the coding region of the *E.coli* β-galactosidase gene. The resulting protein is a hybrid of β-galactosidase and *M. leprae* DNA encoding protein. Screening such

libraries with patient antiserum allows the identification of antigenic determinants.

Expression of mycobacterial genes in *E. coli* has provided an important contribution to the study of protein antigens of *M. leprae* and *M. tuberculosis*. However, the recent developments related to the transfer of genes between mycobacterial species rather than using *E.coli* have an important potential with regard to biochemical aspects (Snapper *et al.*, 1990). Initial attempts to express *M. leprae* genes in *E. coli* indicated that mycobacterial regulatory sequences failed to function in the foreign host. *E. coli* promoter sequences had to be supplied in front of almost every mycobacterial gene in order to achieve useful levels of expression of its protein product in recombinant systems (Jacobs *et al.*, 1986). It was then recognized that studies of gene expression in mycobacteria are important because many mycobacterial genes, including recA are not expressed in *E. coli* (Clark-Curtiss *et al.*, 1985; Das-Gupta *et al.*, 1993) and so presumably have promoters which are not recognized by the *E.coli* RNA polymerase. The structure of such mycobacterial promoters and their interaction with the mycobacterial RNA polymerase are poorly defined. Some promoter sequences are recognized by the *E.coli* machinery but the expression levels are low. *M.leprae* and *M.tuberculosis* genes can be over-expressed in other mycobacterial hosts.

The transcription machinery of the different mycobacteria, thus, seems to be conserved. Thole *et al.* (1985) showed that mycobacterial antigens in mycobacterial expression systems do not rely on the presence of a strong exogenous promoter. The level of expression in *M. smegmatis* can even be much higher than that of the native protein in the pathogen. For instance, the level of expression of the 19 kD antigen from *M. tuberculosis* was much higher as a recombinant protein in *M. smegmatis* as compared to the expression of the native protein in *M. tuberculosis* (Garbe *et al.,* 1993). Another example is the gene encoding the superoxide dismutase enzyme of *M. leprae* and *M. tuberculosis.* They are expressed from their own promoters in *M. smegmatis*, while provision of an *E.coli* promoter was essential for the expression of the gene in *E. coli* (Thangaraj *et al.,* 1990). In addition it was found that the mycobacterial system allowed expression of functionally active superoxide dismutase, incontrast to the enzymatically inactive recombinant product in *E. coli* (Zhang *et al.,* 1991). These findings led to the understanding that high level expression of functional proteins and especially secretion of relatively non-conserved mycobacterial specific proteins requires a mycobacterial host. The introduction of foreign DNA into a mycobacterial host by transformation (Jacobs *et al.,* 1987) was however stimulated in the first place by the goal to generate a new recombinant *M. bovis* BCG vaccine (Aldovini and Young, 1991).

1.5. Mycobacterial antigens

Antigens are substances capable of inducing an immunological response. Their capacity to do this can be enhanced by adjuvant and the response they induce may be humoral, cellular or frequently a mixture of both. In their composition antigen differ in size and chemical make-up. They may be proteins or carbohydrates alone, combined together, or combined with lipids (Stanford, 1983).

The well known complex mycobacterial extract, purified protein derivative (PPD), is a good example of a potent antigen preparation. It has however a number of drawbacks. When used as a screening tool it does not distinguish between individuals with active disease and those who have been exposed to infection but have no clinical disease. It also does not distinguish between individuals vaccinated with BCG or exposed to *M.tuberculosis*. This is one of the many reasons why scientists in the mycobacterial field started to characterize individual antigens of the pathogenic mycobacteria.

A broad spectrum of mycobacterial antigens has been defined including conserved and species specific proteins found in the cytoplasm, the cell wall or extra-cellular in the growth medium. The objective of antigen characterization has been to determine if the defined proteins play a role in the cell mediated immune response to mycobacteria and whether or not recognition of different antigens induces different forms of immune

activation. The cellular immune response to defined antigens has been tested using either protein purified from mycobacterial cultures or expressed as recombinant products in *E. coli.* These products are then used in assays based on induction of delayed type hypersensitivity and T-cell proliferation. In addition to their possible application as sub-unit vaccines they are also useful in the design of specific diagnostic kits. Sequence analysis of antigen encoding genes in concert with synthetic peptide chemistry has a major role in detailed immunological characterization of mycobacterial antigens (Harris *et al.,* 1991).

Difficulties in obtaining sufficient quantities of purified reagents, the requirement for strict containment facilities and slow or no growths in laboratory cultures are significant practical obstacles to large scale growth for biochemical and immunological characterization of native proteins. The mycobacterial heat shock proteins were among the first proteins to be over-expressed in an *E. coli* recombinant system. Thereby overcoming the above mentioned constraints that are linked to the isolation of native proteins. A large number of other antigens are now expressed as fusion proteins in *E. coil* but for some proteins major difficulties are encountered (Matsuo, 1990).

Another approach has been to isolate *M.tuberculosis* secreted proteins from the culture filtrate (Andersen *et al.*, 1991). These released proteins are

generally believed to be responsible for the high efficacy of the live vaccine *M. bovis* BCG. It is now generally accepted that recognition of these secreted molecules may lead to early immunological detection of mycobacteria within the infected macrophages and control of the disease. Sub-unit vaccines based on the mixture of culture filtrate proteins from *M. tuberculosis* have, in a number of studies resulted in protective immunity in animal models of TB (Andersen, 1994). Furthermore, these molecule are recognized strongly during *M. tuberculosis* infection in various animal models as well as in the early stage of pulmonary TB in humans (Boesen *et al.*, 1995; Haslov *et al.*, 1995).

A lot of emphasis has been put on the low molecular mass secreted antigen Esat-6. It is an early and dominant T-cell target during TB infection in experimental animals (Andersen *et al.*, 1995). The immunological properties of Esat-6 have been the subject of a number of studies in animals models and it is known that this antigen is a prominent target broadly recognized early during disease in different species infected with *M. tuberculosis* or *M. bovis*. Esat-6 was originally identified by its potent induction of INF-γ from mouse memory Th-1 type lymphocytes (Andersen *et al,* 1995). Despite the small size of Esat-6, some findings indicate that this molecules contains an unusual high number of T-cell epitopes spanning the entire sequence of the antigen. All peptides from Esat-6 are able to

induce a T cell response in some persons, resulting in levels of IFN-γ comparable to or even higher than that induced by intact molecules. The potential of Esat-6 to discriminate between exposure to environmental mycobacterial and strains causing disease was further evaluated by investigating the distribution of the Esat-6 gene in non-tuberculosis mycobacterial strains that were typically isolated from healthy sensitized cattle (Pollock and Andersen, 1997b). The absence of Esat-6 in the vaccine strain *M.bovis* BCG and its potent immunological properties make it a valuable protein for use in a subunit vaccine or as a candidate for a specific tuberculosis diagnostic test.

We have therefore chosen Esat-6 as one of the model proteins for our study. The two other mycobacterial proteins described below were also used as model proteins. The 45kD protein of *M.leprae* because it is an *M.leprae* specific protein and the thioredoxin protein of *M.tuberculosis* to test the ability to over-express functional enzymes.

The thioredoxin system is composed of thioredoxin (Trx), NADPH and the flavoenzyme thioredoxin reductase (TR). Trx reduction by TR comprises two half reaction, in which the reduction of the FAD prosthetic group of TR by NADPH and electron transfer to active–site in TR occurs first. The

second half of the reaction is the reduction of bound oxidized cysteines on Trx by TR.

Trx is a small , 12-14kD protein that is present in virtually all prokaryotic and eukaryotic cells. The active site of thioredoxin contains two redox active cysteine residues which are conserved in the sequences –WCGPC-. Trx was first isolated from *E. coli* as an in vitro hydrogen donor for the production of deoxyribonucleotides for DNA replication (Laurent *et al.*, 1964). It has been reported that thioredoxin protects living organisms from oxidative stress by scavenging reactive oxygen species as well as regenerating proteins inactivating by such stress (Fernando *et al* ., 1992; Mitsui *et al* ., 1992).

TR from *E. coli* of which both amino acid sequence (Russel and Model, 1988) and crystal structure (Kuriyan *et al.*,1989) have been determined is a dimeric protein with two subunits of 35kD. The enzyme is highly specific for NADPH and both subunits contain FAD and an oxido-reductase active disulfide in the conserved sequence – CATC-.

The thioredoxin of *M.tuberculosis* differs in size from the thioredoxin of *M.smegmatis* (Wieles *et al*, 1995). This property will allow us to differentiate the native thioredoxin from the recombinant product in *M.smegmatis*.

The third model protein is the 45kD antigen of *M.leprae*. This protein is specific for *M.leprae*. It was not detected in other mycobacterial strains including *M.smegmatis* (Rinke de Wit *et al.*, 1993). This specificity was confirmed by a recent study demonstrating frequent recognition of the 45kD antigen by leprosy patients sera but not tuberculosis patient sera from the same leprosy endemic area (Dockrell, unpublished data).

2.Objective

Most of the work that has been done to get protein antigens has a number of drawbacks. This work is concerned to see the possibility of over-expressing proteins of mycobacterial pathogens in non pathogenic mycobacterial hosts.

Specific objective.

. Construction of a vector that allows affinity purification of mycobacterial proteins in a mycobacterial host.

. Cloning of mycobacterial protein encoding genes in an *E. coli* expression vector and the constructed mycobacterial expression vector.

. Comparing the recombinant proteins obtained from both hosts with regard to their immunological and biochemical properties.

3. MATERIALS AND METHODS

3.1. Bacterial strains, plasmids and growth conditions

The bacterial strains, plasmids and growth conditions in this study are listed in Table 1. *E. coli DH5α* was grown in Luria Bertani (LB) media (1% NaCl, 1% Bacto-trypton, 0.5% Yeast extract) supplemented with magnesium salt (10mM) and incubated at 37°C overnight. The culture was grown in LB broth with appropriate antibiotics. Antibiotics were added to the culture at the following concentration: Ampicillin 100μg.ml^{-1}, (Sigma Chemicals Co., St. Louis, USA.) Hygromicin 200μg.ml^{-1} (Boehringer Mannheim GmbH, Mannheim, Germany). *M. smegmatis* was grown at 37°C for 3 days on $7H_{10}$ plates containing $7H_{10}$ agar, OADC (Oil Albumin Dextrose Complex) (Difco Laboratories, Detroit Michigan, USA). and glycerol according to the recommendation of manufacturer. *M. smegmatis* liquid cultures were grown in Dubos media supplemented with Dubos albumin (Difco) and 50μg/ml Hygromicin until the desired OD_{600} was reached.

3.2. DNA Manipulation

3.2.1 Synthetic Oligonucleotide

The ESAT-6 protein encoding gene sequence was used to design deoxyoligonucleotides primers. The forward primer designated FtesatBhis

was constructed with a *Bam*HI restriction site (5'-CCCCGGATCCCATGACAGAGCAGCAGTGG-3'). The reversed primer was constructed with an *Eco*RI restriction site RtesatEco (5'-CTCGGAATTCCCCTATGCGAACATCCC-3'). The gene encoding the 45kDa protein from *M. leprae* was amplified using forward primer containing a *Bam*HI restriction site F45Bam (5'GTAAGGATCCCATGTTCG ACTTCATGGTG-3'). The reverse primer was constructed with an *Eco*RI restriction site R45Eco (5'ACTCGAATTCAAACCGCTTCGTTGAAGG-3'). The DNA fragment polyhistidine tag A,B,C were amplified by using the following primers. The forward primer containing an *Eco*RV restriction site FhisEV (5'-TAAAGATATCGGGGTTCTCATCATC-3') and the reverse primer with a *Hin*dIII restriction enzyme recognition site RhisHind (5-TCCGCCAAAACAGCCA AG-3'). All primers were purchased from Gibco BRL.

Table 1: Bacterial strains; plasmids and growth conditions used.

Strains/Plasmids	Relevant genotype	Growth medium/ maker	Source/ reference
Strains			
E.coli $DH_{5\alpha}$	FendA1hsdR17($r_k$mk)supE44thi-1λ ecA1gyrA96relA1deOR ϕ80 lacZΔM15Δ (lacZyA-argF) U_{169}	LB broth/plate	
M. smegmatis 1-2c		Dubos Broth & $7H_{10}$ plate	
Plasmids			
pTrc HisA	ColE1 ori; ptrc	Amp^r	Invitrogen
pTrc HisB	ColE1 ori; ptrc	Amp^r	Invitrogen
pTrc HisC	ColE1 ori;ptrc	Amp^r	Invitrogen
pTrcHisA/Trx	ColE1 ori; ptrc	Amp^r	Wieles, unpublished
$pSMT_3$	*Mycobacterium-E.coli* Shutlle Vector	Hyg^r	Garbe *et al.*, 1994

Amp^r: ampicillin resistant; Hyg^r: Hygromicin resistant

3.2.2 Polymerase Chain Reaction

A/ Mycobacterial DNA was subjected to PCR in a total volume of 25µl with Ready to go PCR beads (Pharmacia P-L Biochemicals Inc., Uppsala, Sweden). These beads contain DNA-Taq polymerase, dNTP and PCR-buffer. To these beads were added 10µM of each primer and 100 ng mycobacterial DNA and dH_2O. The mixtures were covered by mineral oil. Amplification reaction was as follows: first step denaturing at 95°C for 2' one cycle; second step denaturing 95°C for 15"; primer annealing 60°C for

30"; and primer extension 72°C for 1' for 25 cycles and prolonged primer extensions at 72°C for 7' for one cycle.

B/ pTrcHisB Vector DNA (1μg/μl) was subjected to PCR in a total volume of 50μl with 1μl DNA template, 5μl of 10μM each primer, 1μl of 10μM dNTP, 1μl of Pfu polymerase enzyme and 32μl H_2O.

The amplification was achieved by stage one: denaturing at 94°C for 2' one cycle. Stage two: first step denaturing 94°C for 15" annealing 55°C for 30"; and extension 72°C for 1' for a total of 25 cycles. Step 3: 72°C for 7' one cycle.

C/ pTrcHisA and C, pHisA-Trx, pHisA-TR vector DNA was amplified using the Expand™ High Fidelity PCR system (Boehringer Mannheim) in a total volume of 50μl. The preparation of the PCR mix was carried out by preparing two separate master mixes each volume was 25μl. Mix 1 contained 4μl dNTP (200nM), 5μl of 300nM of each primer, 1μl DNA template (1μg/μl) and 15 μl H_2O. Mix 2 contained 5μl of Expand PCR buffer with $MgCl_2$ and 0.75μl of High Expand ™ Polymerase enzyme and 19.25μl H_2O. The two mixes were pooled together and covered by mineral oil. The following program was used for amplification. First stage denaturing at 94°C for 2'min one cycle; the second stage DNA denaturing at

94°C for 15'', annealing 55°C for 30" primer extension 72°C for 1′ for 10 cycles. The third stage denaturing at 94°C for 15", primer annealing at 55°C for 30″ primer extension 72°C for 1' plus 20" increment elongation for each cycle for a total 15 cycles and prolonged elongation of primer 72°C for 7' for one cycle

All the PCR reactions were carried out using a Hybid Omini-gene PCR-machine.

3.2.3 Isolation and purification of DNA from the gel.

Agaros gel slices were sliced from the gel using a surgical scalpel blade. The gel slices were treated and purified using a Sephaglass S™ kit (Pharmacia Biotech) according to the recommendation of the manufacturers. The fragment of DNA will be used for cloning purpose.

3.2.4 Polynucleotide Kinase treatment of DNA fragments.

The reaction was carried out in 10x One Phor All buffer with the addition of 10mM dNTP, 10mM ATP, 5-10 units polynucleotide kinase, 2-5 units Klenow DNA polymerase (Pharmacia Biotech). The mix was incubated for 45' at 37°C and extracted with Phenol Chlorophorm Isoamyl Alcohol (PCIA 25:24:1). After addition of 0.1 vol. 3M sodium acetate and 2-2.5 vol. Ethanol (100%) the DNA was precipitated washed with 70% ethanol and air

dried. The pellets were dissolved in dH_2O or TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA).

3.2.5 Ligation Reaction

Ligation reactions were performed in a total volume of 20µl that contained insert DNA, vector DNA, T_4 DNA ligase, ATP (10mM) and (10x) One Phor All buffer (Pharmacia Biotech) and incubated overnight at 10°C for 16h.

3.2.6 Plasmid DNA isolation

E. coli colonies were grown on LB plates that contained a selection marker and were randomly picked and incubated in 3ml of LB broth that contained the appropriate antibiotics and incubated overnight at 37°C in a shaking incubator. The plasmid was isolated using the alkali-lysis method Birnboim (Sambrook *et al.,* 1989). The cultures were centrifuged and the pellets resuspended in Quickmix (50mM glucose, 10mM EDTA, 25mM Tris-HCl pH 8.0) and incubated for 5' incubation on ice. 0.2 mM NaOH / 1% SDS was added and incubated for 5' on ice. 3M KAC was added and the tubes were again incubated for 5' on ice. The mixtures were centrifuged for 5'. The supernatant was transferred to a fresh Eppendorf tube and an equal amount of PCIA was added. The DNA was ethanol precipitated with 100% Ethanol, washed with 70% Ethanol and the air-dried pellets were dissolved in TE buffer.

Maxi-prep isolation of plasmid DNA was accomplished according to the protocol of Quiagen(Quiagen, GmbH, Max-Volmer- StraBe4.40724Hiden. Germany).

Mycobacterium plasmid isolation was carried out by taking transformed colonies of *M. smegmatis* picked and grow in 5ml of Dubos broth containing 50μg/ml Hygromicin at 37°C on a shaker. The cultures were treated with 10mg/ml of Cycloserin (Sigma) and 0.1mg/ml Ampicillin and incubated at 37°C overnight on shaker. The mixture was further treated with lysoenzyme solution (10mg/ml lysozyme, 1M Trs-HCl pH 7.5, dextrose) and incubated for 1h at 37°C in a water bath. 10mg/ml proteinase K and 1%, SDS were added and incubated for 1h at 55-60°C. The normal plasmid isolation was carried out by the method described above.

3.3 Preparation of competent cells

3.3.1. Heat shock competent cells.

For the transformation of plasmid DNA to *E.coli* the cells need to be competent. The preparation was based on the observations of Mandel and Higa with slight modifications. *E.coli* $DH_{5\alpha}$ was grown on LB plates containing Mg salt and a single colony was picked and incubated in TYM (2% bacto trypon, 0.5% bacto yeast extract; 100mM NaCl, 10mM $MgCl_2$ or

MgSO4) at 37°C until the $0D'_{600}$ =0.2-0.4. The culture was centrifuged at 4°C for 5′ at 2500 rpm (Herasus centrifuge) and the pellet resuspend with 1 culture volume of ice cold TfbI (30mMKAC, 50mM $MgCl_2$, 100mM KCl 10mM $CaCl_2$, 15% glycerin). This mixture was incubated for 5' on ice and centrifuged at 4°C and 2500 rpm. The pellet was resuspended in 0.1 Volume of TbfII (10mM Na-MOPS pH 7.0, 75mM $CaCl_2$ 10mM KCl, 15% glycerin). The competent cells were aliquoted and placed in the –70°C freezer.

3.3.2 Electrocompetent cells

Electrocompetent E.coli cells were prepared according to Wily and Sons (1994). The *E.coli* strain $DH_{5\alpha}$ was inoculated in LB medium and incubated at 37°C with vigorous shacking until the OD_{600} was between 0.5 to 1. The bacteria were placed on ice >15′ and centrifuged 4000rpm in GSA rotor at 4°C. The pellet was resuspended in cold sterile 10% glycerol and centrifuged at 4000 rpm for 15′ at 4°C. This step was repeated 5 times. After the last centrifugation the pellet was resuspended in 15% glycerol and aliquoted in eppendorf tubes. The electrocompetent cells were snap frozen and placed in the –70°C freezer.

Electrocompetent *M. smegmates* was prepared in a similar way (Snapper *et al.*, 1990). Modifications to the above protocol are: the colonies were

incubated in Dubos media supplemented with Dubos albumin (Difco). The culture was incubated at 37°C until the OD_{600} reached 1.0. The centrifugation was performed at 3000 rpm for 5'.

3.4 Transformation

3.4.1. Heat Shock

The transformation reaction was carried out by mixing the ligation product with 100µl competent *E.coli* $DH_{5\alpha}$ and flicking the tubes and leave the tubes on ice for 20'. Heat shock the bacteria by placing the tubes at 42°C for 1.5' and a further incubation on ice for 5'. 500µl LB broth was added to each tube and incubated for 30' at 37°C. The bacteria were streaked plates containing the antibiotics ampicillin (100µg/ml) or Hygromicin (200µg/ml) depending on the plasmid's selection marker. The plates were incubated overnight at 37°C (Sambrook *et al.*, 1989).

3.4.2. Electroporation.

The transformation mixes contain 50µl of electrocompetent cells and 1µl DNA added into 0.2cm electrode gap cuvette (Bio-Rad Laboratories, Richmond, USA.). The cuvette was incubated at for 10' on ice and electroporated with 1.7V, 25µF and 200W. These settings gave the desired time constant of around 20 (Wily & Sons, 1994). Immediately after giving the pulse 1ml S0C media (2%w/v Bacto-trypron., 0.5% yeast extract,

10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM glucose) was added and incubated for 45min at 37°C in a shaker. The cells were poured on LB plate containing selective media and incubate overnight at 37°C.

Transformation of *M. smegmatis* was done according to Snapper *et al* (1990) with little modification. By taking 100µl electrocompetent cells and 1µl DNA. The mix was put in a electroporation cuvette and electroporated with 2.5V; 25µF and 1000Ω giving a time constant around 20. A Bio-Rad Gene Pulser machine was used for the electroporations. Dubos media was added directly after the pulse. The cells were incubated for 2h at 37°C on a shaker and plated on $7H_{10}$ that contain 50 µg/ml of hygromicin and incubated at 37°C for 3 days.

3.5 Restriction enzyme analysis

Restriction endonucleases were used as specified by the manufacturer (New England Bio lab).

3.6 Gel Electrophoresis

DNA gel electrophoresis.

For DNA Agarose gel electrophoresis Agarose (sigma) (w/v) was mixed with 0.5xTBE buffer from a 10xTBE stock (108 g Tris base, 55g boric acid, and 40ml 0.5M EDTA pH 8.0 for 1 liter) The agarose was melted using a

micro-wave oven. When the temperature of the gel mixture was around 50°C, ethidium bromide (10mg/ml) was added and the gel mixture was poured in a gel tray. 5x sample buffer (50% glycerol w/v, 50mM Tris-HCl pH7.5, 5mM EDTA and 0.5%, bromophenol blue) was mixed proportional with the sample.

SDS-PAGE

For protein electrophoresis a 12% and 15% SDS-PAGE (sodium dodecyl–sulfate poly acrylamide gel electrophoresis) resolution gel was prepared from a 30% acrylamide-bisacrylamide solution (29:1), 1.5M Tris-HCl pH 8.3, 10% Ammonium persulphate (APS) 10% SDS and TEMED. A 4% stacking gel was prepared from 0.5M Tris-HCl pH 6.3. The gel was prepared on a mini gel slabs (Bio-Rad) apparatus. The samples were prepared with 2xSDS sample buffer (100mM Tris-HCl pH 6.8, 200mM dithiotheritol, 4% SDS, 0.2% bromo phenol blue, 20% glycerol). Gel electrophoresis was performed in 5xSDS–running buffer (25mM Tris-HCl 192mM glycine and 1% SDS with pH 8.3). After gel electrophoresis the gel was stained with Coomassie Brilliant Blue(CBB) (0.5%(w/v), CBB, 10% glacial acetic acid, 40% methanol). In order to visualize the proteins the gel was destained with a destaining solution (40% methanol, 7% glacial acetic acid) (Sambrook *et al,* 1989).

Electrophoresis was carried out using an electrophoresis power supply machine (Pharmacia Biotech). DNA was visualized under UV light and the gel was photographed using Polaroid film cassettes.

3.7 Over expression of Proteins

Colonies of *E. coli* were picked and inoculated in a 5ml culture containing Ampicillin and grown overnight at 37°C. The overnight culture was used to inoculate a 500ml culture which was induced using a final concentration of 1mM IPTG (5-bromo-4-chloro-3-indolyl-b-D galactopyranosied) after the culture reached an OD_{600} of 0.5. The growth continued for 3h at 37°C and the bacteria cells were pelleted by centrifugation at 4,000 rpm for 10min at 4°C using a GSR20 rotor in a Sorval centrifuge.

3.8 Protein purification

Protein purification from *E.coli* was done in a denatured way using the Qiagen protein purification system. Briefly, the bacterial pellet was lysed with buffer A (6M Guanidine HCl, 0.1M NaH_2PO_4, 0.01M Tris HCl, pH=8.0). After 1h of incubation at room temperature the mixture was centrifuged at 10,000 rpm for 30min at 4°C. The supernatant was collected and mixed with a 50% slurry of Ni-NTA agarose (Qiagen) that was equilibrated with buffer A. This mixture was incubated for 1h at room temperature to allow recombinant protein binding to the beads. The mix was centrifuged for 5 min at 3000 rpm and the supernatant was removed.

The resin was washed with 10 bed volumes of buffer A. Followed by 2 wash steps using buffer B (8M urea, 0.1M NaH_2PO_4, 0.01M Tris/Hcl pH 8.0) and finally once with buffer C (8M urea, 0.1M NaH_2PO_4 , 0.01M TrisHcl/ pH6.5). 20mM imidazole was added to buffer C for the last stringent wash step. The recombinant protein was eluted from the beads by incubating the resin 5 times with 1 ml elution buffer (250mM imidazole in buffer C). The protein was dialysed against PBS (140mM NaCl, 2.7mM KCl, 0.1mM $Na_2 HPO_4$, 1.8mM K H_2PO_4/ pH 7.3).

4. RESULTS

4.1. Gene amplification

4.1.1 Amplification of the ESAT-6 protein encoding gene from *M. tuberculosis*.

In our case we have constructed the PCR primers in such a way that the forward primer contains a *Bam*HI site and the reversed primer an *Eco*RI site. This allows us to clone the PCR fragment in the *Bam*HI/*Eco*RI sites of any vector containing these two sites. Besides that the primers are chosen in such a way that the inserted gene forms a translational fusion with that part of the plasmid encoding the affinity tag. *M.tuberculosis* DNA was subject to PCR amplification using FtesatB and RtesatEco. After resolution of the PCR amplification products on agarose gel one major band of 300bp was visualized under ultraviolet light. This amplified DNA fragment encodes the 6 kD protein called ESAT-6 (fig 2).

4.1.2. Amplification of the 45kD protein encoding gene from *M. leprae.*

The F45Bam and R45Eco primers constructed with a *Bam*HI and *Eco*RI restriction endonuclease recognition site were used to amplify the 45 kD protein encoding gene. Gel electrophoresis of the PCR amplified product showed a DNA band with the approximate size of 1250 bp (Fig 2). The size of this DNA band corresponds with the expected size for the gene encoding the 45 kD protein of *M.leprae*.

4.1.3. Amplification of the HisB-tag from the pTrcHisB expression vector.

Primers were constructed in such a way that the His-tag of the expression vector pTrcHisB could be cloned in a relatively simple way into the mycobacterial shuttle vector pSMT3. The forward and reversed primers were designed with *Eco*RV and a *Hin*dIII restriction endonucleases recognition site respectively. These primers were used to amplify a 150 bp DNA fragment. This little DNA fragment contains the 6 nucleotide triplets encoding 6 Histidine residues. It also contains a stretch of nucleotides encoding a T7 epitope. Commercially available antibodies directed towards this T7 epitope can be used to show over-expression of your gene of interest. In addition it has a region encoding an entrokinase cleavage site which is followed by a multiple cloning site. The isolated DNA fragment is shown in fig 2.

A similar approach was used to amplify the His-tag of the pTrcHisA and pTrcHisC expression vectors (fig 3). The difference between the three vectors is a minor change of a couple of nucleotides. This change allows you to clone genes in the 3 different reading frames depending on which of the three vectors you choose.

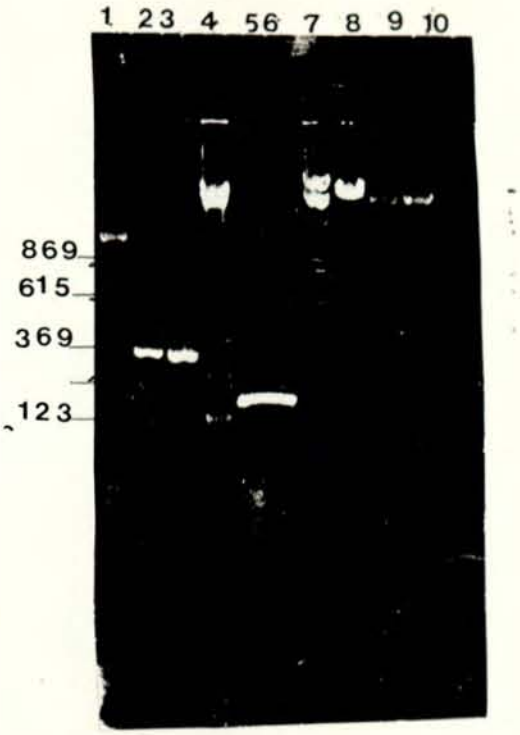


Fig. 2. Result of PCR amplification of mycobacterial DNA and expression vectors on 2% agarose gel. Lane 1: 45 kD protein encoding gene from *M. leprae*; lane 2 and 3: ESAT-6 protein encoding gene from *M.tuberculosis*; lane 4: 123 bp DNA ladder molecular weight marker; lanes 5 and 6: histidin-tag B from the pTrcHisB vector; lane 7: λ DNA *Eco*RI and *Hin*d III digested molecular weight marker(Kbp); lane 8: pSMT3; lane 9 pTrcHisB and lane 10: pGEX-2T. The plasmid DNA's in lanes 8-10 are undigested.

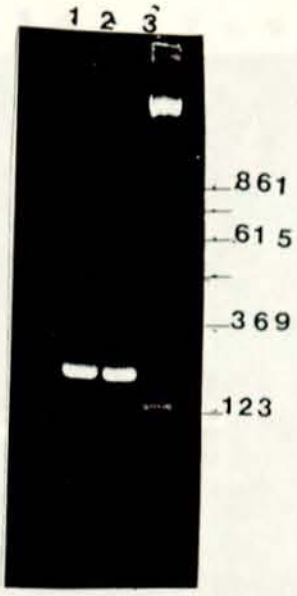


Fig 3. Gel electrophoresis of PCR product of pTrcHisA and pTrcHisC on 2% agarose gel. lane1: pTrcHisA; lane 2: pTrcHisC; lane 3: 123 DNA ladder molecular weight marker (bp).

4.1.4. Amplification of the HisA-tag /Trx and TR of *M. tuberculosis*

The Trx and TR genes of *M. tuberculosis* were obtained from pTrcHisA/Trx and pTrcHis/TR (Wieles, unpublished results). The PCR was performed using the following primers FhisEV and RhisHind. These are the same primers used to obtain the His-tag A,B and C PCR fragments. Using these primers would allow us to directly clone the Trx and TR protein encoding genes on pSMT3. The size of the amplified DNA fragments is 530 and 1200bp. These sizes coincide with the DNA size of Trx and TR plus the 150bp of the HisA-tag. (fig 4).

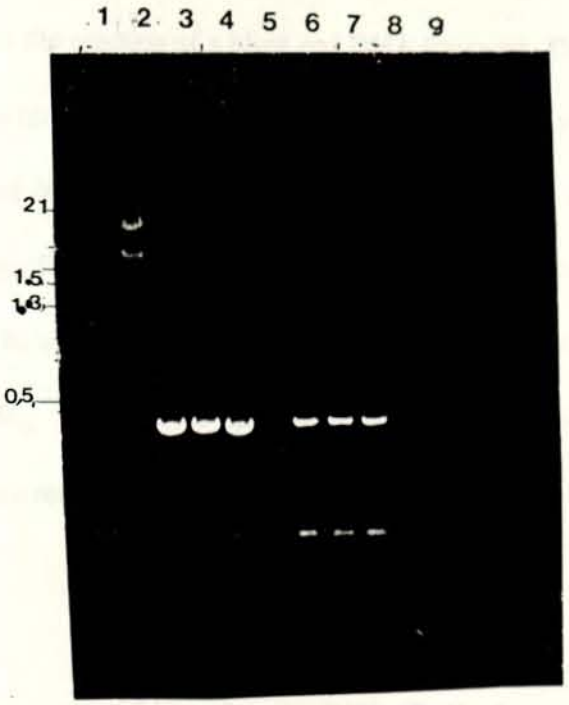


Fig 4. PCR amplification of histidine tag A-Trx and TR from pTrcHisA/Trx and pTrcHisA/TR. Lane 1: control (PCR mix without DNA template. Lane2:λ-DNA molecular weight marker. Lane3,4,5: histidine tag A/Trx encoding gene. Lane7,8,9: histidine tag A/Trx and TR encoding gene.

4.2. Gene cloning

4.2.1. Construction of pDK1

For the construction of our mycobacterial expression vector we combined the properties of two plasmids: pSMT3 and pTrcHis. We decided therefore to clone the his-tag region of pTrcHis in pSMT3. To achieve this pSMT3 was digested with *Bam*HI and treated with polynucleotide kinase and

Klenow. These two enzymes facilitate the filling in of 5-overhangs resulting in the creation of a blunt end DNA fragment. The vector was then digested with *Hind*III. The HisB-tag PCR product was digested with *Eco*RV and *Hin*d III resulting in a DNA fragment with one blunt and one sticky end. The treated pSMT3 vector and the digested HisB-tag were subjected to a ligation reaction. This ligation mixture was transformed to *E.coli* $DH_{5\alpha}$. A number of transformants confirming resistance to Hygromicin resistance were randomly chosen for further restriction enzyme analysis.

The resulting plasmid is termed pDK1 (fig5). A mycobacterial shuttle vector that allows the over-expression of proteins in a mycobacterial host cell with the possibility to affinity purifies over-expressed proteins using the histidine-tag.

4.2.1.1. Mapping of pDK1 using restriction endonuclease digestions.

Individual pDK1 clones were checked by restriction digest analysis using the restriction enzymes *Kpn*I and *Bgl*II. *Kpn*I is absent in pSMT3 but present in the multiple cloning site of pTrcHisB. This means that *Kpn*I should linearize pDK1 if the plasmid indeed contains the HisB-tag fragment.

In the case of *Bgl*II, pDK1 should be cleaved into fragments of the following size: ~ 500, 1295 and 4000bp. The correct plasmid can be distinguished from the empty pSMT3 because the latter has only two *Bgl*II restriction enzyme recognition sites. The additional site in pDK1 appears after introduction of the His-tag. Figure 6 shows that the mycobacterial expression vector, pDK1, is indeed correct.

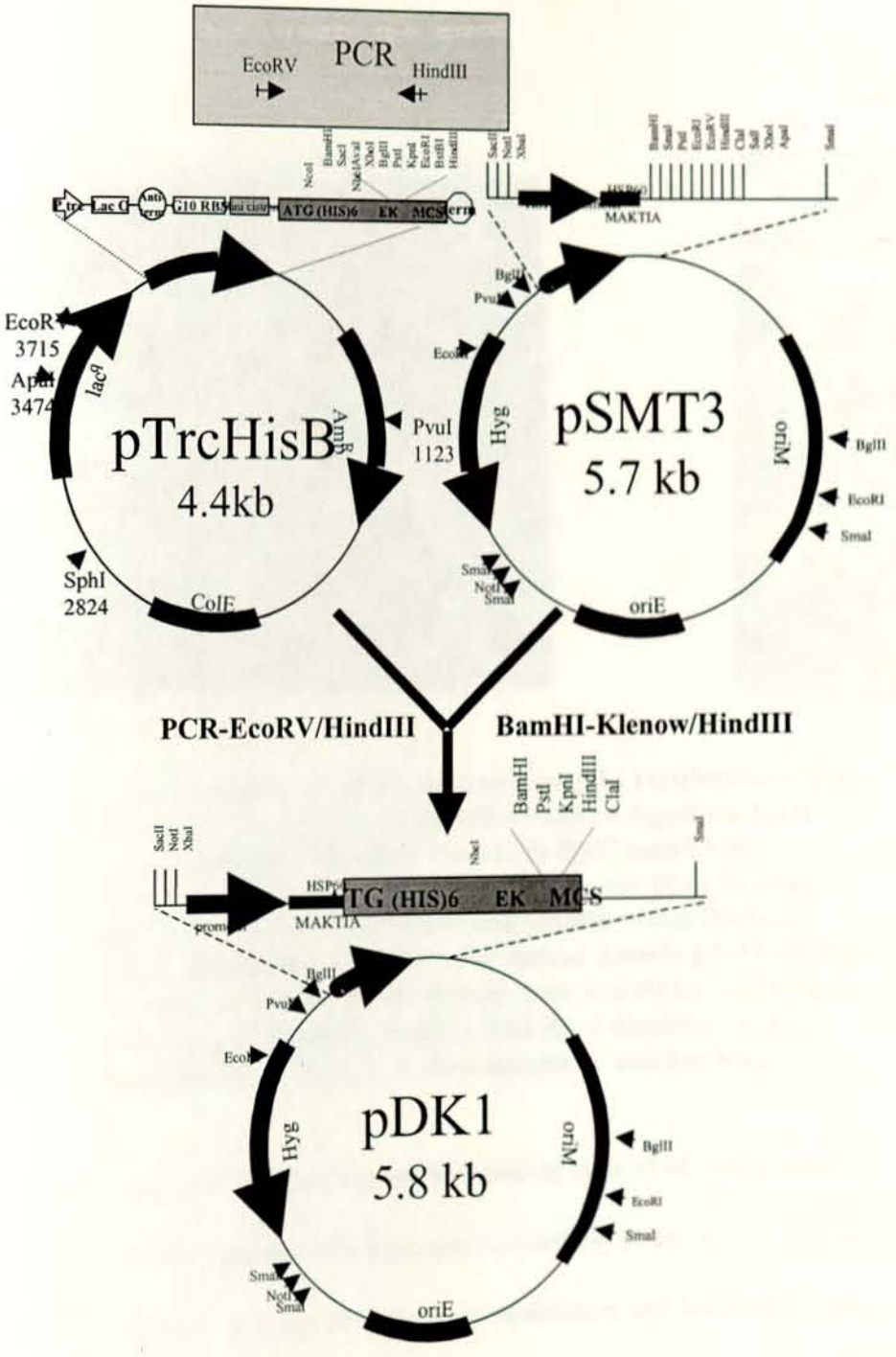


Fig 5. Diagrammatic representation of the construction of pDK1.

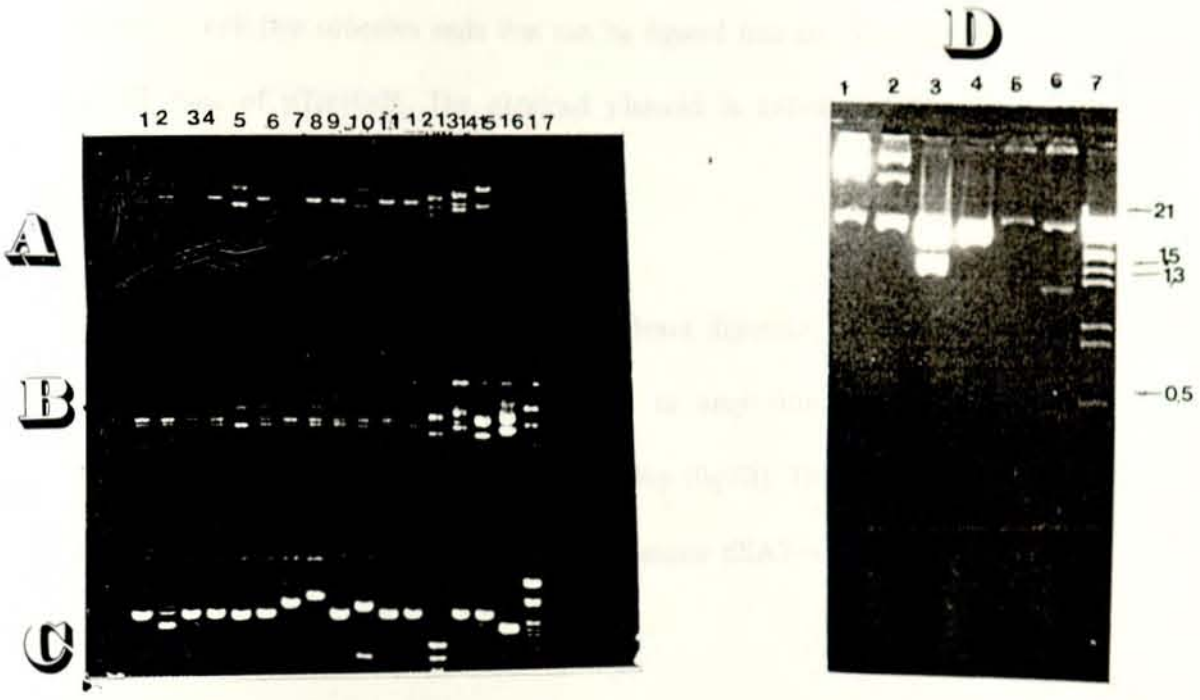


Fig. 6. The correctness of pDK1 was confirmed by restriction enzyme analysis. (A) *Kpn*I restriction endonucleas enzyme digestion, lanes 9,11,12 linearized pDK1 (positive result) (B) *BglII* restriction endonuclease digestion which yield three fragments. (C) Checking of restriction enzyme normal function using pic20H vector DNA.(D) Further characterization of pDK1 with control. Lane1: pTrcHisB *Bgl*II digested lane2: pTrcHisB *Kpn*I digested. lane 3: pSMT3 *Bgl*II digested ., lane 4: $pSMT_3$ undigested., lane5: pDK1 *Kpn*I digested., lane 6: pDK1 *Bgl*II digested ., lane 7: λ DNA molecular marker(Kbp)

4.2.2. Cloning of the Esat-6 protein-encoding gene of *M. tuberculosis.*

Procedures for cloning DNA segments in *E.coli* by insertion into a plasmid or bacteriophage genome are now well established and have been used to isolate proteins that encodes different genes. The pTrcHisB vector was used to clone the Esat-6 protein encoding gene that was amplified by PCR from *M. tuberculosis* genomic DNA. The PCR fragment was digested with the

restriction endonucleases *Bam*HI and *Eco*RI. This results in a DNA fragment with two cohesive ends that can be ligated into the *Bam*HI and *Eco*RI sites of pTrcHisB. The obtained plasmid is called pHis/ESAT (fig7A).

Gel electrophoresis after restriction endonuclease digestion of randomly selected transformants conferring resistance to ampicillin showed the presence of the esat-6 gene whose size is ~300bp (fig7B). These constructs can be used for overexpressing the *M. tuberculosis* ESAT-6 protein in *E. coli.*

The construct for the over-expression of Esat-6 in mycobacteria was constructed as follows. PDK1 was digested with *Bam*HI and *Hin*dIII. The esat-6 protein-encoding gene was excised from pHis/ESAT using *Bam*HI and *Hin*dIII. The fragments were ligated and transformed to *E.coli.* Transformants selected on the basis of hygromicin resistance were analyzed using *Bam*HI and *Hind*III (fig 10B). The resulting pDK2 expression vector, whose size is ~6.1kb (fig 8) will be used for overexpression of ESAT-6 in *M. smegmatis.*

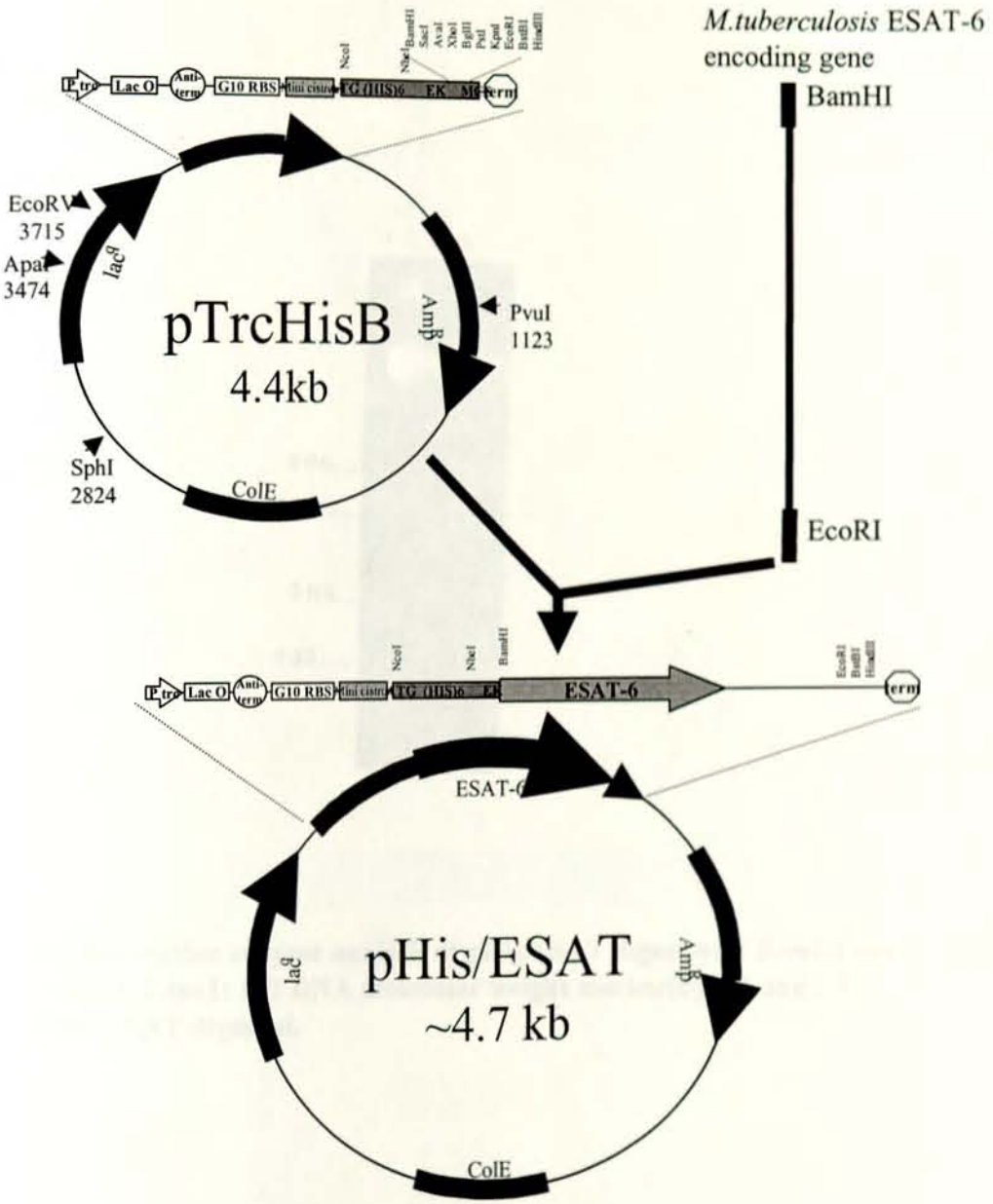


Fig.7 (A) Construction of pHis/ESAT

(B) Restriction enzyme analysis of pHis/ESAT digest with *Bam*HI and *Hin*dIII. Lane1: 123 DNA molecular weight marker(bp). Lane 2,3 :pHis/ESAT digested.

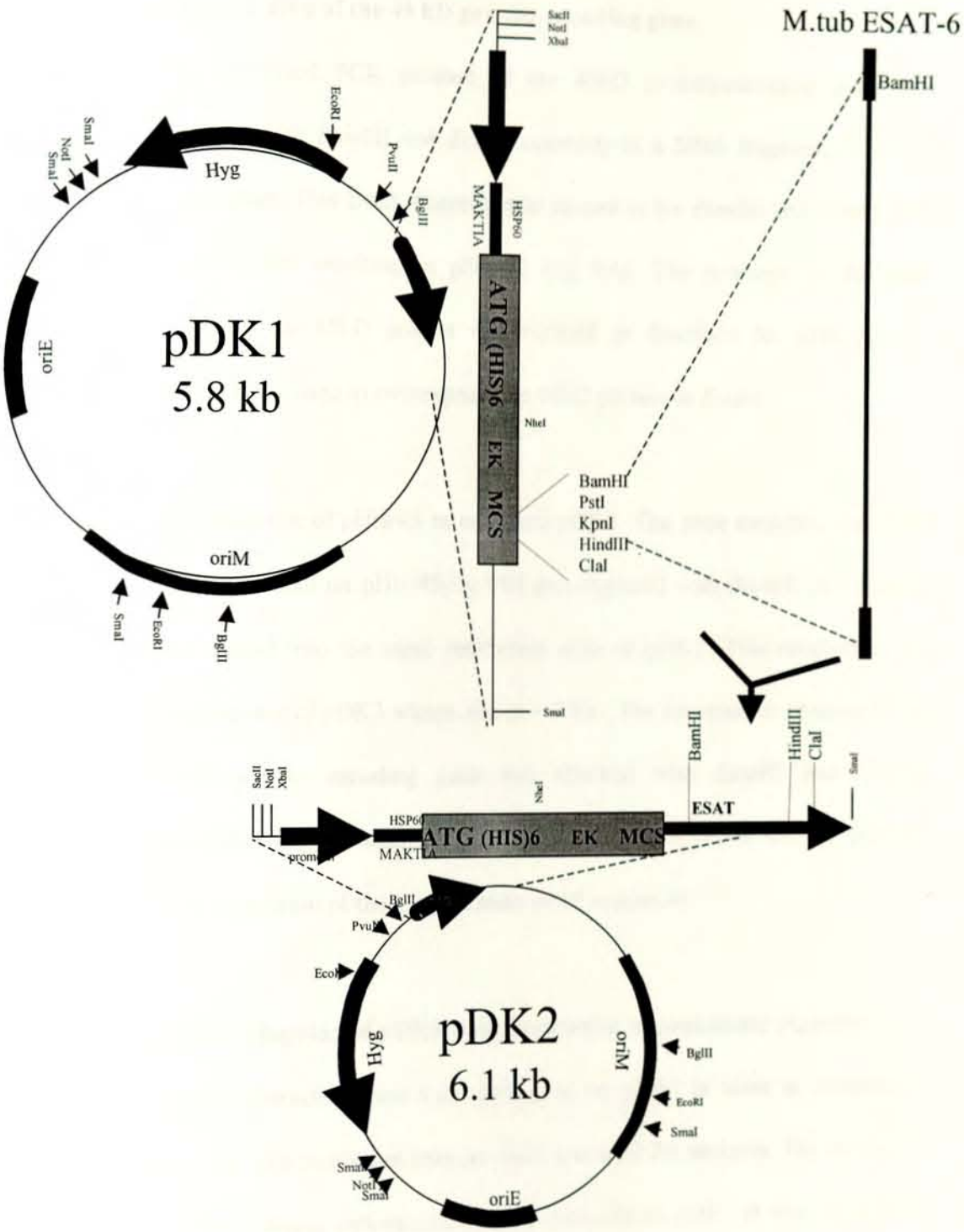


Fig.8.Construction of pDK2 mycobacterial expression vector

4.2.3. Cloning of the 45 kD protein-encoding gene

The amplified PCR product of the 45kD protein-encoding gene was digested with *Bam*HI and *Eco*RI resulting in a DNA fragment with two sticky ends. This DNA fragment was cloned in the *Bam*HI and *Eco*RI sites of pTrcHisB resulting in pHis/45 (fig 9A). The presence of the gene encoding the 45kD protein was verified as described for pHis/ESAT . pHis/45 was used to overexpress the 45kD protein in *E.coli.*

We made use of pHis/45 to construct pDK3. The gene encoding the 45kD protein present on pHis/45(fig 9B) was digested with *Bam*HI and *Hin*dIII and cloned into the same restriction sites of pDK1. This resulted in the construction of pDK3 whose size is ~ 7 kb . The presence or absence of the 45kD protein encoding gene was checked with *Bam*HI and *Hin*dIII restriction enzyme analysis (fig. 10B). The pDK3 vector will be used for over expression of the 45kD protein in *M. smegmatis.*

4.2.3.1. Mapping of pDK3 using restriction endonuclease digestion.

Further characterization was carried out on pDK3 in order to confirm the identity. The restriction enzyme *Sma*I was used for analysis. The number of *Sma*I restriction endonuclease recognition site on pDK1 is four, as a result of 3 sites derived from $pSMT_3$ and one from the pHisB-tag. PDK3 should contain an additional *Sma*I site derived from the 45kDa protein encoding gene. Computer calculations revealed that the following fragments should

be obtained following a *Sma*I digestion: 2970, 1786, 1237, 964 and 66bp Figure 11 shows that this is indeed the case. The last fragment which is only 66 bp is not visible on the gel. But all the other fragments are of the correct size and differ from the pDK1 digest that is used as a control.

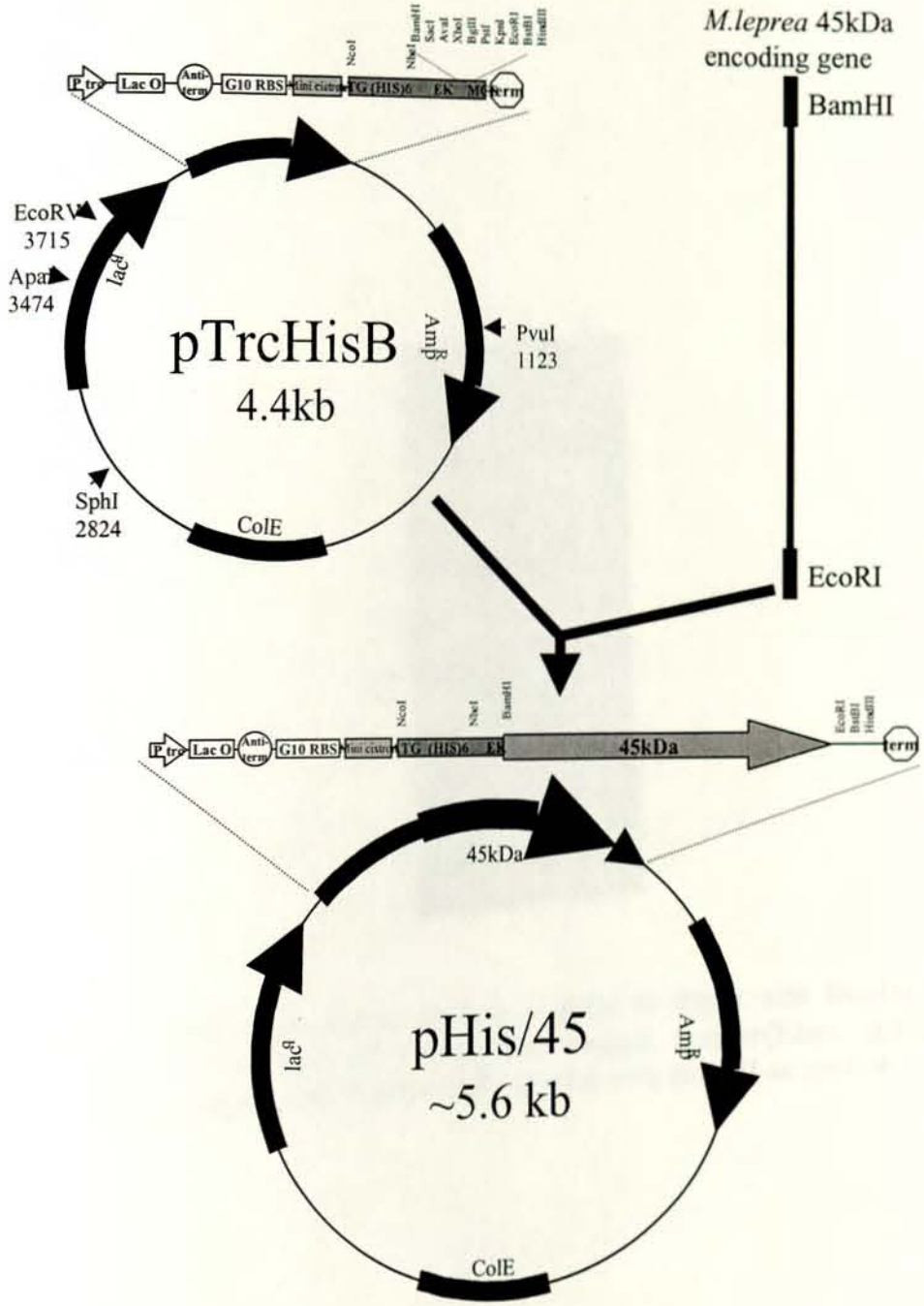


Fig.9 Construction of pHis/45

(B). Restriction enzyme analysis of pHis/ 45 digest with *Bam*HI and *Hind*III. Lane1: λ-DNA molecular weights marker(Kbp). lane2,3,4 pHis/45 digest, lane 5 :pTrcHisB digested with BamHI as control.

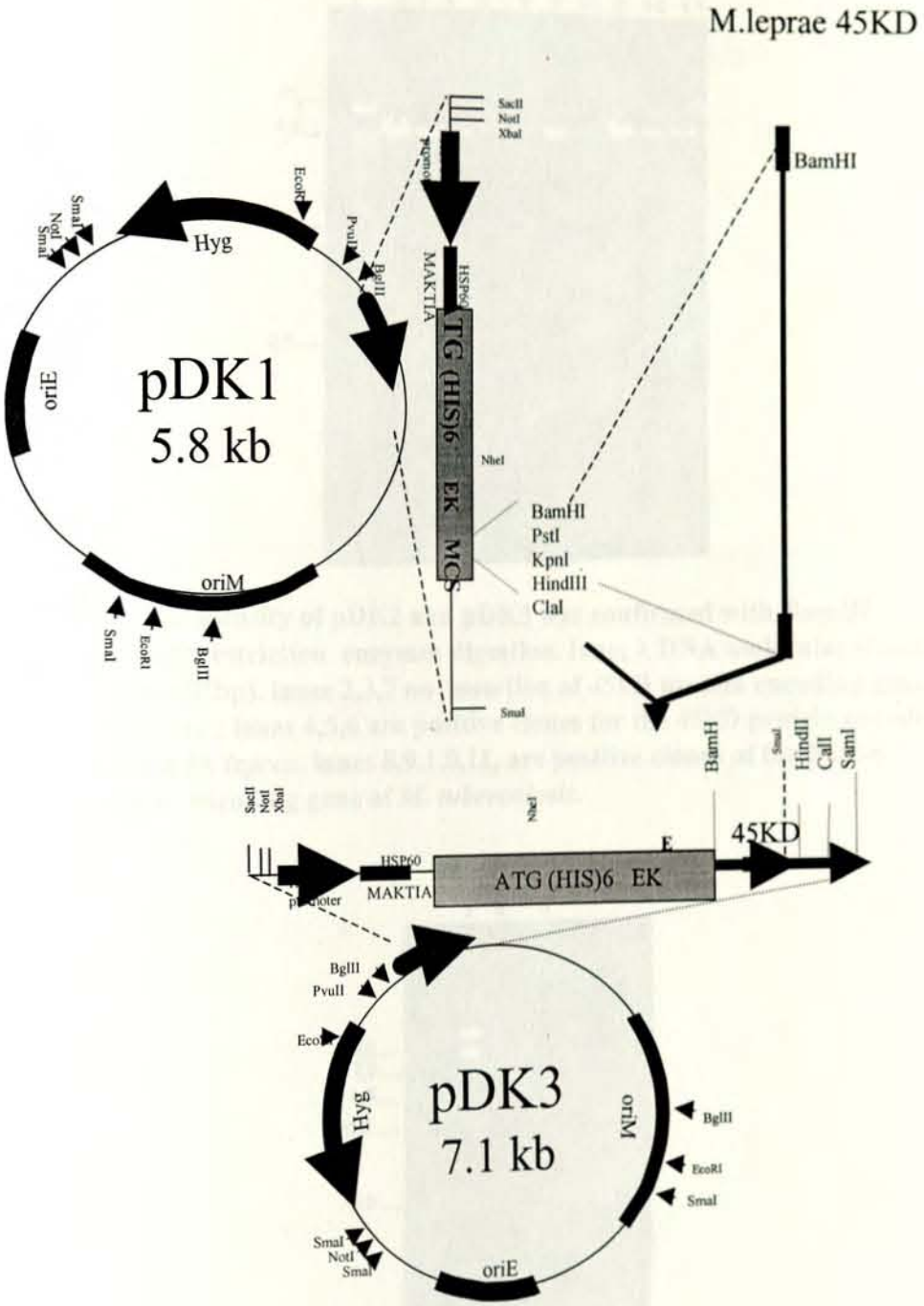
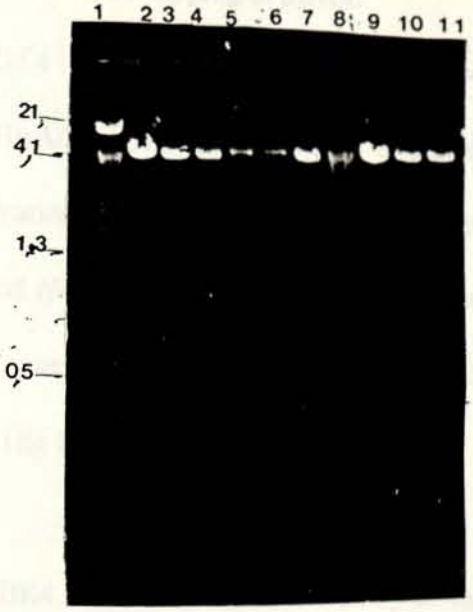


Fig.10 (A) Construction of pDK3 mycobacterial expression vector



(B). The identity of pDK2 and pDK3 was confirmed with *BamHI /HindIII* restriction enzymes digestion. lane; λ DNA molecular weight marker(Kbp). lanes 2,3,7 no insertion of 45kD protein encoding gene of M. leprea.; lanes 4,5,6 are positive clones for the 45kD protein encoding gene of *M. leprea*. lanes 8,9,1,0,11, are positive clones of the Esat-6 protein encoding gene of *M. tuberculosis*.

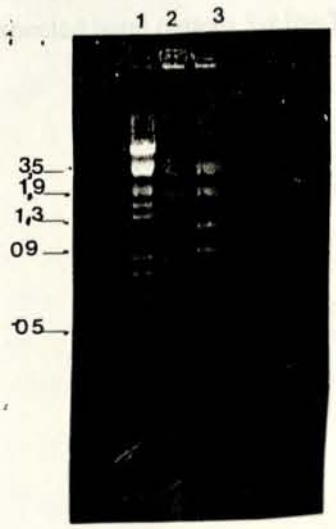


Fig.11. Mapping of pDK3 with *SmaI* restriction endonuclease digestion. Lane 1:λ- DNA molecular weight marker. Lane2: pDK1. Lane3: pDK3

4.2.4. Construction and Mapping of pDK4.

The vector pDK4 was constructed using the following two plasmids: pSMT3 and pHisA/Trx. The latter was used to amplify the Trx encoding gene in combination with the HisA-tag. This PCR product was digested with *Eco*RV and *Hin*dIII that create a blunt and a steaky end respectively. This fragment was cloned in the *Eco*RV and *Hin*dIII sites of pSMT3 creating pDK4 (fig 12A).

The plasmid pDK4 was further characterized by double digestion with the *Bam*HI and *Hin*dIII restriction endonuclease digestion. This results in the excision of the Trx encoding gene whose size is approximately 380bp (fig 12B). The excised band was isolated from gel and further digested with Sau3A to confirm the identity of the excised fragment. The restriction pattern showed the expected band pattern for the Trx gene (data not shown).

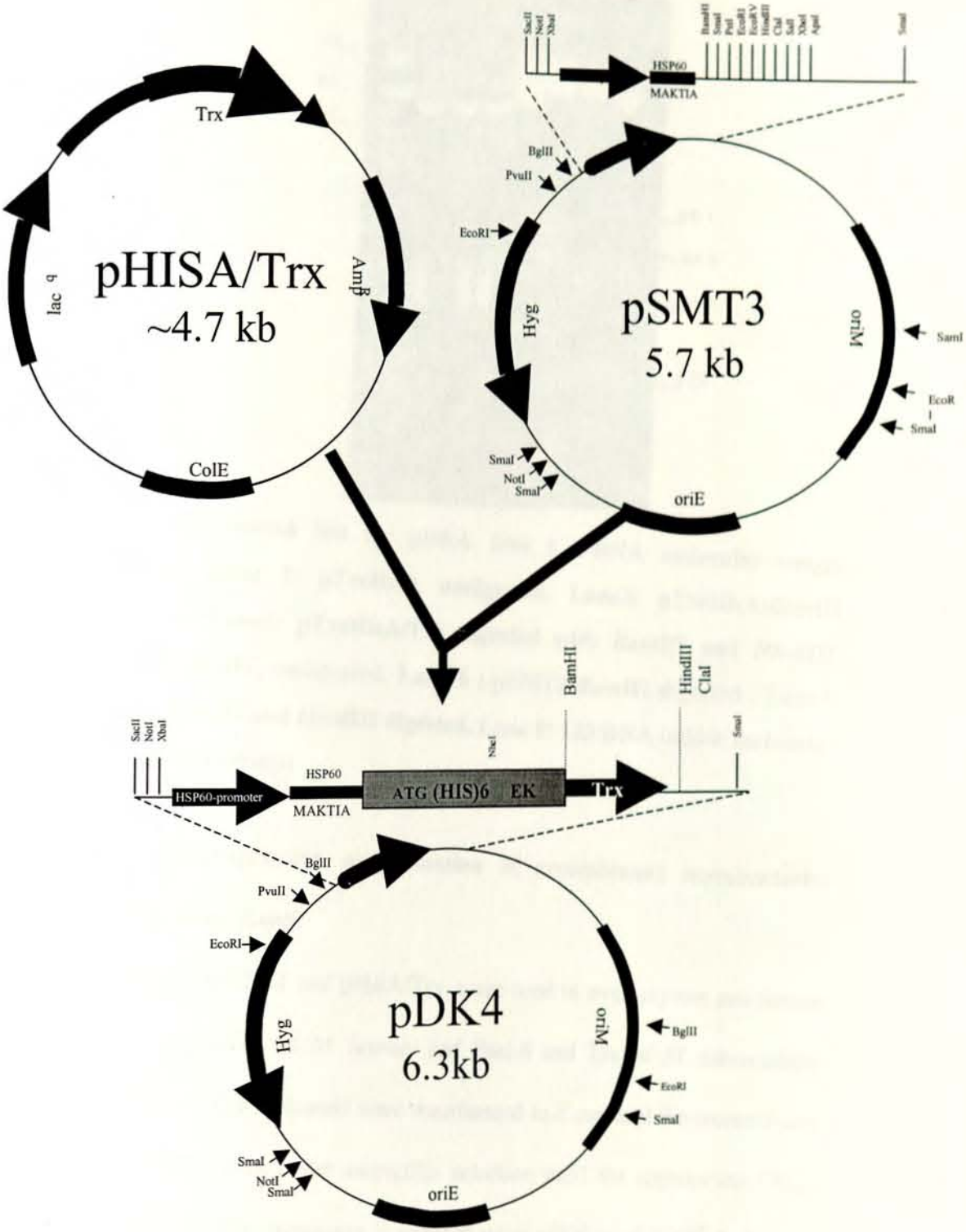
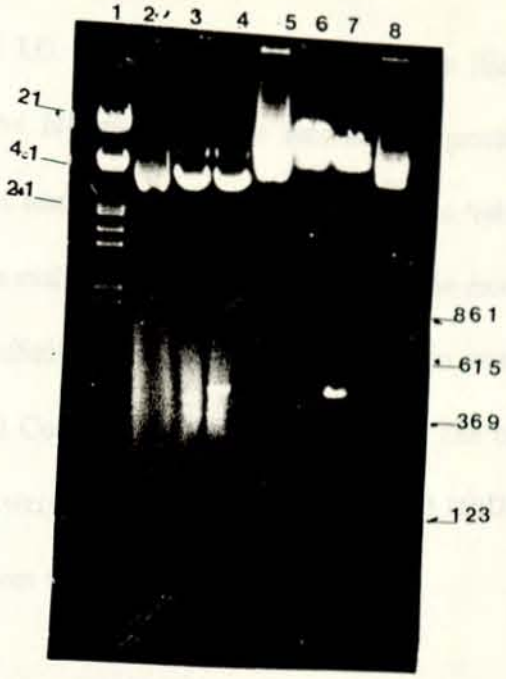


Fig. 12.(A) Construction of pDK4



B) Conformation test for pDK4. lane 1 λ-DNA molecular weight marker. Lane 2: pTrcHisA undigested. Lane3: pTrcHisA/*Bam*HI digested. Lane4: pTrcHisA/Trx digested with *Bam*HI and *Hin*dIII. Lane 5: $pSMT_3$ undigested. Lane 6 : pSMT3 *Bam*HI digested . Lane7: pDK4 *Bam*HI and *Hin*dIII digested. Lane 8: 123 DNA ladder molecular weight marker(bp)

4.3. Overexpression and isolation of recombinant mycobacterial proteins from *E.coli.*

pHis/45, pHis/ESAT and pHisA/Trx were used to over express and isolate the 45 kD protein of *M. leprae,* and Esat-6 and Trx of *M. tuberculosis* respectively. These plasmid were transformed to *E.coli* and the recombinant strains were grown under ampicillin selection until the appropriate OD_{600} were reached. The expression is induced upon addition of IPTG. Induction of pHis/45, pHis/ESAT and pHisA/Trx leads to the production of the

recombinant 45 kD protein, ESAT-6 and Trx. The His-tags which are positioned at the N-terminus of the recombinant protein, facilitate the purification. The histidine- tag consists of 6 histidine residues which have an affinity for metal ions. Using NTA-Ni beads, the recombinant protein can easily be affinity purified. The purified protein was analyzed using SDS-PAGE and Coomassie brilliant blue staining. The obtained products have molecular weights of approximately 50, 10 and 16kD. An additional 4 kD is derived from the HIS-tag (fig. 13).

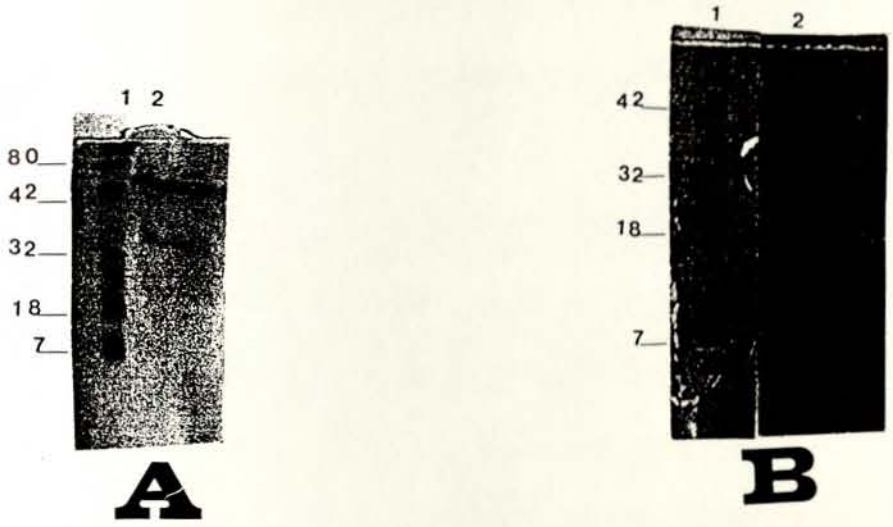


Fig 13. SDS-PAGE followed by Coomassie Blue Brilliant staining . (A) Lane1: molecular weight marker (kD), lane2: 45kD protein of *M. leprea*. (B. lane 1: molecular weight marker(kD) lane 2: Trx protein of *M. tuberculosis*.

4.4 Overexpression and isolation of recombinant mycobacterial proteins from *M.smegmatis.*

The isolation of our model proteins from *M.smegmatis* is still in progress but the preliminary results look promising. All pDK vectors are introduced into the *M.smegmatis* strain and their presence is verified. We are currently trying to find the optimal conditions for overexpression and purification.

5. DISCUSSION

The major importance in the study of microbial antigens is the ability to produce proteins in large quantity. Over the years mycobacterial proteins have been isolated in a number of different ways. One is the isolation of proteins from the pathogenic host. This has a number of drawbacks. *M.leprae* cannot be cultured in vitro whereas *M.tuberculosis* has a very slow replication time. Once enough mycobacteria are collected the purification procedures are tedious. The proteins have to be isolated to purity using a number of different biochemical fractionation methods. With the introduction of recombinant DNA technology and the development of *E.coli* expression systems these obstacles could be overcome.

The gene encoding the protein of interest could now be expressed and isolated from a recombinant host. The use of special fusion proteins systems allowed the affinity purification of the desired protein. Although this system is very useful for a large number of the mycobacterial proteins it can not be used for certain proteins. The reasons for this are numerous. Some proteins cannot be overexpressed in *E.coli* simply because they are toxic for the recombinant host. Good examples are many of the mycobacterial cell wall proteins. Furthermore, proteins that need to be isolated in their native form, for example enzymes for biochemical studies can be problematic as well. First of all, quite a high number of proteins overproduced in *E.coli* end up in

inclusion bodies. This hampers the isolation of the protein of interest in it's native form. Second, the recombinant product might not have the proper posttranslational modification. The lack of sugar or lipid groups that are attached to the protein in mycobacteria but not in *E.coli* could have a negative effect with regard to biochemical studies. Besides that, the lack of posttranslational modification might also alter the immunogenicity of the recombinant protein. It is now generally accepted that these sugar and lipid moieties have great immunological potentials. The ultimate goal therefore should be to produce the recombinant products in the frame that closely resembles their native state. Transcription and translation of mycobacterial genes in mycobacterial hosts should allow more accurate processing of gene products including folding and posttranslational modification than expression in *E. coli*. This hypothesis has been established with the construction of suitable expression vectors that can function normally in a mycobacterial host like *M. smegmatis*.

Our immediate aim was to develop a vector that would permit both the manipulation and amplification of mycobacterial DNA constructs in *E. coli*, and subsequently transfer and replication them in a variety of mycobacteria. In particular we wanted to introduce expression vectors in the fast growing non-pathogenic mycobacterium, *M. smegmatis* and be able to affinity purify the overproduced protein.

We have made use of the properties of the pTrcHis *E.coli* expression vector and the pSMT3 mycobacterial shuttle-vector.

PTrcHis is an *E.coli* expression vector. The pTrcHis vectors contain 6 DNA triples encoding a tract of 6 histidine residues. This His-tag functions as a metal binding domain for purification of recombinant proteins by immobilized Metal affinity chromatography. After expression in the appropriate host the protein can be purified by binding to and elution form Nickel charged agarose beads. The vector has a multiple cloning site with a number of restriction enzyme recognition site. Besides that it encodes for a T7 epitope and a protease cleavage site. The T7 epitope allows the identification of the recombinant protein by making use of commercially available antibodies directed against this epitope. The protease cleavage site which is recognized by the protease entro-kinase allows the removal of the His-tag from the recombinant protein. The pTrcHis system is a set of three vectors A, B and C. The suffix denotes the three reading frames relative to the *Bam*HI cloning site for appropriate translational fusion.

PSMT3 is a mycobacterial shuttle vector. It can be maintained in both mycobacteria and *E.coli* (Garbe *et al.*, 1994). This vector is equipped with a strong mycobacterial promoter and a small DNA piece encoding the hsp-60 N-terminal region. The vector allows the over expression of mycobacterial genes cloned as translational fusion products behind the first 30 nucleotides

of the hsp-60 protein. The mycobacterial promoter is derived from the hsp-60 gene. This promoter is a constitutive promoter but it's activity can be up-regulated by heat-shock. Proteins over-expressed using pSMT3can not be isolated using affinity chromatography.

Therefore, we amplified the complete His-tag from pTrcHis and cloned it in pSMT3. The resulting vector pDK1 should allow us to over-express and affinity purify proteins from mycobacterial hosts.

The histidine purification system overcomes the problems involved with antibody affinity chromatography used in a number of studies to purify recombinant mycobacterial proteins from mycobacterial hosts (Roche *et al.*, 1996). Furthermore, the histidine tag was shown not to an effect on the protein conformation or immunogenicity, suggesting that the vector described may prove useful for the purification of recombinant proteins from fast growing mycobacterial hosts (Triccas *et al.*, 1998).

We used PCR amplification to obtain the His-tags of the pTrcHisA, B and C vectors.

Chemically synthesized oligonucleotides with defined sequences have the potential to fish out a desired fragment a large piece of DNA. The upstream and downstream synthetic fragments are complementary to the 5' and 3'

strands of the DNA respectively and form duplex molecules with specific parts of the target DNA. Not the entire synthetic oligonucleotide needs to bind to the target DNA to achieve amplification. This allows the incorporation of specific restriction endonuclease recognition sites in the primers, which facilitates the cloning of a specific PCR fragments.

For the amplification of a number of our fragment we used the pfu polymerase and High Expand™ Fidelity polymerase enzymes. These enzyme have much higher fidelity compared to Taq-DNA polymerase. Taq-DNA polymerase has no associated 3'-5' exonuclease activity to confer a proofreading function, and the error rate due to base mismatch incorporation during DNA replication is rather high (Strachan and Read1997). Recently the problem of infidelity of DNA replication has largely been overcome by using these high efficient enzymes that minimize mismatch incorporation of nucleotides. High fidelity is required for this His-tag because a single mismatch can result in the loss of a restriction enzyme site or a triplet encoding a histidine. The sequence facilities at our Institute are limited. We do realize that our PCR fragments need to be sequenced in order to make sure that the products we clone do not contain any mismatches. This will certainly be done as soon as all reagents are made available. But making use of high-fidelity enzymes gives us the certainty that the chances of mismatch incorporation are close to nil.

In this study we have used three model proteins.

One is the ESAT-6 protein, an *M.tuberculosis* specific protein with potent immuno-stimulatory capacity. This protein is difficult to overproduce in *E.coli*. Our aim is to test whether overproduction in a mycobacterial host would be more efficient.

The second model protein is the 45 kD protein of *M.leprae*. This protein is specific for *M.leprae*. This specificity is a useful property. *M.smegmatis* lacks the gene encoding the 45kD protein excluding the possibility of contamination with the hosts protein.

The third protein is Trx from *M.tuberculosis*. This is a redox-enzyme. Over-expression of this protein in *M.smegmatis* allows us to determine whether functional and enzymatically active proteins can be produced using our system.

In conclusion, the genes encoding our model proteins were cloned in the pTrcHis vector for over-expression in *E.coli*. The Trx protein and the 45kD could be overproduced in relatively high quantities. The ESAT-6 protein turned out to be quite problematic with very low levels of recombinant protein.The same genes were cloned in our newly constructed pDK1 vector. These new constructs termed _pDK2, pDK3, pDK4, containing the genes

encoding ESAT-6, 45kD and Trx respectively were transferred to the mycobacterial host *M.smegmatis* 1-2c. We are currently overproducing and purifying the proteins from the *M.smegmatis* host.

As soon as we have obtained our model proteins from both hosts we will compare them with regard to differences in immunogenicity, enzyme activity and biochemical property, e.g. differences in posttranslational modification.

We think our vector might offer several advantages as a cloning vehicle for the production of recombinant proteins. Several studies have highlighted the superiority of recombinant proteins purified from mycobacterial hosts compared to the *E. coli* derived products, as assessed by structural and immunological analysis (Garbe *et al.*,1993; Triccas *et al.*, 1996).

Several recent examples (Yuan *et al.*, 1995; Zhang *et al.*,1992) leading to the identification of genes involved in the drug resistance or encoding new therapeutic targets testify to the power of this approach.

We can recommend that the expression of mycobacterial proteins in rapidly growing mycobacteria can be further improved by the addition of stronger mycobacterial promoters in these constructs. In this study we have made use of the hsp-60 promoter. Very recently a paper describing work similar to

ours was published. Instead of using the hsp-60 promoter these researchers used the acetamidase promoter. This is a highly inducible promoter which has clearly some advantage over the promoter we use in our system.

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APPENDIX

Restriction enzyme recognition site, used buffer, Temp.

*Bam*HI	G↓GATCC	*Bam*HI buffer	37°C
*Eco*RI	G↓AATTC	*Eco*RI buffer	37°C
*Eco*RV	GAT↓ATC	NEB2	37°C
*Hin*dIII	A↓AGCTT	NEB2	37°C
*Sma*I	CCC↓GGG	NEB4	25°C
*Bgl*II	A↓GATCT	NEB3	37°C
*Kpn*I	GGTAC↓C	NEB1	37°C

Declaration

I, the undersigned, declare that this thesis is my original work, has not been presented in any other university for a degree and all sources of materials used in this work has been duly acknowledged.

Name: Dawit Kidane

Signature: