Functional Genome Analysis of *Mycobacterium tuberculosis*

vorgelegt von
Diplom-Ingenieur Biotechnologe
Helmy Rachman
aus Berlin

von der Fakultät III Prozesswissenschaften
der Technischen Universität Berlin
zur Erlangung des akademischen Grades

Doktor der Naturwissenschaften
- Dr.rer.nat. -

genehmigte Dissertation

Promotionsausschuss:

Vorsitzender: Prof. Dr. Lauster
Berichter: Prof. Dr. Stefan H. E. Kaufmann
Berichter: Prof. Dipl-Ing. Dr. Ulf Stahl

Tag der wissenschaftlichen Aussprache: 12. November 2003

Berlin 2004
D 83

Functional Genome Analysis of
Mycobacterium tuberculosis

submitted by
Diplom-Ingenieur Biotechnologist
Helmy Rachman
from Berlin

from the Faculty III School of Process Sciences and Engineering
of Technische Universität Berlin
for the obtainment of the academic degree

Doktor der Naturwissenschaften
- Dr.rer.nat. -

approved dissertation

doctoral commitee:

chairman: Prof. Dr. Lauster
examiner: Prof. Dr. Stefan. H. E. Kaufmann
examiner: Prof. Dipl-Ing. Dr. Ulf Stahl

Date of defense: 12 November 2003

Berlin 2004
D 83

_____

_____

_____

_____

1. Introduction

1.1 Characteristics and taxonomy of *M. tuberculosis*

The mycobacteria are acid-fast, aerobic, nonsporeforming, nonmotile bacteria. They have cell wall rich in lipid content, which is responsible for the ability of the organisms to withstand staining by usual aniline dyes (1). Therefore, common stains for bacteria like the Gram stain are relatively ineffective. Methods that promote the uptake of dyes are available. However, once mycobacteria are stained, they are not easily decolourised even with acid alcohol. Therefore, they are described as acid fast (2). They often stain irregularly and appear beaded or granular; during growth on certain culture media, some of the mycobacteria appear non-acid fast. The taxonomy of *Mycobacterium tuberculosis* (*M. tuberculosis*) as adopted from The Institute of Genomic Research (TIGR) (http://www.tigr.org/) is as follows:

Kingdom:	Bacteria
Intermediate Rank 1:	Actinobacteria
Intermediate Rank 2:	Actinobacteridae
Intermediate Rank 3:	Actinomycetales
Intermediate Rank 4:	Corynebacterineae
Intermediate Rank 5:	Mycobacteriaceae
Genus :	Mycobacterium
Species :	tuberculosis

The mycobacteria are considered transitional forms between eubacteria and actinomycetes: some of the latter, of the genus *Nocardia*, are weakly acid-fast, whereas some mycobacteria may exhibit branching. Accordingly, the mycobacteria have been classified in the order Actinomycetales. Mycobacteria may appear to be gram-positive, but they take the stain weakly and irregularly and without requiring iodine treatment to retain it (3).
In general, the growth rate for mycobacteria is slow and requires two weeks or longer for detection by use of traditional mycobacteriology media.

Robert Koch discovered in 1882 the tubercle bacillus as the cause of tuberculosis. Wilhelm Zopf named the organism *Bacterium tuberculosis* in 1883. Three years later Jorgen Lehmann and Ernst Neumann changed this name to *Mycobacterium tuberculosis*, probably because the slow-growing nature of the organism and the rough appearance in culture made it look funguslike. The organism grows in culture as slowly developing, rough colonies with a characteristic buff colour. The variations in hue and texture of colonies of tubercle bacilli on a given medium are readily learned but difficult to describe (4). On the most favourable culture media but without other optimal conditions, colonies are recognisable in less than 3 weeks. Characteristic microcolonies may be readily recognised in less than one week by the microscopic observation of growth on Middlebrook 7H11 plates. Although a definitive identification can not be made on the basis of colony morphology, the development of a colony with serpentine cord formations resulting from the production of cord factor is sufficient presumptive evidence to warrant initiation of drug therapy directed against *M. tuberculosis* (5).

The typical cell morphology of *M. tuberculosis* as seen in acid-fast stains is a thin, slightly curved bacillus measuring 0.3 to 0.6 µm by 1 to 4 µm, deeply red staining (strongly acid fast), with a distinct beaded appearance. In culture media, the cells may range from coccoid to filamentous. Strains differ in their tendency to grow as discrete rods or as aggregated long strands, called serpentine cords.

1.1.1 Cell wall

The most striking chemical feature of the mycobacteria is the abundance of lipids in the cell wall (up to 60% of its dry weight), much of which is attached to polysaccharides, which include glucan, mannan, arabinogalactan, and arabinomannan (6). This property accounts for the hydrophobic character of the organisms and probably also for some of the other unusual properties of mycobacteria, such as their relative impermeability to stains, acid-fastness, unusual resistance to killing by acid and alkali, and resistance to the bacteriocidal action of antibiotics (7-9). The glycolipids and proteins are located in a firmly attached outer layer of the wall, and the external location of

the lipid accounts for the hydrophobic character of the cells. Electron microscopy reveals a rather thick wall, and large lamellar mesosomes are common. Glycogen granules and polymetaphosphate (volutin) bodies are also seen. Various lipid fractions are defined by the conditions used for their extraction from dried organisms. Among the lipids extracted with neutral organic solvents are true waxes (esters of fatty acids with fatty alcohols) and glycolipids (mycosides: lipid-soluble compounds with covalently linked lipid and carbohydrate moieties) (10;11).

Although many different fatty acids are found in mycobacteria, the mycolic acids appear to be unique to the cell walls of these organisms, nocardiae, and corynebacteria (12;13). These very large, saturated α-alkyl, β-hydroxyl fatty acids are found in both waxes and glycolipids. A large arabinogalactan, covalently attached to the peptidoglycan and to about 30 mycolic acid residues, forms a bridge between the rigid layer and the outer, lipophilic layers of the cell wall. Three classes of mycolic acids have been found in *M. tuberculosis*, with cyclopropane, methoxy, or keto groups, and with slight variation in the chain length (mostly C_{78} and C_{80}). Mycolyl acetyl trehalose is a precursor of cell wall mycolic acid (and probably of cord factor); the disaccharide solubilises the very hydrophobic, unusually long-chain fatty acid (14;15). Cord factor, extracted from virulent mycobacteria by petroleum ether, has been identified as a mycoside 6,6'-dimycolyltrehalose. Several lines of evidence relate it to virulence (16;17). The toxicity of cord factor has been related to profound disturbances of microsomal enzymes, mitochondria, and metabolism in the livers of mice.

1.1.2 Nutrients

Unlike most other pathogenic bacteria, which are facultative aerobes or anaerobes, the tubercle bacillus is an obligate aerobe microbe (18-20). It can grow in simple synthetic media, with glycerol or other compounds as the sole carbon source and ammonium salts as the nitrogen source. Asparagine or amino acid mixtures are usually added to promote the initiation and improve the rate of growth (21). In fact, Sauton medium, a minimal medium for mycobacteria, contains glycerol and asparagine as the only carbon and

nitrogen source, respectively (22-24). In ordinary synthetic liquid media, the bacilli grow as adherent clumps that form a surface pellicle. Some detergents, such as Tweens, cause the bacilli to grow in more dispersed form, although still not as single cells.

Mycobacteria generally show a marked nutritional preference for lipids; egg yolk is a prominent constituent of the rich media used for diagnostic cultures (25). Thus, although the tubercle bacillus is very sensitive to inhibition by long-chain fatty acids, it is stimulated by them at very low concentrations (26). A satisfactory concentration is maintained by adding to the medium serum albumin, which binds the fatty acids with sufficient affinity to maintain a low free concentration (27). Growth of tubercle bacilli in culture media is characteristically slow: the shortest doubling time observed, in rich media, is about 12 hours.

1.2 Pathogenesis of *M. tuberculosis*

Pulmonary tuberculosis is the most important life-threatening bacterial disease in the world today. The World Health Organisation has estimated that about 1.7 billion people (one-third of the world's population) are infected with *M. tuberculosis* (28;29). Approximately 2 million individuals die from tuberculosis and more than 8 million new cases arise annually. Moreover, the incidence of tuberculosis is increasing due to the human immunodeficiency virus (HIV) pandemic and the emergence of multidrug-resistant strains of *M. tuberculosis*. The pathogenesis of tuberculosis is unusual because, unlike many other infections, the development of active disease can involve a delay between infection and clinical disease ranging from several weeks to several decades. While active disease may arise almost immediately after infection in about 5% of exposed individuals, most of the others will develop a latent infection in which the tubercle bacilli can persist *in vivo* without causing any clinical symptoms (30). Active disease may develop decades later either as a recrudescence of the initial infection (endogenous reactivation) or because of a secondary infection with a different tuberculosis strains (exogenous reinfection) (31-34). The consequences of inhaling or ingesting tubercle bacilli depend on both the virulence of the organism and the resistance of the host

_____

(as well as the size and the location of the inoculum). At one extreme, organisms with little virulence for the particular host disappear completely, leaving no anatomic trace behind. At the opposite extreme, the bacilli flourish within macrophages as well as extracellularly, are disseminated widely, and cause death within a few months (29). Humans exhibit a range of responses. The initial infection in a tuberculin-negative immunocompetent individual most often produces a self-limited lesion, but sometimes the disease progresses, presumably because of low resistance or a large inoculum. Because of the delicate balance of resistance, involving local as well as systemic factors, healing and progressing lesions may coexist in the same individual, and the disease often has a chronic, cyclic course (especially without chemotherapy). This pattern stands in marked contrast to the acute course of those infections that instigate well-defined humoral immunity (35).

Histologically, the tubercle bacillus evokes two types of reaction. Exudative lesions are seen in the initial infection or in the individual in whom the organism proliferates rapidly without encountering much host resistance. Acute or subacute infection inflammation occurs, with exudation of fluid and accumulation of polymorphonuclear leukocytes around the bacteria (36-38). Productive (granulomatous) lesions form once the individual has developed delayed hypersensitivities to tuberculoprotein. The macrophages then undergo a dramatic modification on contact with tubercle bacilli or their products, becoming concentrically arranged in the form of elongated epithelioid cells, to form the tubercles characteristic of this disease. In the center of the tubercles, some of these cells may fuse to form one or more giant cells, with dozens of nuclei arranged at their periphery and viable bacilli often visible in their cytoplasm. Outside the multiple layers of epithelioid cells is a mantel of lymphocytes and proliferating fibroblast, leading eventually to extensive fibrosis (39;40). In early lesions, tubercle bacilli are localised primarily within macrophages, in which they multiply (at least for a time). In more advanced lesions, only extracellular bacilli are prominent, probably because the activated macrophages destroy the intracellular bacilli more efficiently.

After inhalation of tubercle bacilli, the initial lesion appears as an area of nonspecific pneumonitis, usually located in a well-aerated peripheral zone. It

_____

is only after delayed hypersensitivity develops, in 2 to 4 weeks, that granulomatous inflammation supervenes and the characteristic tubercles (from Latin word *tuberculum*, small lump) are formed. In the meantime, bacilli are carried to the draining lymph nodes and then, by way of the lymph and blood, throughout the body of the host. The pulmonary focus and the granulomatous lesion in the hilar lymph node together are known as the primary Ghon complex (41). The next stage in the inflammatory response consists of caseation necrosis (from Latin word *caseus*, cheese), in which the necrotic centers of tubercles remain semisolid rather than softening to form pus, presumably because the enzymes that usually liquefy dead cells and tissues are inhibited (42). Primarily, tuberculosis in children usually stabilises and heals. Caseous lesions heal by fibrosis and calcification, which may result in extensive scar formation and shrinkage. The healed and frequently calcified primary complex lesions are referred to as the Ghon complex, which may be recognised in the chest radiographs for the reminder of the person's life. In a small proportion of individuals, however, the infection is not brought under control, and the primary lesions become progressively larger, coalesce, and liquefy. When this material is released, a cavity is formed in the lung, and there may be extension of the disease via the bronchi (43;44).

Most tuberculosis in adults is secondary to reactivation of long-dormant foci remaining from the primary infection (29). The foci are located mostly in the posterior apical or subapical portions of the lung, whose persistent infection, after hematogenous spread, may be attributable to the high pO_2 resulting from their favourable ventilation-perfusion ratio. Viable tubercle bacilli are rarely found in the healed lesions on the primary complex. By the time disease is recognised, liquefaction of the caseous lesion usually has occurred, and a cavity has provided a favourable site for the rapid proliferation of the bacilli. These may then be transmitted to other individuals via droplet nuclei produced by aerosols of infected sputum and to other parts of the lungs by bronchogenic spread. Almost every organ of the body may be the site of extrapulmonary tuberculosis. The most common locations are the genitourinary system, bones and joints, lymph nodes, pleura, and peritoneum. Extrapulmonary disease commonly develops as a result of reactivation of dormant lesions seeded during the primary infection. Disseminated, or miliary,

tuberculosis may follow the rupture of a caseous lesion into a pulmonary vein (45-47).

1.3 Host responses against *M. tuberculosis*

The importance of genetic factors in host resistance has been unequivocally established by Lurie's development of inbred lines of rabbits with a high or a low tendency to acquire progressive tuberculosis from experimental infection (48). Multiple factors are evidently involved: some lines are most resistant to initiation of disease by small inocula, whereas in others the resistance primarily influences the rate of progress of the disease. In humans, it seems likely that there are similar racial differences in resistance associated with different lengths of exposure of the race to the selective pressures of an environment with widespread tuberculosis (49). Epidemiologic evidence on the frequency of tuberculosis in various populations has long suggested that malnutrition, overcrowding, and stress decrease resistance to the disease (50;51). But this kind of evidence does not firmly establish a casual relation because these conditions are generally also associated with a high rate of infection (52;53).

Among persons infected with the tubercle bacillus, as detected by a positive tuberculin test, only a small proportion develops overt disease, and even before the advent of chemotherapy, only about 10% of these progress to fatal disease. The transition from infection to mild or to severe disease depends strongly on various factors besides the presence of the bacilli (54;55). Hence, in an earlier era, when nearly everyone eventually became tuberculin-positive, tuberculosis could almost be regarded as one of the "endogenous diseases". Although many of the observed variations in the response to infection may involve differences in the size, the site, or the virulence of the inoculum, there is also no doubt that resistance in human varies more strikingly with tuberculosis than with most infectious diseases.

M. tuberculosis is an intracellular pathogen in host macrophages. The success of *M. tuberculosis* as a pathogen relies on its ability to survive within macrophages, which at the same time represent the critical host defense cells against mycobacteria. Upon entry, primarily via the respiratory tract, tubercle

---

bacilli are engulfed by alveolar macrophages via a variety of receptor-ligand interactions. In macrophages they reside within early endosome-like phagosomes in which they are able to persist and even multiply (56;57). The *M. tuberculosis*-containing phagosomes are hampered in maturation and fail to fuse with lysosomes which enable the macrophages to kill the bacilli (58;59). The early endosome-like phagosomes allow the bacilli to access the transferrin-iron complex (60;61).

The responses to *M. tuberculosis* include the production of a series of cytokines derived by various host cells including macrophages and T cells (29). Central to the protective immune response is the production of the cytokine IFN-γ by $CD4^+$ T helper 1 (Th1) type and $CD8^+$ T cells. Macrophage activation by IFN-γ stimulates the antimycobacterial mechanisms, most notable the expression of inducible nitric oxide synthase (iNOS). This enzyme cleaves a terminal guanidino group from L-arginine, releasing the nitric oxide radical, NO, and a by-product, L-citrulline. NO is quickly oxidised to other reactive nitrogen intermediates (RNI), including nitrogen dioxide and peroxynitrite (62-65). These reactive radicals are able to attack cellular components of *M. tuberculosis* such as transition metals, thiol groups, Fe-S groups, lipids, and DNA bases leading to general growth inhibition or even killing of the intracellular pathogen. Another paramount aspect of IFN-γ activation of macrophages is the change of iron metabolism. Since iron is a vital component for both host and microbial pathogen, the iron restriction to intracellular pathogens is believed to add to the host defense strategies. Macrophages usually acquire iron via binding of their cell surface receptor TFR (transferrin receptor) to extracellular Fe-TF (iron-transferrin) complex. The fact that activation of macrophages causes the downregulation of TFR expression is appreciated as a host defense strategy to restrict iron availability to pathogens (66;67).

As an intracellular pathogen, *M. tuberculosis* is equipped with strategies to counteract the host defense. Efforts have been made by several groups to identify the virulence factors required by *M. tuberculosis* for host invasion, survival within the host cells, long-term persistence, and manifestation of disease. Gene disruption to generate knockout mutants and subsequent

infection studies in macrophages or mice have been employed to investigate the relevance of certain genes to virulence (68-71).

1.4 Diagnosis of *M. tuberculosis*

A provisional diagnosis of tuberculosis is usually made by demonstrating acid fast bacilli in stained smears of sputum or of gastric washings (containing swallowed sputum) (72). For rapid screening of smears, some laboratories employ fluorescence microscopy following staining with the fluorochrome dye mixture rhodamine-auramine (73-75).

To control tuberculosis, it is still necessary to find a rapid and sensitive diagnostic method. The standard diagnostic techniques include skin tests, X-rays, DNA techniques, and serodiagnosis in addition to the traditional methods (smear and culture) (76). In Western Europe laboratories fatty acid chromatography is also routinely used (77). Skin tests based on tuberculin protein derivatives cannot distinguish tuberculosis infection, tuberculosis vaccination and exposure to environmental Mycobacteria (78-80). The serological diagnostic approach in the diagnosis of mycobacterial infections has been studied for a long time, and its reproducibility and reliability have been reported. Many mycobacterial antigens have been evaluated: cellular extracts, proteins, and glycolipids from the mycobacterial cell wall. In addition to the lipoarabinomannan antigen, the acylated trehalose family has been the most frequently investigated group of glycolipids (81-83). The main obstacles encountered in serodiagnosis of mycobacterial infection are its low specificity and non-standardisation. The sensitivity and specificity of serological tests depend on what types of antigens are used. Higher specificity is achieved with a purified antigen. Studies using enzyme-linked immunosorbent assay (ELISA) detecting mycobacterial antigen showed promising results.

Tuberculin hypersensitivity is best evaluated by intradermal injection of 0.1 ml of an appropriate dilution of standardised PPD into the most superficial layers of the skin of the forearm (Mantoux test) (84-86). The average diameter of induration (and not simply erythemal) at the injected site is measured at 48 hours, and reactions of 10 mm or more are generally considered to be positive (87;88). Persons infected with cross-reactive mycobacteria frequently

exhibit weak reactions (4-9 mm in diameter). Ordinarily, however, the population is quite sharply divided into tuberculin-positive or -negative. Other commonly used methods of skin testing involve multiple punctures of the skin by means of various mechanical devices. These methods are less accurate than the Mantoux test, and positive or doubtful results should be checked by intradermal technique.

1.5 Therapy of tuberculosis

Until the discovery of streptomycin in 1945, the treatment of tuberculosis was limited to rest, good nutrition, and artificial collapse of the lung. Unfortunately, the value of streptomycin was restricted by its toxicity to the eighth cranial nerve and by the frequent emergence of resistant tubercle bacilli during therapy. Some principles of drug therapy were learned rapidly: (i) long-term treatment was necessary to minimise the rate of relapse, (ii) combinations of two or more drugs delayed the emergence of resistant organisms, (iii) some combinations worked better than others, and (iv) important drug characteristics included the ability to penetrate macrophages and kill mycobacteria in their acidic environment.

The older drug regiments included streptomycin (SM), para-aminosalicylic acid (PAS), isoniazid (INH), ethambutol (EMB) replacing PAS or rifampin (RMP), in various combinations. These regiments were usually continued for 18 to 24 months. More rapid action of the newer regiments has been ascribed to the unique ability of pyrazinamide (PZA) to kill tubercle bacilli in the acid environment of macrophages, excellent intracellular penetration of INH, RMP, and PZA, and the rapid bacteriocidal activity of RMP during the periodic bursts of metabolic activity of otherwise dormant bacilli. Dormant bacilli are not susceptible to killing by any available drug. Ethionamide, cycloserine, and capreomycin are available as reserve drugs for instances of drug intolerance or bacillary resistance (89-93).

The emergence of drug-resistant mutants in patients with tuberculosis is favoured by several features of the disease: the numerous bacteria in the lesions, especially in the walls of cavities, their further multiplication during the required long periods of therapy, and the limited degree of host resistance,

which provides the rare mutant cell with a good opportunity to replicate before being eliminated by host defenses. Hence, specifically resistant strains often appear in patients treated with any one of the drugs used singly.

1.6 Vaccines

The current tuberculosis vaccine, the Bacille Calmette-Guerin (BCG), was derived from an isolate of *Mycobacterium bovis* (which causes bovine tuberculosis) that had been attenuated by laboratory passage in the early years of this century (94;95). It has been pre viously suggested that differences in efficacy might have arisen as a result of changes in the vaccine strain over time. The subculture of BCG in different laboratories resulted in a series of genetic deletions and the evolution of a number of BCG substrains. DNA microarray comparisons of genomes of *M. bovis* and contemporary BCG strains with that of a virulent reference *M. tuberculosis* strain revealed that 129 genes encompassing 16 regions in the genome of *M. tuberculosis* have been deleted during passages of *M. bovis* to result in the attenuated *M. bovis* BCG substrains. BCG has been used for over 5 decades to immunise over 3 billion people in immunisation programs against tuberculosis, but its variable efficacy, seen in a number of carefully controlled clinical trials, has suggested that the capacity of BCG to prevent adult primary tuberculosis is severely limited. Moreover, since BCG is a live attenuated vaccine, its use is contraindicated in human immunodeficiency virus-infected and other immunocompromised patients, who have the highest risk of developing tuberculosis. Clearly, the development of a new, more effective vaccine would greatly facilitate worldwide control of this ancient plague. As a consequence, the search for a new vaccine against tuberculosis has greatly intensified over the last decade, with several experimental vaccines already have shown some promise in preclinical testing (96). In recent years, many new candidate tuberculosis vaccines, including recombinant BCG strains, attenuated tuberculosis auxotrophs, various subunit formulations, and DNA vaccines, have been developed and are being actively tested in animals (97-103). Several of these immunogenic preparations have been shown to elicit protective responses that approach the protective efficacy of BCG when

tested in primary infection models (104-106). However, the therapeutic effectiveness of these new tuberculosis vaccines in postexposure models is much less certain.

Protective immunity to mycobacteria is dependent on several effector mechanisms working together, particularly at the infection's local microenvironment. These factors include the local cell population and its cytokine production and expression of adhesion and costimulatory molecules. Local cytokines produced by inflammatory cells are thought to direct macrophage activation and T-cell development to a Th1 or Th2 pattern on the following days of infection, leading to control of antibacterial activity. Although protective immunity against tuberculosis is essentially mediated by CD4$^+$ T cells, CD8$^+$ T cells are also required for resistance against *M. tuberculosis* infection (107;108). It is plausible that immunisation strategies stimulating both CD4$^+$ and CD8$^+$ T cells should lead to an improved protection against *M. tuberculosis* infection. In general, immunisation with soluble proteins stimulates mostly CD4$^+$ T cell responses, whereas DNA or viral vaccines induce stronger CD8$^+$ T cell responses.

1.7 DNA array technology

The increasing rate at which genomes are being sequenced is attracting attention to functional genomics (http://www.tigr.org/tdb/), an area of genome research that is concerned with assigning biological functions to DNA sequences. More precisely, the completion of the sequencing of any given genome immediately raises the essential and formidable task of defining the role of each gene, and of understanding the interactions between sets of genes in that genome. These tasks can be carried out in various ways, including protein prediction, homology searching, and expression analysis. The advent of DNA microarray technology allows simultaneous monitoring of the expression levels of numerous genes (109;110). Thus, this technique permits the quantification of specific genes and their expression patterns in a comprehensive genome-wide framework. Although expensive relative to other quantitative hybridisation and amplification methods, the high-throughput capacity makes it a cost-effective technique for a variety of applications.

These analyses can be made within the same species and between different, but similar, species. In addition, arrays can be designed specifically to deduce simultaneously the sequence of multiple different genes. This technology is being more and more widely applied in biological and medical research to address a wide range of questions (111). The principle of a DNA microarray experiment, as opposed to the classical northern blot analysis, is that mRNA from a given cell line or tissue is used to generate a labelled sample, sometimes termed the "target", which is hybridised in parallel to a large number of DNA sequences, immobilised on a solid surface in an ordered array. The arrayed material has generally been termed the "probe" since it is equivalent to the probe used in a northern blot analysis. Tens of thousands of transcript species can be detected and quantified simultaneously. During recent years, DNA microarray technology has been advancing rapidly (112). The development of more powerful robots for arraying, new surface technology for glass slides, and new labelling protocols and dyes, together with increasing genome sequence information for different organisms, including humans, will enable us to extend the quality and complexity of DNA microarray experiments.

1.7.1 DNA array types and production

Although many different microarray systems have been developed by academic groups and commercial suppliers, the most commonly used systems today can be divided into two groups, according to the arrayed material: complementary DNA (cDNA) and oligonucleotide microarrays (113-116). Probes for cDNA arrays are usually products of the polymerase chain reaction (PCR) generated from cDNA libraries or clone collections, using either vector-specific or gene-specific primers, and are printed onto glass slides or nylon membranes as spots at defined locations. Spots are typically 100-300 µm in size and are spaced about the same distance apart. Using this technique, arrays consisting of more than 30,000 cDNAs can be fitted onto the surface of a conventional microscope slide. For oligonucleotide arrays, short 20-25mers are synthesised in situ, either by photolithography onto silicon wafers (high-density oligonucleotide arrays from Affymetrix,

_____

http://www.affymetrix.com) or by ink-jet technology (developed by Rosetta Inpharmatics, http://www.rii.com, and licensed to Agilent Technologies) (117). Alternatively, presynthesised oligonucleotides can be printed onto glass slides. Methods based on synthetic oligonucleotides offer the advantage that because sequence information alone is sufficient to generate the DNA to be arrayed, no time-consuming handling of cDNA resources is required. Also, probes can be designed to represent the most unique part of a given transcript, making the detection of closely related genes or splice variants possible. Although short oligonucleotides may result in less specific hybridisation and reduced sensitivity, the arraying of presynthesised longer oligonucleotides (50-100mers) has recently been developed to counteract these disadvantages. However, the high cost of commercially available, in situ-synthesised oligonucleotide arrays can make them inaccessible for academic laboratories, and purchase of large numbers of long oligonucleotides also incurs significant cost.

Because spotted microarrays can be designed and constructed in academic facilities, they allow a greater degree of flexibility in the choice of arrayed elements, particularly for the preparation of smaller, customised arrays for specific investigations. As a result, cDNA gridded arrays have so far been the technique most frequently used in academic labs. The first glass slide arrays were produced at Stanford (http://cmgm.stanford.edu/pbrown/index.html) in Pat Brown's laboratory. Brown's website also contains detailed specifications for building an arrayer and associated software.

However, a disadvantage of spotted microarrays is the variation in the size of the spot and the amount of DNA contained in every spot. Also, variations in length and composition of sequence and variations in post-processing and hybridisation result in less consistency and reproducibility within and across experiments than the high-density oligonucleotide arrays. In high-density oligonucleotide arrays, several different oligonucleotides interrogate each gene, in part accounting for the improvement in the overall consistency and reproducibility of results. On the other hand, these chips are relatively inflexible because their construction requires the design and manufacture of specific sets of masks that will determine the nucleotides to be synthesised *in situ*, a process that must be repeated entirely if any changes are made.

_____

Filter arrays have the advantage of being relatively affordable and needing no special equipment to use, although potential users should be aware that large format phosphorimager screens may be required with larger (22 cm^2) filters (118). Filters are also useful for scarce RNA (for example from microdissected tissue) (119-123). The major disadvantage of filters is that comparison of expression between two samples requires hybridisation of each sample to separate duplicate filters, or to a single filter that must be stripped and hybridised sequentially. Commercial filter arrays of clone sets are available from different companies. For those considering an investment, a detailed comparison of cost, species range and complexity is warranted. Important considerations also include whether the clones used to produce the arrays are restreaked and sequence-verified, whether DNA or lysed colonies are arrayed, and the number of known genes and ESTs. When considering the cost of commercial filters, it is important to bear in mind the additional cost of obtaining individual clones for follow-up experiments, especially as the expression of a substantial number of the genes may vary in a typical experiment.

In addition, arraying of unsequenced clones from cDNA libraries can be useful for gene discovery. However, with prices for oligonucleotide synthesis falling all the time, spotted long oligonucleotide arrays could be a viable alternative for the future.

Another important difference between *in situ* synthesised, high-density oligonucleotide arrays (Affymetrix) and spotted arrays lies in target preparation. In both cases, mRNA from cells or tissue is extracted, converted to DNA and labelled, hybridised to the DNA elements on the surface of the array, and detected by phosphoimaging or fluorescence scanning. The high reproducibility of *in situ* synthesis of oligonucleotide chips allows accurate comparison of signals generated by samples hybridised to separate arrays. In the case of spotted arrays, the process of gridding is not accurate enough to allow comparison between different arrays. The use of different fluorescent dyes (such as Cy3 and Cy5) allows mRNAs from two different cell populations or tissues to be labelled in different colours, mixed and hybridised to the same array, which results in competitive binding of the target to the arrayed sequences. After hybridisation and washing, the slide is scanned using two

different wavelengths, corresponding to the dyes used, and the intensity of the same spot in both channels is compared. This results in a measurement of the ratio of transcript levels for each gene represented on the array. To be able to compare a large number of samples, the same reference RNA - sometimes a mixture of all the samples of one experiment or a commercially available standard - can be used.

1.7.2 Application of DNA arrays

A main characteristic of pathogenic microorganisms is their ability to cause tissue damage and consequently disease in their host. For decades, scientists have used a wide variety of methods to study how microorganisms interact with the host to cause damage and the mechanisms by which the host protects itself from microorganisms. This information has been invaluable in the design of safe, effective and reliable diagnostics, therapeutics and vaccines.

The analysis of genetic polymorphisms among isolates from the same species is a way of distinguishing between different strains, studying the transmission dynamics of the microorganisms and exploring the relationship between genetic and phenotypic polymorphisms. In addition, genetic polymorphisms among isolates from the same or different species can be used to understand the population genetics and evolutionary relationship between closely related species. Both spotted microarray and high-density oligonucleotide array technologies have been used to study small-scale genomic deletions among clinical isolates (124).

One of the earliest applications of microarrays for pathogen analysis was to determine which of a number of known alleles were present at a specific genomic locus, such as those conferring resistance to antimicrobial agents. For example, mutations in coding regions of the reverse transcriptase and protease genes of human immunodeficiency virus were detected using high-density oligonucleotide arrays in order to predict resistance to antiviral drugs (125;126). A similar approach has been used to detect mutations in the *rpoB* and *katG* genes and the 16S rRNA of *M. tuberculosis* (127). Interestingly, in addition to detecting mutations associated with drug resistance, the pattern of

_____

hybridisation to these arrays discriminated between different mycobacterial species (128). These results suggested that array data specifically collected for one purpose (drug susceptibility testing) can be interpreted to reveal additional insights (speciation). In similar fashion, spotted microarrays were used to compare the genomes of 15 different *Helicobacter pylori* isolates, which showed that 22% of the genes were dispensable in one or more strains. Microarrays can also be used to compare the genomic content of similar species. For example, spotted microarrays were used to compare *Mycobacterium tuberculosis*, *Mycobacterium bovis* and the family of Bacille Calmette-Guerin (BCG), the antituberculosis vaccine produced by attenuating *M. bovis.* It has been postulated that the differences between *M. tuberculosis* and BCG could be exploited to develop diagnostics to distinguish between the immunity induced by infection and vaccination (95). Microarray-based comparative genomics may also be used to understand the evolutionary relationship between different species, such as the pathogen *Streptococcus pneumoniae* and the commensals *Streptococccus mitis* and *Streptococccus oralis* (129).

Integrating clinical data and genomic deletion data is another promising application of microarrays. In a recently published study, *M. tuberculosis* cDNA high-density oligonucleotide arrays were used to identify segments of DNA that are present in the sequenced strain (*M. tuberculosis* H37Rv) but missing from clinical isolates (130;131). On average, 0.3% of the genome was deleted in each isolate, and a total of 1.7% of the genome was polymorphic among 19 clinical isolates studied from 16 different clones. The distribution of deletions varied between different clones, yet was conserved among clonally related organisms, suggesting that genomic deletion patterns may be a useful marker for molecular epidemiological studies. The most provocative finding in this study was that strains with a large amount of deleted DNA were less likely to cause cavitary pulmonary disease than strains with fewer deletions. The establishment of an infection or disease in humans by a microorganism is an exceptional event. By understanding why certain strains of a particular organism are pathogenic or under which conditions they cause disease, it may be possible to develop new tools for disease prevention or management. The complex interaction between host and pathogen is now being explored

_____

using microarrays (132-134). The basic model consists of an *ex vivo* measurement of gene expression of host cells before and after they are infected with a microorganism. By following the pattern of gene expression at different times, it is possible to elucidate which host genes are up- or downregulated over the course of infection. Identification of genes that are differentially regulated and the characterisation of their functions provide a promising window on the understanding of pathogenicity.

The specific pattern of gene response also provides evidence of the scope of the host response to infection. Relatively few genes were induced when the gene expression profile of human colorectal and colonic epithelial cells was studied after infection with *Salmonella dublin* (135). These included cytokines, kinases, transcription factors, and HLA class I genes. Similarly, few human bronchial epithelial genes were induced after infection with *Bordetella pertussis* (136). These included genes encoding proinflammatory cytokines with chemoattractant activities, which may explain the cellular infiltrate seen in patients with pertussis.

Directly comparing gene expression profiles of host cell infected by wildtype and mutant pathogen strains has been used to explore host-pathogen interactions. This approach was used to identify a role for the *Salmonella* response regulator *phoP* in human macrophage cell death (137).

A central challenge to all pathogens is adapting to changing environmental conditions. This adaptation involves regulating gene expression in response to different environmental signals. In general, it has been assumed that the expression of genes that are needed in a particular situation will be upregulated, whereas unnecessary functions in the same situation will be downregulated. The same principle can be applied to virulence genes, which are also subject to regulatory mechanisms that ensure expression in the appropriate host environment (138). The power of microarrays is their ability to provide a whole-genome perspective on these responses.

Elucidating the repertoire of genes co-expressed at specific times or under certain environmental and physiologic conditions is a novel approach to identifying their functions. This approach is based on the assumption that genes with similar expression patterns are likely to have the same functions (guilt-by-association). This method has been used to hypothesise the

functions of hundreds of uncharacterised genes in yeast, *Drosophila melanogaster*, mice, and human.

Microarrays have also been used to identify virulence genes that promote colonisation or host damage. The approach is based on the assumption that virulence-associated genes are likely to be co-regulated (139).

Because genes with similar expression patterns may be regulated by the same regulatory genes, microarray technology can also be used to identify the *cis-* and *trans*-regulatory elements that control the expression network. For example, a group of regulatory genes controlled by an autoinduced peptide in *Streptococcus pneumoniae* was identified using microarrays (140). The aim of drug development is to design compounds targeting disease-causing gene products to achieve disease modification or compounds targeting disease-result gene products to alleviate symptoms (141). Many of the drugs sold today were designed in biochemistry laboratories. The drug discovery process started with the knowledge of biochemical pathways implicated in a pathophysiological process or by empirical observations. An enzyme, preferably catalysing a rate-limiting step in this pathway, was characterised and then tested with various potential inhibitory molecules. Once a good candidate molecule was found, medicinal chemists optimised the compound. Currently, the advent of molecular biology techniques and the availability of sequences from humans and several organisms have changed the field of drug discovery and development. Microarrays are also a useful tool for investigating the mechanisms of drug action. The basic approach is to compare the expression of normal and diseased cells, with or without drug exposure. The expression profile of a cell exposed to a specific drug has been called drug-specific gene expression signature.

In a similar fashion, spotted microarrays were used to determine the gene expression signature of *M. tuberculosis* exposed to isoniazid (INH). This drug is used in treatment against tuberculosis and blocks the mycolic acid biosynthetic pathway. The results confirmed biochemically derived observations that INH induces a set of genes that encodes polypeptide components of the FAS-II complex (142).

The analysis of the gene expression signatures includes data that may be useful to delineate and predict adverse events. If the expression of a drug

_____

candidate target closely matches the pattern generated by a known toxic agent, it can be assumed that the drug candidate would probably be toxic, a fact that can be confirmed by *in vitro* experimental analysis (143).

The whole-genome perspective of microarrays makes them promising tools for sifting through the genome to identify candidate genes as targets for drug development. However, expression data have limitations because mRNA levels may not reflect protein levels, and expression of a protein may not always have a pathological consequence. Therefore, traditional pathology and toxicity studies will remain necessary (144).

1.7.3 Improvement of DNA array sensitivity

Diseased tissue contains a mixture of normal tissue, inflammatory cells, necrotic tissue and, in cancer samples, areas of different grade. Similarly, healthy tissue also includes a range of cell types. All of these elements can combine to produce a complex RNA expression profile. Microdissection capability is thus critical for microarray studies involving tissues and is also useful for associated technologies such as comparative genomic hybridisation (145-147). Current protocols for fluorescent labelling of RNA demand large quantities of RNA, which impedes the use of microdissected RNA on glass slide arrays. Laser-based microdissection offers a means of more rapidly obtaining pure material than conventional techniques. The commercially available laser capture microdissection microscope (http://www.arctur.com) is thus a valuable adjunct to microarray studies. Strategies for using limited material include PCR amplification of total cDNA before labelling, or the generation of P^{33}-labelled nucleic acids (or targets) for filters and glass slides, as these require relatively small amounts of total RNA.

As DNA array technology has advanced, more sensitive and quantitative methods for target preparation have had to be developed. In cases in which the quantity of RNA is not limited, incorporation of nucleotides coupled to fluorescent dyes during synthesis of the first strand of cDNA is the method of choice, as it provides the most linear relationship between starting material and labelled product. However, most protocols require between 25-100 µg total RNA, which is often not readily available in studies using primary cells or

_____

tissues. Various procedures have been developed to increase sensitivity and reduce the amount of RNA required. One strategy is target amplification by *in vitro* transcription (148). In addition, several rounds of *in vitro* transcription can be combined with cDNA synthesis to enhance the amplification even further (149). Using these protocols, it is even possible to profile the transcripts of a single cell (150). Another strategy is post-hybridisation amplification using labelled antibodies or molecules carrying large numbers of fluorophors (151). Several studies have used target-amplification techniques to compare the expression profiles of defined cell populations extracted from tissue sections by laser-capture microdissection. However, suitable controls are required to ensure that amplification has not introduced significant experimental bias into the target preparation. This problem has been particularly evident in the expression profiling of tumour samples. In the case of solid tumours, obtaining pure populations of tumour cells for microarray analysis would require microdissection. However, a recent study using grossly dissected breast cancer specimens has demonstrated a way to circumvent the problem of sample heterogeneity (152). Expression profiles from whole solid tumours can be compared to profiles from potential untransformed infiltrating cell types, such as lymphocytes or endothelial cells, to identify a subset of genes with expression patterns that are specific to the tumour cells. Subsequent data analysis and sample clustering can then be carried out only on this "intrinsic gene subset", which in the case of the recent study was sufficient for tumour classification (152).

1.7.4 Data analysis

A typical array experiment generates thousands of data points and creates serious challenges for storing and processing data. Thus some of the greatest challenges lie not in generating these data but in the development of computational and statistics tools to analyse the large amounts of data. Informatics can be categorised as either "tools" or "analysers". Tools include software that operate arraying devices and perform image analysis of data from readers, databases to hold and link information, and software that link

data from individual clones to web databases. Some involve fairly straightforward software but are nevertheless quite extensive (153-157).

The Brown laboratory has made available software for operating custom-built arrayers (http://derisilab.ucsf.edu/arraymaker.shtml).

The quality of image analysis programs is crucial for accurate interpretation of signals for slides and filters. In addition, an important ancillary task is to design the experiments so that the efficiency and reliability of the obtained data can be improved (158-163).

1.8 Proteome analysis of *M. tuberculosis*

Proteomics combines large-scale protein separation and sensitive protein identification, and is widely used in studies covering two-dimensional gel electrophoresis (2-D PAGE) reference maps, differential protein expression, as well as protein interactions (164;165). For a number of years, individual *M. tuberculosis* proteins have been studied in order to identify novel vaccine candidates (166-168). Several studies have focused on proteins released to the culture medium, the culture filtrate proteins, as this protein fraction has been shown to be a potent vaccine against tuberculosis in animal models (169).

Proteome analysis by 2-D PAGE and sensitive protein identification methods is a complementary approach to transcriptional analysis. It has been reported that measurement of mRNA and protein levels show a relatively low correlation, reflecting that two different biological parameters are measured. Proteins are generally more stable than mRNA, and the amount of protein in a spot of a 2-D PAGE gel represents the final gene product and reflects transcriptional rate, potential post-transcriptional modifications and the stability of the protein (170).

Earlier attempts to resolve mycobacterial proteins resulted in a resolution of about 50-170 proteins (171-175). Using immobilised pH gradients in the first dimension of 2-DE the amount of proteins which can be detected was increased to 700 protein species (176;177). Recent development of this methodology allows the identification of around 1800 distinct protein species (178;179). This was used to perform subtractive protein analysis. The

comparison of gene expression in different biological situations allows the correlation of biological effects with protein composition. In the next future the results from proteomics and transcriptome studies are expected to complement each other very well in an effort to elucidate the pathogenicty of *M. tuberculosis* and to develop vaccines and drugs against tuberculosis.

In addition to experimental proteome analysis of *M. tuberculosis*, bioinformatics approaches based on the comparison of complete genome sequences of various organisms have been currently used to predict functional linkages of genes in the genome of *M. tuberculosis*. Such approaches can provide aid to make prediction of the functions of non-annotated genes by establishing their protein networks (180-184).

1.9 Aims of the doctoral thesis

This doctoral thesis work is aimed at performing genome-wide analyses of comparative genomics and gene expression profiling of *M. tuberculosis*, the causative agents of tuberculosis, applying DNA array technology.

Based on the genome sequence of *M. tuberculosis* H37Rv, filter-based DNA arrays are produced representing all known open reading frames (ORFs).

In the comparative genomic study the DNA arrays are used to compare the genome content of *M. tuberculosis* H37Rv with other mycobacterial strains. The observed variations in the genome contents of these strains may provide support for the elucidation of phenotypic differences between the investigated strains and can be explored to develop novel diagnosis method.

In the transcriptome study the DNA arrays are used to monitor gene expression profiles of *M. tuberculosis* during infection in human lung and in murine bone marrow-derived macrophages. The transcriptomes obtained from this study may provide information about the mechanisms used by *M. tuberculosis* to persist and establish disease in host. The genes which are upregulated in this study may serve as candidates for drug targets.

2. Materials and Methods

2.1 Production of DNA arrays

2.1.1 Primer design and PCR amplification of ORFs

PCR primers were mainly constructed to amplify the first 300 bp of each predicted ORF described in the annotated genome sequence of *M. tuberculosis* H37Rv. For ORFs which are less than 300 bp, PCR primers were designed to amplify the whole length of each ORF. Five hundreds primer pairs were redesigned using PRIMER3 software:

(http://www.genome.wi.mit.edu/genome_software/other/primer3.html)

to amplify a fragment of the corresponding ORFs resulting in PCR products with the length of ~300 bp or less, since the initial primer pairs did not yield PCR products in proper amount or in the expected size. The melting temperature for the primer design was set to 60°C.

PCR reactions were performed in 100 µl reaction in 1 x PCR buffer containing 10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% Tween 20 and 1 M Betaine (Sigma) with 200 µM dNTP mix and 0.3 M primer mix (forward and reverse) in the presence of 2 U Taq DNA polymerase (MBI Fermentas) and 1 ng of gDNA of *M. tuberculosis* H37Rv. The PCR reaction mixes were pipetted into 96-well PCR plates (Biozymes) using pipetting robot Hydra (Robbins Scientific) with 96 syringes. The reactions were carried out in the thermal cyclers with 96-well heating block (MJ Research).

After an initial 2-min denaturation at 95°C, 50 PCR cycles were run as follows: 95°C for 45 s, 52°C for 45 s, and 72°C for 30 s. The final elongation was carried out at 72°C for 10 min. The quality of PCR products were assessed using three different methods. The first method employed the horizontal slab agarose gel apparatus. Aliquots (1 µl) of each PCR product were run on 1.2% agarose gels in 1 x TAE buffer (89 mM Tris-borate [pH 8.3], 2 mM Na_2EDTA) to confirm the presence of a PCR product migrating at the expected size. The second method used to analyse the PCR products employed the relatively new developed horizontal slab agarose gel apparatus with four electrodes (instead of two electrodes in the conventional apparatus) causing the DNA to run diagonally. The agarose gels used here were relatively thin and small, allowing yet to analyse 96 or even 384 PCR products in one gel run. Aliquots

_____

(1 µl for 96-well and 0.5 µl for 384-well agarose gel) of PCR products were loaded into the wells of 1.2% agarose gel using the pipetting robot Hydra (Robbins Scientific) and the loaded gel was run in 1 x TBE buffer. The third method employed the Agilent 2100 Bioanalyzer™ (Agilent), a high-resolution electrophoresis system. This instrument separates DNA fragments through microfabricated channels with real-time fluorescence detection. DNA samples were first diluted with injection buffer according to the manufacturer's protocol of DNA 7500 assay kit and then analysed in parallel with an external DNA size ladder included in the kit. One hundred PCR products were chosen randomly for the measurement with the Bioanalyzer™.

2.1.2 DNA array printing

Prior to spotting, the PCR products were denatured by adding NaOH solution to the final concentration of 0.1 N. The denatured PCR products were spotted on 22 x 22 cm^2 positively charged nylon membranes (Hybond, Amersham Biosciences) by using a customised spotting robot at Max-Planck Institute for Molecular Genetics Berlin, Germany, Lehrach's Department. The printing of PCR products on DNA arrays was performed using gadget with 384 pins.

Two replicates of each PCR product were spotted on each DNA array to improve reproducibility. As negative control, two replicates of PCR product from an *Arabidopsis thaliana* cDNA clone were also spotted on each DNA array. Some spot positions were kept empty and were used also as negative control.

After spotting process, the DNA arrays were subjected to UV crosslinking (Stratalinker UV Crosslinker, Stratagene). The DNA arrays were then washed in washing buffer containing 0.1 x SSC and 0.5% SDS at room temperature, semidried and stored at 4℃.

_____

2.2 Comparative Genomic of *M. tuberculosis* complex strains

2.2.1 Bacterial strains and culture condition

M. tuberculosis H37Rv, *M. bovis* (clinical isolate from Lungenklinik Heckeshorn and biochemically characterised as *M. bovis*), *M. bovis* BCG Copenhagen (ATCC no. = 27289), *M. tuberculosis* Beijing (RIVM no. = 17919), *M. tuberculosis* Haarlem (RIVM no. = 9401431), *M. africanum* (clinical isolate from Lungenklinik Heckeshorn and biochemically characterised as *M. africanum*), and *M. canettii* (RIVM no. = 17728) were grown to late logarithmic phase in Middlebrook 7H9 media supplemented with 10% (v/v) ADC (Difco Laboratories) and 0.05% (v/v) Tween 80 (Sigma), at 37°C with shaking at 90 r.p.m in screw-capped bottles.

2.2.2 DNA Isolation

Two different methods were applied to isolate gDNA from mycobacterial strains. The first method was modified from the one previously described and making use of the combination of methanol, chloroform, and phenol as extraction agents and chaotropic guanidium salt (185).

2.2.2.1 Modified guanidium method

The cells from a 15-ml culture were harvested by centrifugation at 4000 x *g* for 10 min and lysed with 2 ml of a 1:3 mixture of chloroform/methanol. The lysate was then mixed with 2 ml of Tris-equilibrated phenol (Sigma) and 3 ml of RLT buffer (Qiagen) containing 0.5% sarcosyl and β-mercaptoethanol added to a final concentration of 1% prior to use. The suspension was vortexed for 1 min or until the formation of interface occurred and centrifuged at 4000 x *g* for 1 h to separate the aqueous phase from the organic phase. The upper phase was collected after centrifugation, and the gDNA was precipitated with isopropanol. The pellet was dissolved in 500 μl extraction solution from GFX Genomic Blood DNA Purification Kit (Amersham Biosciences) and further purified using GFX column according to the manufacturer's instruction. Two sets of gDNA were prepared using this

method. The third set of genomic DNA preparation was performed using lysozyme essentially as described previously (186).

2.2.2.2 Lysozyme-SDS-Proteinase K method

The cells were harvested by centrifugation at 4000 x *g* for 10 min and resuspended in 1 x TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA). The cells were heat inactivated for 20 min at 80°C and treated with lysozyme at 37°C for 16 h. Lysis was performed with a sodium dodecyl sulfate-proteinase K mixture at 65°C for 1 h or until lysis was obtained. The lysate was treated with NaCl and cetyltrimethylammonium bromide-NaCl at 65°C for 10 min, and chloroform extraction was performed. The supernatant was precipitated with isopropanol, and after washing, the pellet was resuspended in TE buffer and the mixture was heated for 10 min at 65°C (185;186).

2.2.3 Target DNA labelling

Prior to labelling reaction, gDNA was sonicated for 4 min to obtain fragment size ranging from 400 bp to 1000 bp. The optimisation of sonication time was performed by sonicating 1 μg gDNA of *M. tuberculosis* H37Rv for 0, 1, 2, 4, and 8 min. The size of the resulting DNA fragments was analysed using Bioanalyzer^{TM}.

Each DNA target for hybridisation of DNA arrays was generated in a Klenow reaction with random hexamers using 500 ng of the sonicated gDNA as template in the presence of 50 μCi [^{33}P]-α-dCTP (Amersham Biosciences) according to the manufacturer's instruction (HexaLabel Plus; DNA Labeling Kit, Fermentas). The sonicated gDNA was denatured for 10 min at 95°C and immediately cooled on ice for 2 min. The Klenow reaction was performed at 37°C for 30 min. The unincorporated [^{33}P]-α-dCTP was removed from the labelled DNA target using G-50 column (Amersham Biosciences).

2.2.4 Hybridisation of DNA arrays

DNA arrays were prehybridised at 60°C for at least 1 h in 20 ml hybridisation buffer containing 1 M NaCl, 1% SDS, 10 mM Tris-HCl [pH 8.0] and 1 mM EDTA.

The radioactive-labelled DNA target and 30 µg salmon sperm DNA (Sigma) was denatured at 95 °C for 10 min, cooled immediately on ice for 2 min and then transferred to 8 ml hybridisation buffer which was preheated at 60 °C. The hybridisation was performed at 60 °C overnight. The DNA arrays were washed three times in washing buffer (0.5% SDS, 0.1 x SSC) at 70 °C, each for at least 30 min.

After washing, the DNA arrays were wrapped with clear food wrap film (Saran Wrap) and exposed to a phosphorimaging screen (Fuji) for 3 days. Array images were scanned using Fuji BAS 2500 phosphorimaging instrument at 50 µm pixel resolution.

The resulting image files were analysed using customised software "Genespotter" developed by Microdiscovery (Microdiscovery, Germany; www.microdiscovery.de) (187;188).

Some spots were excluded from analysis because of high local background intensities, DNA array abnormalities, or weak intensities as determined primarily by the background signal observed for hybridisation to the Arabidopsis-specific control gene spots and empty spots.

2.2.5 Validation of DNA sequence variation

2.2.5.1 Primer design

Based on the result of BLAST search, a region on gDNA of *M. tuberculosis* H37Rv which showed sequence variation was chosen for the primer design using PRIMER3. The primer sequences for DNA sequence analysis are:

Rv0050: forward: 5´-TCGTTGTCTACGGCTGTGTG-3´

       reverse: 5´-GACCGATCGGGATGGTAAT-3´

Rv2090: forward: 5´-GTAGCCGGGAGTTATCACCA-3´

       reverse: 5´-AGAACGAGCGGAACCACAT-3´

Rv3281: forward: 5´-TATGGGCTCCAAAGACATGG-3´

       reverse: 5´-CAGTATCTCGATGTGGGGCT-3´

2.2.5.2 PCR reactions

PCR reactions for the sequence analysis of Rv0050, Rv2090, and Rv3281 were performed using HotStarTaq Master Mix Kit (Qiagen) in 50-µl volume in the presence of 1 M Betaine (Sigma), 5 ng gDNA and 0.3 M primer mix (forward and reverse). After an initial 15-min activation of HotStarTag DNA polymerase at 95°C, 50 PCR cycles were run as follows: 95°C for 45 s, 60°C for 45 s, and 72°C for 1 min. The final elongation was carried out at 72°C for 10 min. An aliquot of 3 µl of each PCR product was run on 1.2% horizontal slab agarose gel in 1 x TAE buffer (40 mM Tris-acetate [pH 7.6], 1 mM Na_2EDTA). The rest of the PCR products was purified using QIAquick PCR Purification Kit (Qiagen) and subjected to sequencing.

2.2.5.3 Analysis of DNA sequence

DNA sequences obtained from sequencing of PCR products for Rv0050, Rv2090, and Rv3281 were compared with the corresponding sequences from TubercuList (http://genolist.pasteur.fr/TubercuList/) using the alignment program "Genestream" (http://vega.igh.cnrs.fr/bin/align-guess.cgi).

2.3 Gene expression profile analysis of *M. tuberculosis* H37Rv infecting *in vitro* grown murine bone marrow-derived macrophages

2.3.1 *In vitro* culture condition for *M. tuberculosis* H37Rv

M. tuberculosis H37Rv was grown to mid-logarithmic phase in 100 ml Middlebrook 7H9 media supplemented with 10% (v/v) ADC (Difco Laboratories) and 0.05% (v/v) Tween 80, at 37°C with shaking at 90 r.p.m in screw-capped bottles. Fifty ml of this culture was subjected to RNA isolation, whereas the rest of the culture was used to infect macrophages.

2.3.2 Isolation of mycobacterial cells from macrophages

Murine bone marrow-derived macrophages were generated using L929 culture supernatant from bone marrow precursor cells isolated from C57BL/6 mice as described previously (189). Macrophages were cultured in DMEM

containing 10% FCS and 5% horse serum at 37℃/7% CO_2 either without treatment (resting) or upon activation using 1000 U/ml murine recombinant IFN-γ 24 h prior to infection. For infection, macrophages were incubated with *M. tuberculosis* H37Rv at an of 5:1 MOI for 2 h and washed excessively. Mycobacteria were taken from cultures growing in 7H9 medium at the logarithmic growth phase (CFU around 2 x 10^8/ml), washed twice in PBS/0.05% Tween 80 and resuspended in cell culture medium. Infected macrophages were cultured for 3 days (resting macrophages) or 1 and 3 days (activated macrophages). Mycobacteria were isolated by scraping the infected macrophages in ice cold PBS/0.05% Tween 80 and subsequent lysis by putting the lysates through fine syringe as described previously (189). Lysates were diluted 1:10 in PBS/0.05% Tween 80 and mycobacteria were harvested through a spin at 1800 x *g* for 15 min. Pellets were washed in PBS/0.1% NP40 and in 0.5 M KCl/0.01% NP40 before RNA was isolated.

2.3.3 RNA extraction

The cell pellet from 50 ml of mid-logarithmic phase grown culture of *M. tuberculosis* H37Rv or from infected macrophages was resuspended in 5 ml phenol and 5 ml chloroform/methanol (3:1), and vortexed for 1 min or until the formation of interface occurred. RNA was extracted with 4 ml RLT buffer (RNeasy Kit, Qiagen) containing 0.5% sarcosyl and 1% β-mercaptoethanol (added prior to the use of buffer). The suspension was centrifuge at 4000 x *g* for 1 h to separate the aqueous phase from the organic phase. The aqueous layer was precipitated in ethanol, RNA was redissolved in 400 μl RLT buffer and further purified using RNeasy columns (Qiagen) according to the manufacturer's instructions.

2.3.4 RNA quantification

The size distribution and the quantity of isolated total RNA samples were determined using a high-resolution electrophoresis system, the Agilent 2100 Bioanalyzer[TM] (Agilent). This instrument separates RNA fragments through microfabricated channels with real-time fluorescence detection. RNA samples were first diluted with injection buffer according to the manufacturer's protocol

_____

and then analysed in parallel with an external RNA 600 size ladder (Ambion). Clear and sharp 23S and 16S ribosomal RNA peaks indicated relatively intact RNA.

2.3.5 Target cDNA synthesis and labelling

Total RNA of *M. tuberculosis* H37Rv from *in vitro* culture (500 ng) or 3 µg total RNA from infected macrophages was mixed with 5 pmoles mtGDPs (mycobacterial genome-directed primers) (190) and heated at 70°C for 10 min, followed by immediate cooling to 4°C (on ice). RNA was then reverse transcribed using RevertAid^{TM} First Strand cDNA Synthesis Kit (MBI Fermentas) in 20 µl reaction containing 200 U RevertAid^{TM} M-MuLV reverse transcriptase, 20 U RNase Inhibitor, 0.5 mM each of dATP, dGTP, and dTTP, 0.5µM dCTP, and 50 µCi of [^{33}P]-α-dCTP (Amersham Biosciences) in 1 x RT buffer. The reaction was carried out at 25°C for 10 min and subsequently at 42°C for 2 h. The unincorporated radioactive nucleotide was removed from the labelled cDNA by a G-50 column (Amersham Biosciences).

2.3.6 Hybridisation of DNA arrays

DNA arrays were prehybridised at 50°C for at least 1 h in 10 ml hybridisation buffer (ULTRArray^{TM} Hybridization Buffer, Ambion). The radioactive-labelled cDNA probe was denatured at 95°C for 10 min, cooled immediately on ice for 2 min and then transferred to the hybridisation buffer. The hybridisation was performed at 50°C overnight. The DNA arrays were washed three times in washing buffer (0.5% SDS, 0.1 x SSC) at 55°C, each for 30 min. After washing, the DNA arrays were wrapped with clear food wrap film (Saran Wrap) and exposed to a phosphorimaging screen for 5 days. Array images were scanned using Fuji BAS 2500 phosphorimaging instrument at 50 µm pixel resolution. The feature extraction from the resulting image files was carried out using the software "Genespotter" (www.microdiscovery.de).

2.3.7 Data analysis of DNA arrays

Each array experiment was performed in triplicate. The raw data of signal intensities was normalised as follows. After exclusion of negative control spots

(spots without DNA or containing PCR product from a gene of *Arabidopsis thaliana*), the logarithmic value of each spot and the average from all these logarithmic values were calculated. The average value was then subtracted from each logarithmic value to obtain the normalised data. The normalised intensity data were used as basic values to calculate the fold of up- and downregulation of genes in each experiment. The data analyses were performed using the customised software from the company "Microdiscovery" (www.microdiscovery.de) (187;188).

The hierarchical clustering of the DNA array data was carried out using the clustering program in the following website:

<div align="center">http://rana.lbl.gov/EisenSoftware.htm (191).</div>

2.4 Gene expression profile analysis of *M. tuberculosis* infecting human lung

2.4.1 Lung tissue and human material

Tuberculosis patients suffering from (often multidrug-resistant) extensive tuberculosis lung disease underwent surgery at the Central Tuberculosis Research Institute in Moscow, Russia, for removal of parts of their lungs. The use of the resected tissue for further immunological and genetic analyses has been approved by Ethics Committees in both Moscow and Berlin. Tissue was only used for this study after informed consent of the patient. Following surgery the tissue was then separated into three different regions relative to the main cavernous granuloma: cavity wall of granuloma, pericavital tissue and macroscopically normal lung tissue from distant parts of the removed tissue.

Eleven tuberculosis patients have been included in this study, and one patient suffering from lung cancer as tissue control, with a mean age of 39 ± 15.3 years, a male:female ratio of 8:4. In the removed cavity tissue, CFU analyses revealed a mean densitiy of mycobacteria in the tissue of 6.3×10^8/g (minimum 5.5×10^3/g, maximum 3.7×10^9/g). All tissue specimens were analysed histologically for the presence of mycobacteria by Ziehl-Neelsen

_____

(ZN) and by immunohistological staining using a polyclonal serum against *Mycobacterium bovis* BCG (pAbBCG).

2.4.2 *In vitro* culture condition for *M. tuberculosis* clinical isolates

Immediately after removal from patient lung, the samples from the human lung granuloma, pericavity, distant lung, and lung cancer tissue were homogenised. An aliquot of the homogenate from granuloma was inoculated into 5 ml Middlebrook 7H9 media supplemented with 10% (v/v) ADC (Difco Laboratories) and 0.05% (v/v) Tween 80 (Sigma). The culture was grown to mid-logarithmic phase at 37°C with shaking at 90 r.p.m in screw-capped bottles. This culture was then transferred to 100 ml Middlebrook 7H9 media supplemented with 10% (v/v) ADC (Difco Laboratories) and 0.05% (v/v) Tween 80 (Sigma). The culture was grown to late logarithmic phase and the cells were harvested from 50 ml of this culture. The rest of homogenate from granuloma, homogenates from pericavity, distant lung, and lung cancer tissue along with the cell pellet from *in vitro* culture were immediately further processed for RNA extraction.

2.4.3 RNA extraction

The RNA extraction was performed using the same protocol as in macrophage infection in section 2.3.3.

2.4.4 RNA quantification

The size distribution and the quantity of isolated total RNA samples were determined using a high-resolution electrophoresis system, the Agilent 2100 Bioanalyzer™ (Agilent).

2.4.5 Target cDNA synthesis and labelling

Prior to labelling reactions, total RNA was subjected to amplification. For this purpose, 200 ng total RNA of *in vitro* cultured *M. tuberculosis* and 2 µg total RNA from human lung granuloma, pericavity, distant lung or lung cancer were applied for amplification. The development of the amplification method is described in section 2.5.

Total RNA (500 ng) from *in vitro* cultured *M. tuberculosis* or 8 µg amplified total RNA from human lung granuloma, pericavity, distant lung or lung cancer were mixed with 5 pmoles mtGDPs and heated at 70°C for 10 min, followed by immediate cooling to 4°C (on ice). The labelling reaction was performed using the same protocol as in the macrophage infection in section 2.3.5.

2.4.6 Hybridisation of DNA arrays

Hybridisation, washing and scanning of DNA arrays were performed using the same procedures as in the macrophage infection in section 2.3.6.

2.4.7 One-step RT-PCR

The primer sequences used for one-step RT-PCR are listed below:

Rv0379: forward: 5´-CAAGGTGATCGACATCATCG-3´
         reverse: 5´-GGCCTCATCTTGAACGACAC-3´
Rv0696: forward: 5´-TACCAGTACGGAACAACGCA-3´
         reverse: 5´-CAGAAGTGGCCGAGTAAGGA-3´
Rv1166: forward: 5´-GCACCCCAGAGCACTGATAC-3´
         reverse: 5´-CGGATCTTGTAGGTCACCGT-3´
Rv1699: forward: 5´-AGGACAATTGTTGACGGCTC-3´
         reverse: 5´-GTGATATGGGGGATCACCTG-3´
Rv1790: forward: 5´-AGGACAATTGTTGACGGCTC-3´
         reverse: 5´-GTGATATGGGGGATCACCTG-3´
Rv3496: forward: 5´-ACCATCGTGGGCTACTTCAC-3´
         reverse: 5´-TCTTTCACCTCGTCCCATTC-3´

Prior to use in one-step RT-PCR reations, RNA samples were treated with DNase I (Invitrogen). After heat inactivation of DNase I, RNA was purified using RNeasy columns (Qiagen) according to the manufacturer's instructions. The one-step RT-PCR reactions were performed using Qiagen One-Step RT-PCR Kit (Qiagen). Each reaction contained 10 pg total RNA, 0.4 µM gene-specific primer pair, 400 µM dNTP, 1 M Betain, 20 U RNase Inhibitor (MBI Fermentas) and Qiagen One-Step RT-PCR enzyme mix in 1 x reaction buffer. Reverse transcription took place at 50°C for 1 h without prior heat

denaturation of total RNA. After activation of HotStarTag DNA polymerase by heating at 95°C for 15 min, the PCR reactions were carried out for 50 cycles (94°C for 45 s, 58°C for 1 min, and 72°C for 1 min), followed by final extension of 72°C for 10 min.

As negative control reaction to confirm the absence of DNA contamination, PCR reactions were performed with 20 pg of each total RNA sample, 1 U HotstarTag DNA polymerase (Qiagen), 0.3 µM gene-specific primer pair, 200 µM dNTP, 1 M Betain, in 1 x reaction buffer. PCR conditions were the same as those for one-step RT-PCR.

A 15-µl aliquot of each RT-PCR or control PCR reaction was subjected to electrophoresis on a 1.2% agarose gel in 1 x TAE buffer. The PCR products for Rv0379 and Rv1166 were sequenced.

2.4.8 Data analysis of DNA arrays

The DNA array data were analysed using the same procedure and software as in macrophage infection in section 2.3.7.

The hierarchical clustering of the DNA array data was carried out using the clustering program in the following website:

<div align="center">

http://rana.lbl.gov/EisenSoftware.htm (191).

</div>

2.5 The development of RNA amplification system for mycobacteria

Culture medium and conditions, RNA isolation and quantification, and cDNA synthesis and labelling were performed using the same procedures as described in macrophage infection in section 2.3.

2.5.1 Double-stranded cDNA synthesis

Total RNA of *M. tuberculosis* H37Rv or *M. bovis* BCG Copenhagen (200 ng), 100 pmoles T7 primer (5′-GGCCAGTGAATTGTAATACGACTC-ACTATAGGGAGGCGGGGGGGG-3′), and 5 pmoles mtGDPs were heated at 70°C for 10 min, followed by immediate cooling at 4°C (on ice). Total RNA was then reversed transcribed using Powerscript MMLV-RT (Clontech) in 1 x

RT buffer in the presence of 10 mM DTT, 1 mM dNTP mix (1 mM each dATP, dCTP, dGTP, dTTP), and 80 U RNase Inhibitor (MBI Fermentas). The RT reaction was first performed at 25°C for 10 min to allow a better annealing of mtGDPs to total RNA and then continued at 42°C for 3 h.

The second-strand synthesis was accomplished by applying the second-strand cDNA synthesis kit (Roche) according to the manufacturer's protocol. Briefly, the RNA on the RNA-cDNA duplex was digested into small fragments by RNase H. These RNA fragments served as primers for DNA polymerase. The resulting second-strand cDNA fragments were joined together by T4 DNA ligase. To fill up the DNA strand gap at the 3′-end of the second-strand cDNA, T4 DNA polymerase was employed. The second-strand cDNA synthesis was performed at 16°C for 3 h. The double-stranded cDNA was precipitated in the presence of Pellet Paint Co-Precipitants (Novagen) according to the manufacturer's instruction.

2.5.2 Spiked RNA experiments

Mycobacterial RNA was spiked with human lung total RNA (Ambion) with the ratio of 1:50. This RNA mix was then subjected to amplification.

2.5.3 *In vitro* dscDNA transcription

The transcription of dscDNA was carried out using MEGAscript™ II High Yield Transcription Kit (Ambion). One-third of the amount of the produced dscDNA was used as template for transcription reaction which occurred in the presence of 10 mM NTP mix (10 mM each ATP, CTP, GTP, UTP), 2 µl enzyme mix, and 200 U T7 RNA polymerase (Ambion) in 1 x transcription buffer at 37°C overnight (~15 h). The resulting RNA was purified using RNeasy column (Qiagen). The quality and quantity of amplified RNA was assessed with Bioanalyzer™.

3. Results

3.1 Production of DNA arrays

The quality of PCR products were assessed using three different methods. The first method employed the horizontal slab agarose gel apparatus. An aliquot (1 µl) of each PCR product was run on 1.2% agarose gel. Figure 1 shows an example of the quality assessment of PCR products using this method. As can be seen in Figure 1, some PCR reactions failed to yield any product or gave several amplification products in one reaction. Around 70% of the 3925 PCR reactions gave one amplification product in each reaction with the expected size (~300 bp). The PCR reactions with product failure were repeated using different reaction conditions or new primer pairs amplifying other regions in the ORFs.
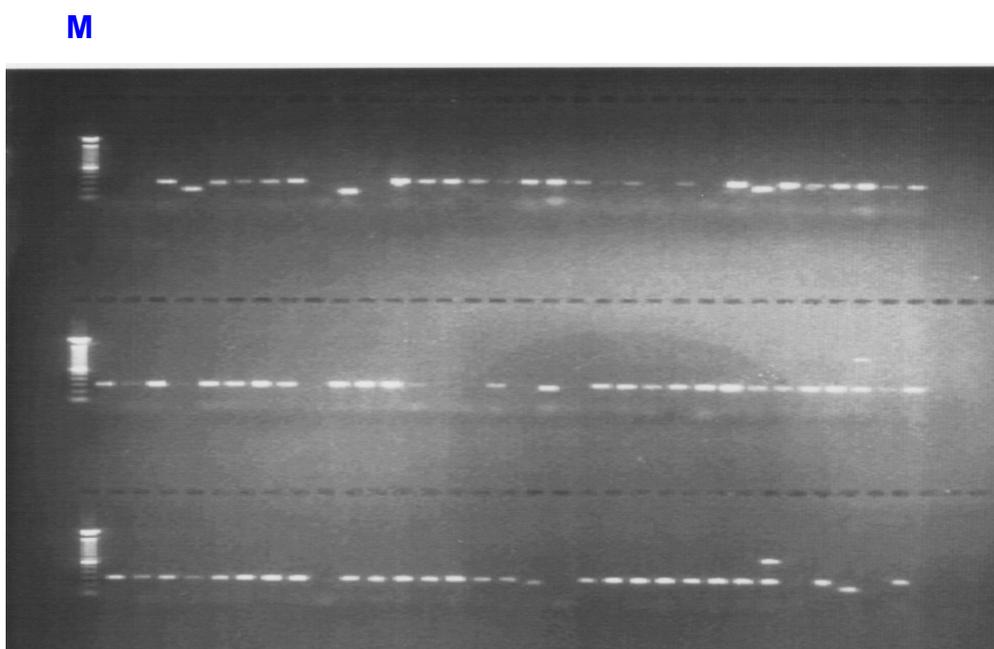
Fig. 1 PCR products of some arbitrarily chosen ORFs of *M. tuberculosis* H37Rv run on horizontal slab agarose gel. On the first well on the left 100-bp DNA marker (M) was loaded.

The second method used to analyse the PCR products employed the relatively new developed horizontal slab agarose gel apparatus with four electrodes (instead of two in the conventional apparatus) causing the DNA to

run diagonally. The agarose gels used here were relatively thin and small, allowing yet to analyse 96 or even 384 PCR products in one gel run (Fig. 2). This equipment system allows thus the high throughput performance of PCR product analysis. The use of this equipment was also intended to contribute to the establishment of this novel method.
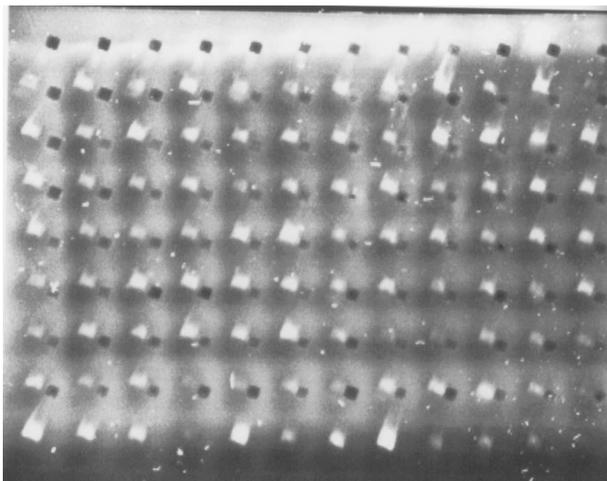
Fig. 2 High throughput analysis of PCR products in 96-well agarose gel.

Such system is especially eligible for detecting the presence of PCR products but not for determination of their size.

The third method employed the Agilent 2100 Bioanalyzer[TM], the first commercially available system utilising chip-based nucleic acid separation technology. It separates nucleic acid fragments by capillary electrophoresis in a chip with microfabricated channels and automates the detection as well as on-line data evaluation (Fig. 3). One hundred PCR products were chosen randomly for the analysis. According to the data evalution of this equipment, the concentration of the PCR products ranged between 15 to 80 µg/µl.
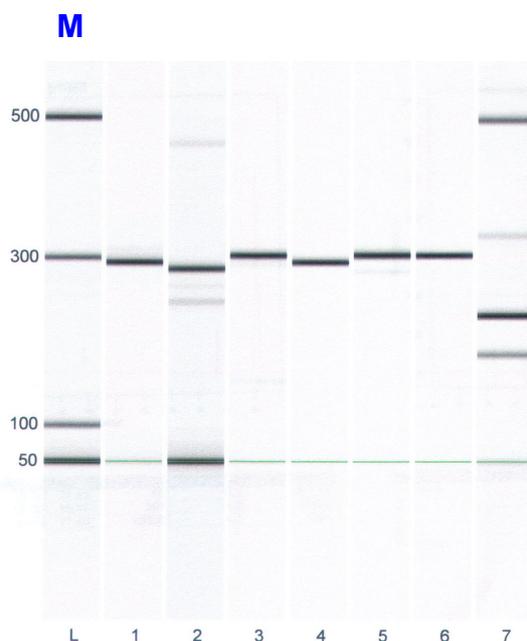
Fig. 3 The result of Bioanalyzer^TM for some arbitrarily chosen PCR products for DNA array production. The first column on the left represents the DNA marker (M).

3.2 The quality assessment of DNA arrays

To asses the quality of the produced DNA arrays, three arrays were hybridised with gDNA of *M. tuberculosis* H37Rv. Before generating radioactive-labelled DNA target, gDNA was sonicated to obtain a certain size distribution. To optimise the length of time used to sonicate gDNA, an experiment was conducted in which gDNA of *M. tuberculosis* H37Rv was sonicated for different lengths of time. The sonicated gDNA was analysed using Bioanalyzer^TM (Fig. 4). A time of 4 min resulting in gDNA fragments between 400 bp and 1000 bp was chosen for further experiments.

The DNA arrays were hybridised with radioactive-labelled DNA targets and the hybridised arrays were analysed using Fuji BAS Phosphoimaging system. An example of the hybridisation results is shown in Figure 5.

_____
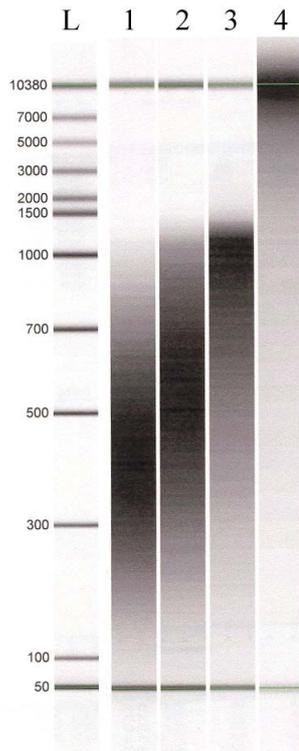
Fig.4 The result of Bionalyzer^{TM} of sonicated gDNA of *M. tuberculosis* H37Rv. L = DNA ladder; 1 = after 8 min sonication; 2 = after 4 min sonication; 3 = after 2 min sonication; 4 = after 1 min sonication.
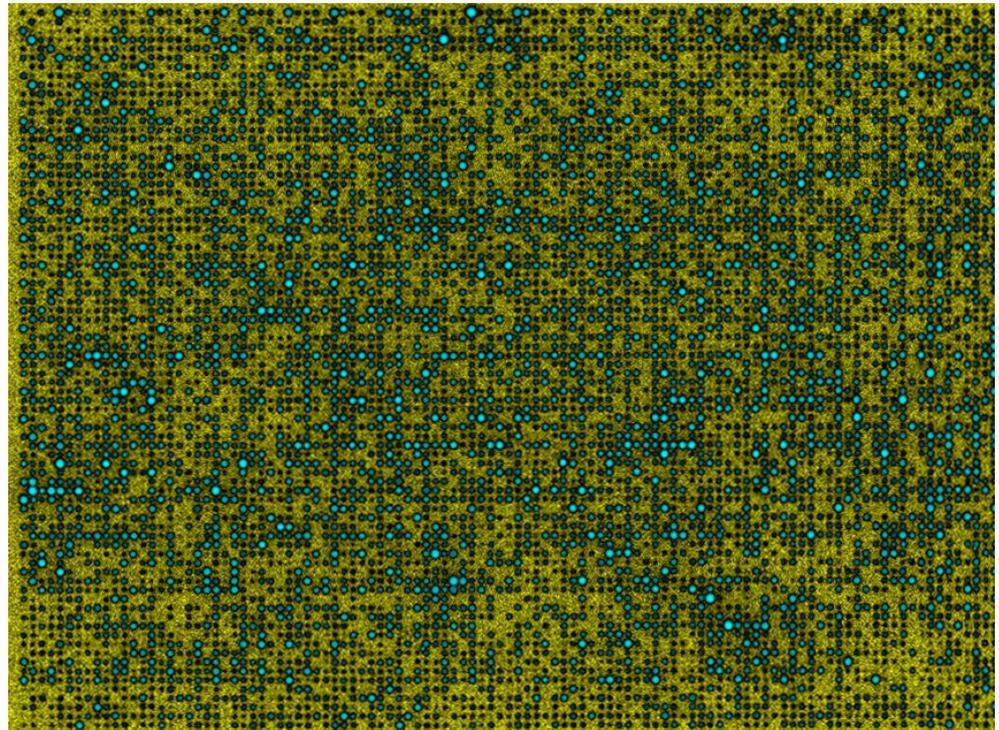
Fig. 5 A DNA array hybridised with radioactive-labelled DNA target generated from sonicated gDNA of *M. tuberculosis* H37Rv. The figure was generated using "VisualGrid" (http://www.gpc-biotech.com).

The quality of DNA array production, especially the spotting and hybridisation process, were assessed by double spotting of each PCR product on DNA arrays. The correlation coefficients of double spotting of DNA arrays in all experiments varied between 0.95 to 0.99.

A   **B**
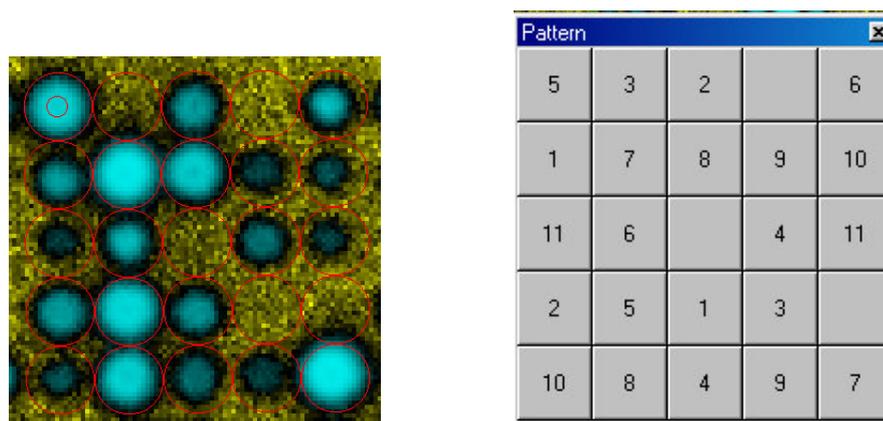
Fig. 6 (A) An example of a grid on a DNA array generated by "VisualGrid". The red circles mark the areas on the array in which the intensities were read by the software. (**B**) The spotting pattern used for depositing PCR products on DNA arrays. The numbers represent the spotting sequence of the 384-well plates in which the PCR products were kept during spotting process.
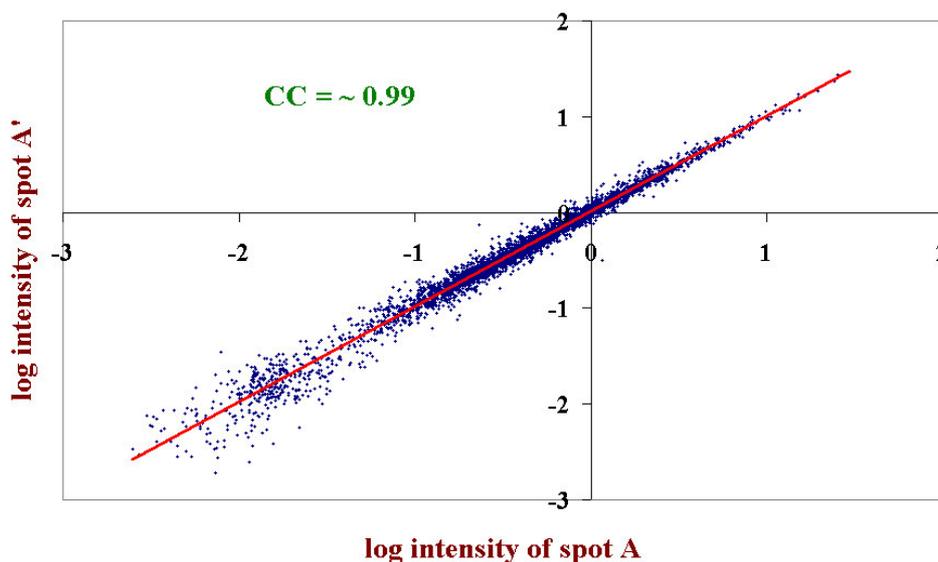
Fig. 7 An example of scatter plot for double spotting of a DNA array. X axis displays logarithmic value of signal intensity of a spot (A) on DNA array representing an ORF and Y axis displays that of another spot (A') on the same DNA array representing the same ORF. The red line is drawn to display the correlation coefficient of the two sets of signal intensities which amounts ~0.99 in this example.

3.3 Comparative Genomic of *M. tuberculosis* complex strains

To identify the genes which are deleted or show low sequence homology in the genome of *M. bovis* and *M. bovis* BCG Copenhagen as compared to that of *M. tuberculosis* H37Rv, the normalised intensities from data set of *M. tuberculosis* H37Rv were subtracted from the corresponding normalised

intensities from the data sets of *M. bovis* and *M. bovis* BCG Copenhagen. A cumulative curve was set up using the resulting subtracted data sets with X axis representing the amount of ORFs having the subtracted normalised intensities less than or equal to the corresponding value and the Y axis representing the subtracted normalised intensities (Fig. 8A).

The deletion analysis was performed by gradually increasing the threshold value as determined based on the subtracted normalised intensities of *M. bovis* BCG Copenhagen starting with the first one hundred ORFs with the lowest values of differences of normalised intensities. All the 100 ORFs belong to the recently published deleted genes in *M. bovis* BCG Copenhagen (95) as compared to *M. tuberculosis* H37Rv. When the threshold value was increased so that the number of genes with the lowest values of differences of normalised intensities was increased to 135 (Fig. 8B), in addition to 125 known deleted genes, 5 ORFs of IS6110 and 5 ORFs which have not been reported recently to be deleted in *M. bovis* BCG Copenhagen as compared to *M. tuberculosis* H37Rv were detected. These 5 ORFs were Rv0050, Rv0278c, Rv0279c, Rv2090, and Rv3281. Rv0278c and Rv0279c belong to the PE-PGRS gene family.

To confirm the DNA array result for these five ORFs, the whole DNA sequence of each of the five ORFs was compared with the whole genome sequence of *M. bovis* AF2122/97 using the BLAST search program (http://www.sanger.ac.uk/Projects/M_bovis/blast_server.shtml) in the Sanger Institute. All five ORFs exhibited some region with differences in DNA sequence between *M. tuberculosis* H37Rv and *M. bovis* AF2122/97.

_____
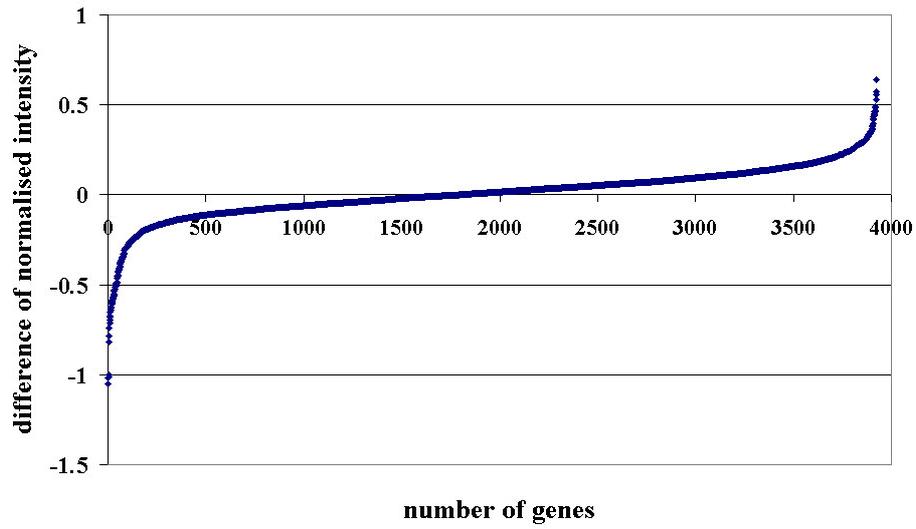
Fig. 8A Cumulative curve of *M. bovis* BCG Copenhagen.
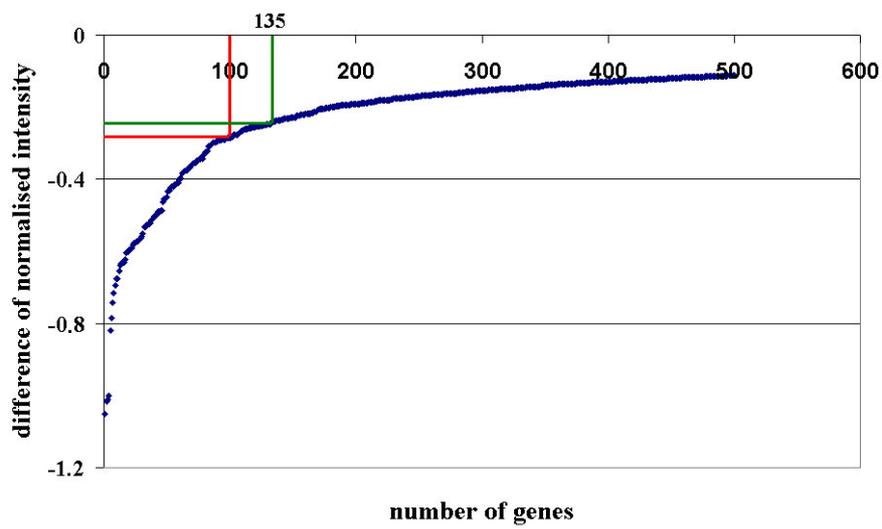
Fig. 8B Cumulative curve of *M. bovis* BCG Copenhagen for the first 500 ORFs with the lowest differences of normalised intensities.

A

```
GACGGCGCCTTGAAGGGCACGTCGAACGAGACTTTCCCCAAACCGACCGAGGTCGGTGGT
GACGGCGCCTTGAAGGGCACGTCGAACGAGACTTTCCCCAAACCGACCGAGGTCGGTGGT
GACGGCGCCTTGAAGGGCACGTCGAACGAGACTTTCCCCAAACCGACCGAGGTCGGTGGT

TATGCCGGTGTGCCGCCGCCGCC---GCCGCCGCCG--G-AGGTACCACCTTCGGAGACC  H37Rv
TATGCCGGTGTGCCGCCGCCGCC   GCCGCCGCCG  G AGGTACCACCTTCGGAGACC
TATGCCGGTGTGCCGCCGCCGCCTCCGCCGCCGCCGTCGGAGGTACCACCTTCGGAGACC  AF2122/97

GTCATCCAGCCCACGGTCGAAATTGCGCCGGGGATTACCATCCCGATCGGTCCCCCGACC
GTCATCCAGCCCACGGTCGAAATTGCGCCGGGGATTACCATCCCGATCGGTCCCCCGACC
GTCATCCAGCCCACGGTCGAAATTGCGCCGGGGATTACCATCCCGATCGGTCCCCCGACC
```

B

```
GCCCGCACCCGATCCGATGCGTGGCGACCCGC-CGCACCCG-GCTCCGCCGCGCTTGC--  H37Rv
GC  G ACCC AT   A GC  GG  A CCGC  CG   C CG GC  C G C   G T GC
GCGAGAACCC-ATGTCA-GCCAGGTTAACCGCGCGTTCGCGAGCGCTGGCAAGATAGCCC  AF2122/97

G-AT-CGC-CACTGGACCCAACAAGTGGCGACCCGCTGCACCCGGCTCCGCCGCGCTTGC
G AT C C CAC  GA CC A   GTGGCGACCCGC GCACCCGGCTCCGCCGCGCTTGC
GCATGCCCGCACCCGATCCGATGCGTGGCGACCCGCCGCACCCGGCTCCGCCGCGCTTGC

GATCGCCACTGGACCCAACAAGTGGCGACCCGCTGCACCCGGCTCCGCCGCGCTTGCGAT
GATCGCCACTGGACCCAACAAGTGGCGACCCGCTGCACCCGGCTCCGCCGCGCTTGCGAT
GATCGCCACTGGACCCAACAAGTGGCGACCCGCTGCACCCGGCTCCGCCGCGCTTGCGAT
```

C

```
GTGACGGGAACGAGACGAACAATCCGGCAGAAGT-GAGTGACGGGAACGAGACGAACAAT  H37Rv
GTGA   GA  GAG   GAAC A CC G  GA GT GAGTGA   GGAACGA ACGAA
GTGAGTCGAGTGAGCGGAACGAACCTGT-GA-GTCGAGTGAGCGGAACGA-ACGAAGTGA  AF2122/97

CCGGCAGAAGTGAGTGACGGGAACGAGACGA-ACAATCCGGCCCCTGTGAGTCGAGT-GA
  G C G A  GAG  ACG  AAC A  CG  A AA    G   C G GA  CGAG   GA
GTGACGGGAACGAG--ACG--AACAATCCGGCAGAAGTGAGTGACGG-GAA-CGAGACGA

GCGGAACGAACGAAGTGAGTGACGGGAACGAGACGAACAATCCGGCCCCTGTGAGTCGAG
  C    CG     GAAGTGAGTGACGGGAACGAGACGAACAATCCGGCCCCTGTGAGTCGAG
ACAATCCGGCAGAAGTGAGTGACGGGAACGAGACGAACAATCCGGCCCCTGTGAGTCGAG

TGAGCGGAACGAACGAAGTGAGTGACGGGAACGAGACGAACAATCCGGCCCCTGTGACCG
TGAGCGGAACGAACGAAGTGAGTGACGGGAACGAGACGAACAATCCGGCCCCTGTGACCG
TGAGCGGAACGAACGAAGTGAGTGACGGGAACGAGACGAACAATCCGGCCCCTGTGACCG
```

Fig. 9 The result of BLAST search for **(A)** Rv0050, **(B)** Rv2090, and **(C)** Rv3281. "H37Rv" corresponds to the DNA sequence of *M. tuberculosis* H37Rv and "AF2122/97" corresponds to the DNA sequence of *M. bovis* AF2122/97.

Primer pairs were constructed to amplify the regions with DNA sequence differences for Rv0050, Rv2090, and Rv3281. The PCR products of Rv2090 and Rv3281 showed differences in size whereas those of Rv0050 did not. The PCR products were sequenced and the obtained DNA sequences were aligned with the corresponding DNA sequence from *M. tuberculosis* H37Rv. For Rv0050 *M. tuberculosis* H37Rv showed 9 bp deletion as compared to *M. bovis* and *M. bovis* BCG Copenhagen. For Rv2090 and Rv3281 *M. bovis* and *M. bovis* BCG Copenhagen showed partial deletion as compared to *M. tuberculosis* H37Rv. The three primer pairs were applied to amplify the gDNA of other members of *M. tuberculosis* complex: *M. africanum*, *M. tuberculosis* Beijing, *M. tuberculosis* Haarlem and *M. canettii.*
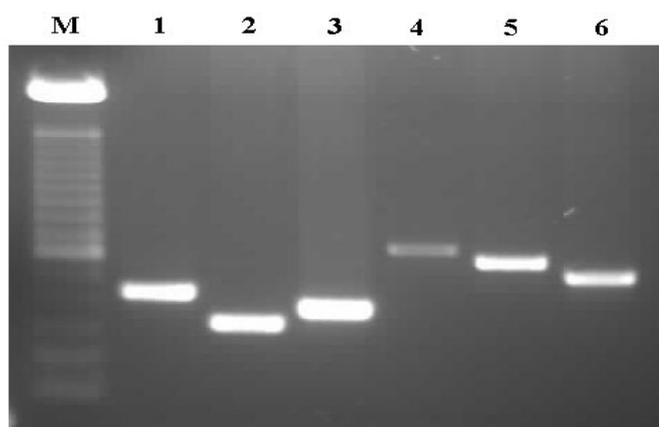
Fig. 10 PCR products of gDNA of *M. tuberculosis* H37Rv (lanes 1 and 4), *M. bovis* BCG Copenhagen (lanes 2 and 5), and *M. bovis* (lanes 3 and 6) using primer pairs for Rv2090 (lanes 1–3) and Rv3281 (lanes 4–6). M: 100-bp marker.

Further differences in DNA sequences could be observed. The sizes of deletions and their positions were summarised in Table 1.

_____

Gene name	*M. africanum*	*M. tb* Beijing	*M. bovis*	*M. bovis* BCG Cop.	*M. canettii*	*M. tb* Haarlem
Rv0050	9 bp; 1893-1894	9 bp; 1893-1894	9 bp; 1893-1894	9 bp; 1893-1894	6 bp; 1893-1894	no deletion
Rv2090	57 bp; 116-172	no deletion	57 bp; 116-172	115 bp 107-221	no deletion	no deletion
Rv3281	no product	72 bp; 94-165	135 bp; 102-236	62 bp; 232-293	162 bp; 138-299	62 bp; 407-468

Table 1 The list of sequence variations on Rv0050, Rv2090, and Rv3281 with the corresponding deletion sizes and positions. The sequence comparison was performed between *M. tuberculosis* H37Rv and the listed strains in the table. *M. tb* = *M. tuberculosis*; BCG Cop. = BCG Copenhagen

A

```
GACGGCGCCTTGAAGGGCACGTCGAACGAGACTTTCCCCAAACCGACCGAGGTCGGTGGT
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GACGGCGCCTTGAAGGGCACGTCGAACGAGACTTTCCCCAAACCGACCGAGGTCGGTGGT


TATGCCGGTGTGCCGCCGCCGCCGCCGCCGCCG---------GAGGTACCACCTTCGGAG  H37Rv
::::::::::::::::::::::::: ::::::                ::::::::::::::::::
TATGCCGGTGTGCCGCCGCCGCCGCCTCCGCCGCCGCCGTCGGAGGTACCACCTTCGGAG  BCG


ACCGTCATCCAGCCCACGGTCGAAATTGCGCCGGGGATTACCATCCCGATCGGT
:::::::::::::::::::::::::::::::::::::::::::::::::::::::::
ACCGTCATCCAGCCCACGGTCGAAATTGCGCCGGGGATTACCATCCCGATCGGT
```

_____

B

```
CGGCTCCGCCGCGCTTGCGATCGCCACTGGACCCAACAAGTGGCGACCCGCTGCACCCGG
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
CGGCTCCGCCGCGCTTGCGATCGCCACTGGACCCAACAAGTGGCGACCCGCTGCACCCGG
```

```
CTCCGCCGCGCTTGCGATCGCCACTGGACCCAACAAGTGGCGACCCGCTGCACCCGGCTC   H37Rv
:::::::::::::::::::::::::::::                                    BCG
CTCCGCCGCGCTTGCGATCGCCACTGG---------------------------------
```

```
CGCCGCGCTTGCGATCGCCACTGGACCCAACAAGTGGCGACCCGCTGCACCCGGCTCCGC

------------------------------------------------------------
```

```
CGCGCTTGCGATCGCCACTGGTGCTACTGGACGGCGCCAGC
                    :::::::::::::::::::::::
--------------------TGCTACTGGACGGCGCCAGC
```

C

```
ACGAACAATCCGGCAGAAGTGAGTGACGGGAACGAGACGAACAATCCGGCCCCTGTGAGT
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
ACGAACAATCCGGCAGAAGTGAGTGACGGGAACGAGACGAACAATCCGGCCCCTGTGAGT
```

```
CGAGTGAGCGGAACGAACGAAGTGAGTGACGGGAACGAGACGAACAATCCGGCCCCTGTG   H37Rv
:::::::::::                                                     BCG
CGAGTGAGCG--------------------------------------------------
```

```
AGTCGAGTGAGCGGAACGAACGAAGTGAGTGACGGGAACGAGACGAACAATCCGGCCCCT
            :::::::::::::::::::::::::::::::::::::::::::::::::::
------------GAACGAACGAAGTGAGTGACGGGAACGAGACGAACAATCCGGCCCCT
```

Fig. 11 The result of sequence alignment between the PCR product sequence of **(A)** Rv0050, **(B)** Rv2090, and **(C)** Rv3281 using gDNA of *M. bovis* BCG Copenhagen as template and their corresponding sequence from TubercuList. "H37Rv" corresponds to the DNA sequence of *M. tuberculosis* H37Rv (TubercuList) and "BCG" corresponds to the DNA sequence of *M. bovis* BCG Copenhagen (PCR product sequence).

A   **B**


C

Fig. 12 Real-time RT-PCR of **(A)** Rv0050, **(B)** Rv2090, and **(C)** Rv3281 using total RNA isolated from granuloma as template. On each figure the curve pair on the left represents reaction in the presence of total RNA whereas the curve pair on right represents reaction in the absence of total RNA (negative control). Each of the curve pairs represents reaction duplicates.

3.4 Gene expression profile analysis of *M. tuberculosis* H37Rv infecting *in vitro* grown murine bone marrow-derived macrophages

3.4.1 DNA array hybridisation

Hybridisation of DNA arrays with the target cDNA generated from RNA samples of *M. tuberculosis* isolated from (i) *in vitro* culture, (ii) resting and (iii) activated macrophages 3 days after infection delivered more than 70% positive signals. However, in the case of RNA samples taken at day 1 after infection of activated macrophages less than 5% positive signals could be observed. This indicates that most of the genes of *M. tuberculosis* H37Rv

_____

were inactive or transcribed in very low levels at day 1 after uptake by activated macrophages.
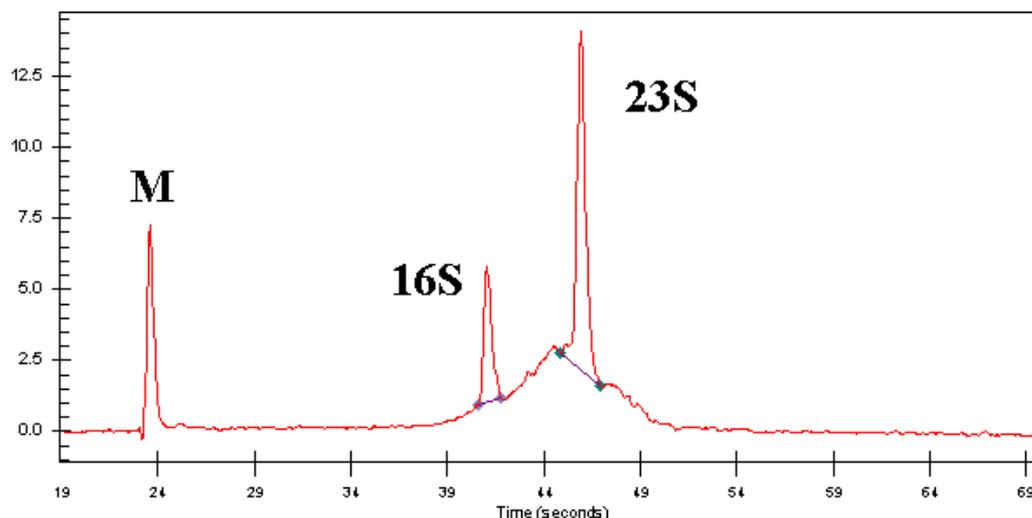

Fig. 13 Bioanalyzer^{TM} result for total RNA of *M. tuberculosis* H37Rv grown *in vitro* culture. The two major peaks represent the two ribosomal RNAs: 16S and 23S rRNA.

Although the same amount of total RNA from day 1 and 3 after infection was applied, much fewer positive signals were observed at day 1 (5% <). Three days after infection of activated macrophages, mycobacteria resumed growth. The transcription processes were reactivated as indicated by hybridisation results with more than 70% positive signals at day 3 after infection. Intracellular *M. tuberculosis* was quickly isolated by mild detergent treatment and collecting free mycobacteria by centrifugation to avoid changes in transcriptomes. The mycobacterial pellet was immediately subjected to total RNA extraction yielding total RNA from *M. tuberculosis* H37Rv and the contaminating macrophage RNA. The quality and quantity of this RNA was assessed using Bioanalyzer^{TM} (Fig. 13).

Figures 14 and 15 depict the distribution of the logarithmic values of signal intensities of *in vitro* grown *M. tuberculosis* H37Rv before and after normalisation, respectively.
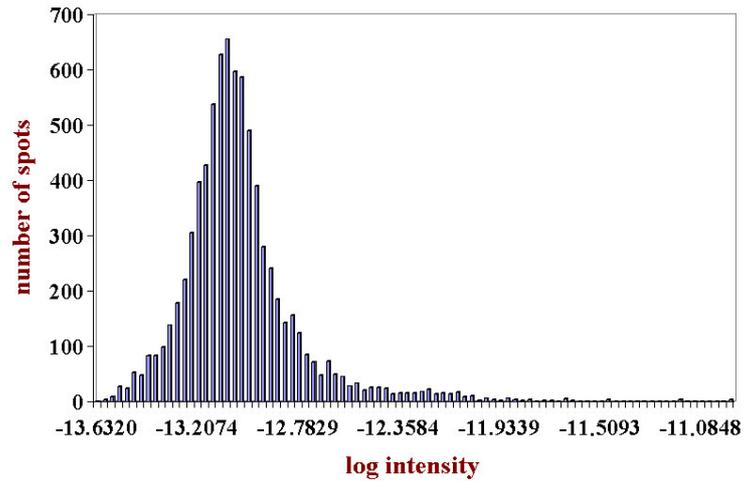
**Fig. 14** Distribution curve of logarithmic value of signal intensities on a DNA array in example of *in vitro* grown *M. tuberculosis* H37Rv. The main Gaussian curve peaks at ~ -13.
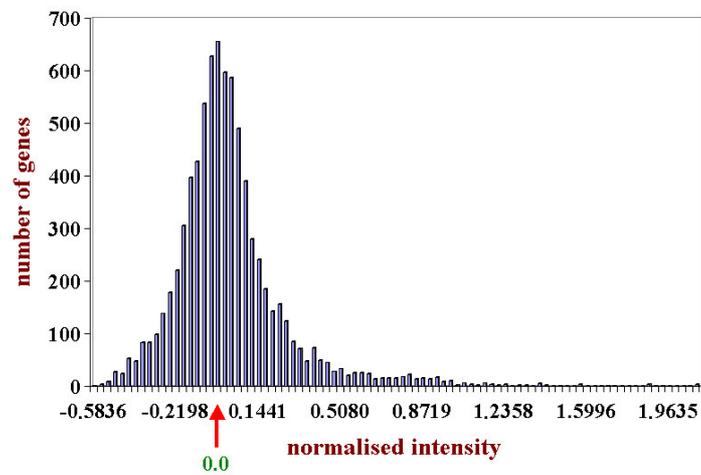


Fig. 15 Distribution curve of the same DNA array data presented in Figure 14 after normalisation of intensity values. The normalisation shifts the peak of the main Gaussian curve to zero without changing the overall distribution pattern.

_____
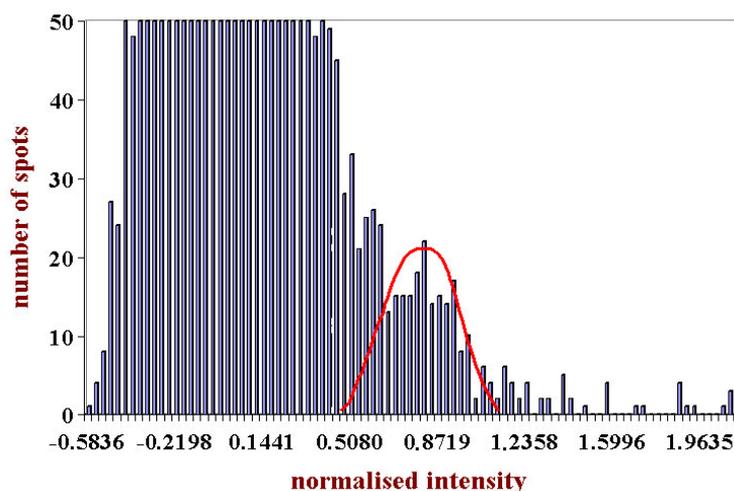
Fig. 16 Magnification of the distribution curve from Figure 15. A second Gaussian curve exists as indicated by the red bell-shaped curve, which represents signal intensities of higher values and peaks at ~0.8. The presence of a Gaussian curve for higher signal intensities indicates that the high signal intensities resulted from higher levels of the corresponding transcripts rather than from hybridisation artefacts.

The distribution of the log intensities peaked at ~ -13 (Fig. 14) and the normalisation calculation brought all normalised spot intensities against zero (Fig. 15).

It should be noted that normalisation did not change the overall profile of signal intensity distribution. In addition to the major Gaussian curve that culminated around zero, a second minor peak can also be observed for higher signal intensities (Fig. 16), which indicates that the high intensities resulted from higher levels of the corresponding transcripts rather than from hybridisation artefacts.

Figure 17 showed a scatter plot generated from a data set of normalised signal intensities of *M. tuberculosis* H37Rv isolated from activated macrophages (X axis) vs that isolated from resting macrophages (Y axis). The red line was drawn to represent the CC of the 2 data sets, which amounted ~0.65. The 2 green lines above and below the red line represented the cut-off for the changes of transcription levels more than threefold.
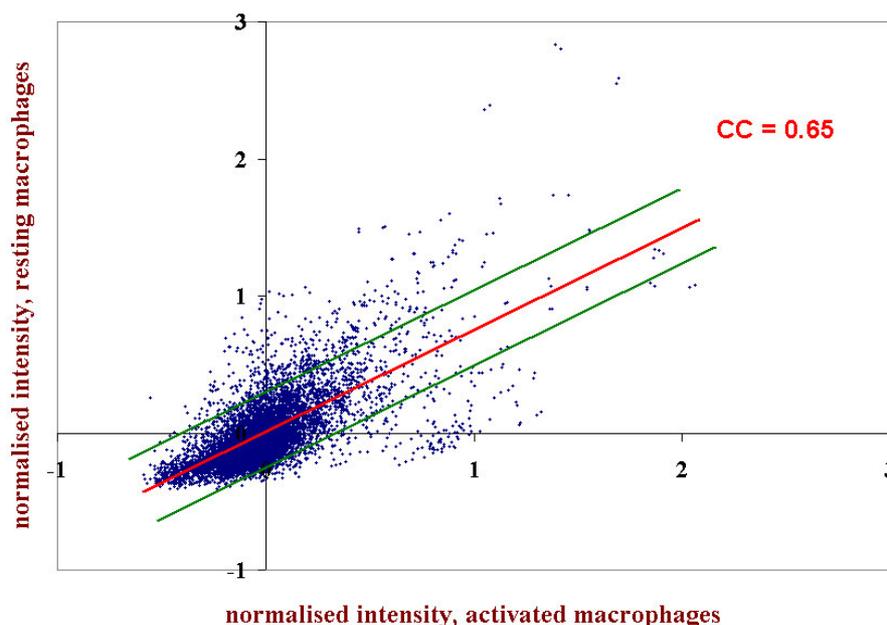
Fig. 17 Scatter plot of *M. tuberculosis* H37Rv infecting activated (X axis) and resting macrophages (Y axis). The spots lying below the lower green line represent the genes of *M. tuberculosis* H37Rv that are upregulated at least threefold in activated as compared to resting macrophages. The spots lying above the upper green line represent the genes of *M. tuberculosis* H37Rv that are upregulated at least threefold in resting as compared to activated macrophages.

3.4.2 Iron metabolism

Two genes, Rv2385 (*mbtJ*) and Rv2386c (*mbtI*), which are associated with the biosynthesis of hydroxyphenyloxazoline-containing siderophore mycobactins were upregulated upon activation of macrophages with IFN-γ. The gene product of *mbtI* is thought to play a role in the conversion of chorismate to salicylate (the starter unit for mycobactin siderophore construction), whereas that of *mbtJ* is a putative acetyl hydrolase which is possibly required for N-hydroxylation of the two lysine residues at some stage during mycobactin assembly. In contrast, the transcription of *bfrB* (Rv3841) was strongly reduced in activated macrophages. The product of *bfrB* is involved in the biosynthesis of bacterioferritin, which serves as iron storage molecule. Among the genes of *M. tuberculosis* H37Rv that were downregulated during infection in activated macrophages was a series of

genes encoding proteins that require iron as their cofactor such as oxidoreductases and oxygenases.
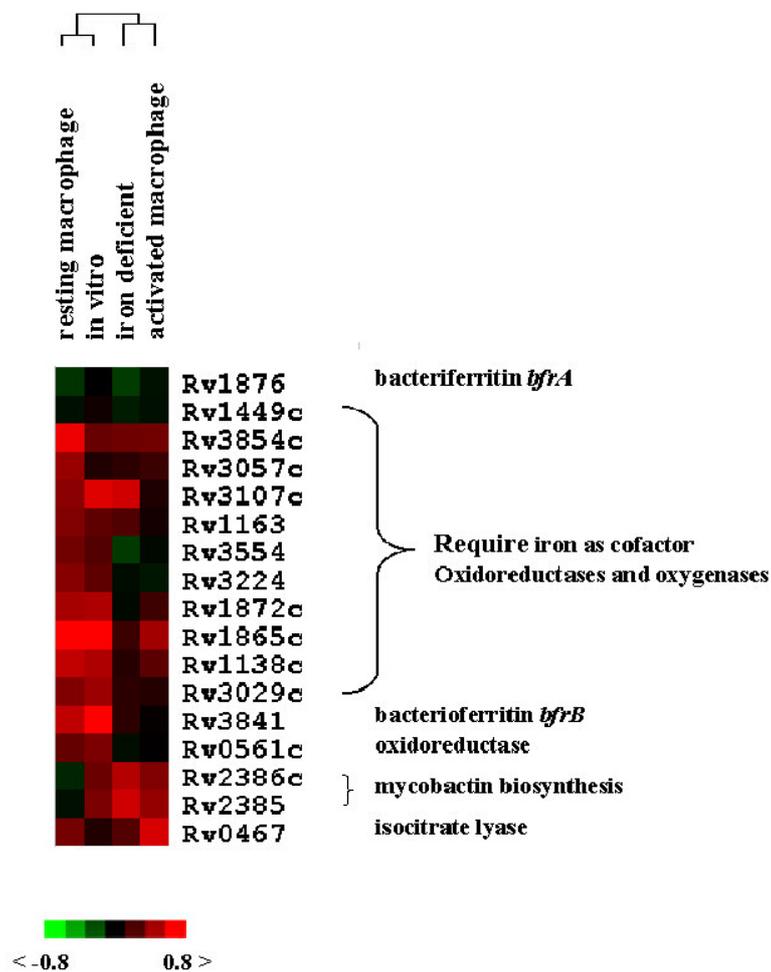
Fig. 18 Dendrogram and matrix displaying the hierarchical clustering of 17 selected genes of *M. tuberculosis* H37Rv gene expression data generated from 4 different growth environments using algorithm based on the average linkage method. The matrix below the dendrogram displays the relative gene expression levels of individual samples. Columns represent individual growth conditions and rows represent individual genes ordered according to hierarchical clustering. The colour scale represents relative gene expression levels.

To investigate the impact of iron deprivation on the gene expression profile of *M. tuberculosis*, *in vitro* culture of *M. tuberculosis* H37Rv were depleted for iron by deferoxamine.

_____
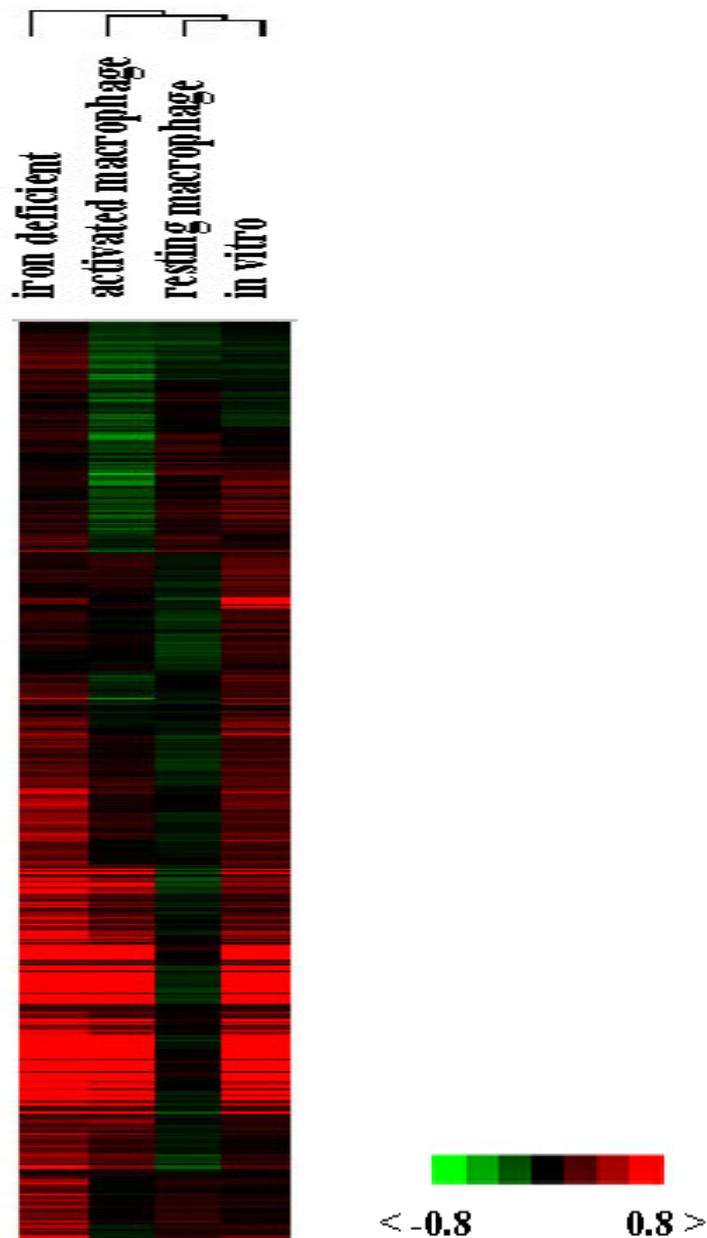
Fig. 19 Dendrogram and matrix displaying the hierarchical clustering of all genes of *M. tuberculosis* H37Rv gene expression data generated from 4 different growth environments using algorithm based on the average linkage method. On the matrix only a part of the whole gene set is shown.

The transcription level of Rv2385 and Rv2386c was also elevated under iron limitation *in vitro*. Correspondingly, the transcription of *brfB* (Rv3841) was repressed. Of the 12 downregulated genes which require iron as cofactor, 7 genes showed reduced transcription levels during growth under iron depletion and in activated macrophages (Fig. 18).

Hierarchical clustering of the *M. tuberculosis* H37Rv selected genes involved in iron metabolism or depending on iron revealed similar expression profiles during growth in activated macrophage or iron deprivation *in vitro* (Fig. 18). Figure 19 displays the dendrogram and matrix of average linkage hierarchical clustering when all genes in the genome of *M. tuberculosis* H37Rv were taken into account in establishing the hierarchical clustering. In Figure 19 only a part of the whole matrix is shown to demonstrate that the hierarchical clustering method applied here was able to bring together the genes with similar gene expression profiles in four growth conditions.

3.4.3 Cell membrane and cell wall synthesis

DNA array results in this study revealed strong induction of some genes associated with the biosynthesis of cell wall and cell membrane components (Figure 20 and website). Among these were Rv1170, Rv2157, and Rv2482 which are involved in mycothiol, peptidoglycan and phospholipid biosynthesis, respectively. These genes were already highly expressed in resting macrophages and this expression level was maintained during infection of activated macrophages.
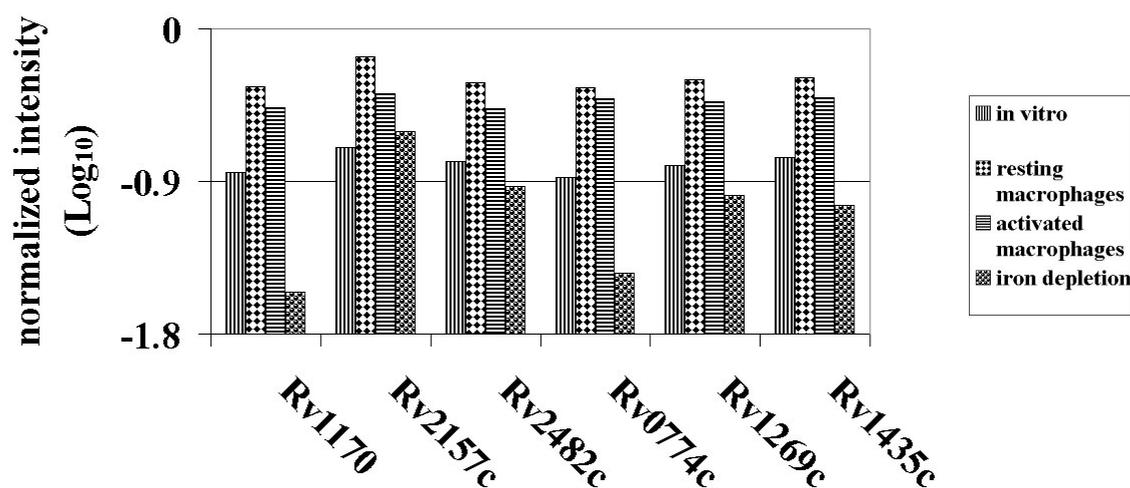
Fig. 20 Relative transcription levels of genes, whose products are involved in the biosynthesis of cell wall and cell membrane components under 4 growth conditions. The relative transcription levels are represented by their corresponding normalised intensities in logarithmic scale.

The expression of these genes was at basal levels during growth *in vitro* with or without iron deprivation indicating that these genes were induced by stimuli other than iron restriction. Another gene that exhibited similar expression pattern is *galU* (Rv0993). Some genes which are involved in the synthesis of lipoproteins such as *lpqV* (Rv1064) and *lppZ* (Rv3006) were upregulated 3 days after infection of activated macrophages but not in resting macrophages. The expression levels of these genes were independent on iron availability.

Some genes encoding exported proteins, a series of conserved membrane proteins and proteins that are involved in signal peptide cleavage were highly induced during infection of resting and activated macrophages (see website). Rv2962c and Rv1699 were also specifically induced in activated macrophages.

3.4.4 Biosynthesis of amino acids
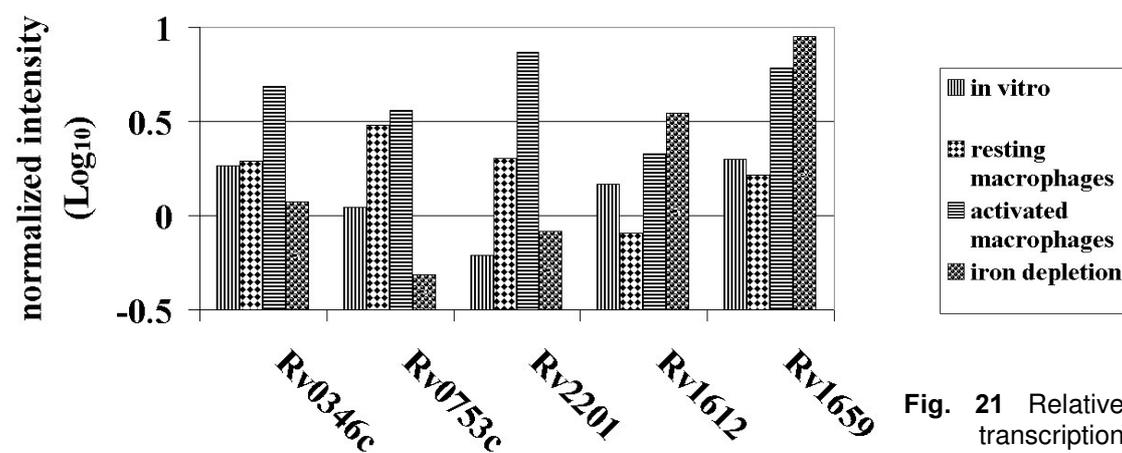
Fig. 21 Relative transcription levels of genes, whose products are involved in the amino acid metabolism under 4 growth conditions as described in the legend. The relative transcription levels are represented by their corresponding normalised intensities in logarithmic scale.

In resting macrophages *M. tuberculosis* resides and multiplies in phagosomes requiring synthesis of a large number of macromolecules. Some genes that are involved in amino acid biosynthesis such as Rv0753c in valine and pyrimidine metabolism and Rv2201 in asparagine synthesis were upregulated in resting macrophages suggesting that the phagosomal environment may be

restricted in amino acids (Fig. 21 and website). The high expression levels of these genes were maintained during infection of activated macrophages. In activated macrophages additional genes involved in amino acid biosynthesis were upregulated including Rv1612 and Rv1659 relevant for the biosynthesis of tryptophan and arginine, respectively. This suggests a general limited amino acid availability in activated macrophages. The notion that intraphagosomal mycobacteria encounter an amino acid deprived condition is further supported by the finding that Rv0346c thought to be involved in transport of L-asparagine across the membrane was upregulated during infection of macrophages.

3.4.5 Pyrophosphatase

The expression of *ppa* (Rv3628) was induced 3 days after infection of activated macrophages.

3.4.6 Lipid metabolism
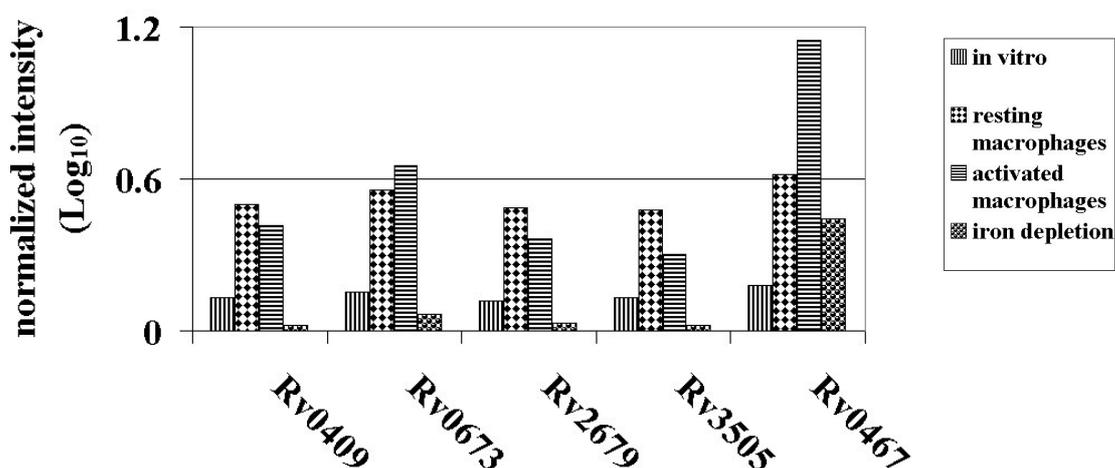
**Fig. 22** Relative transcription levels of genes, whose products are involved in lipid metabolism under 4 growth conditions. The relative transcription levels are represented by their corresponding normalised intensities in logarithmic scale.

The amount of isocitrate lyase transcript (Rv0467) was observed to be only slightly increased during infection of resting macrophages, whilst activation of

macrophages drove the infecting *M. tuberculosis* to produce more isocitrate lyase transcript. Some genes involved in fatty acid oxidation including Rv0215c, Rv0673, Rv0852, Rv2679, and Rv3505 were induced during infection of resting and activated macrophages (Fig. 22 and website). Another gene, Rv0409, whose product catalyses the conversion of acetate to acetyl-CoA was also induced in intracellular mycobacteria.

3.4.7 DNA damage repair

The high expression of *M. tuberculosis* genes responsible for DNA damage repair and neutralization of reactive radicals including Rv1020, Rv1329c, Rv2090, Rv2836c and *bpoB* (Rv1123c) were observed in this study during macrophage infection (Fig. 23 and website). Of the *sod* genes, only the expression level of *sodC* (Rv0432) was elevated in activated macrophage.
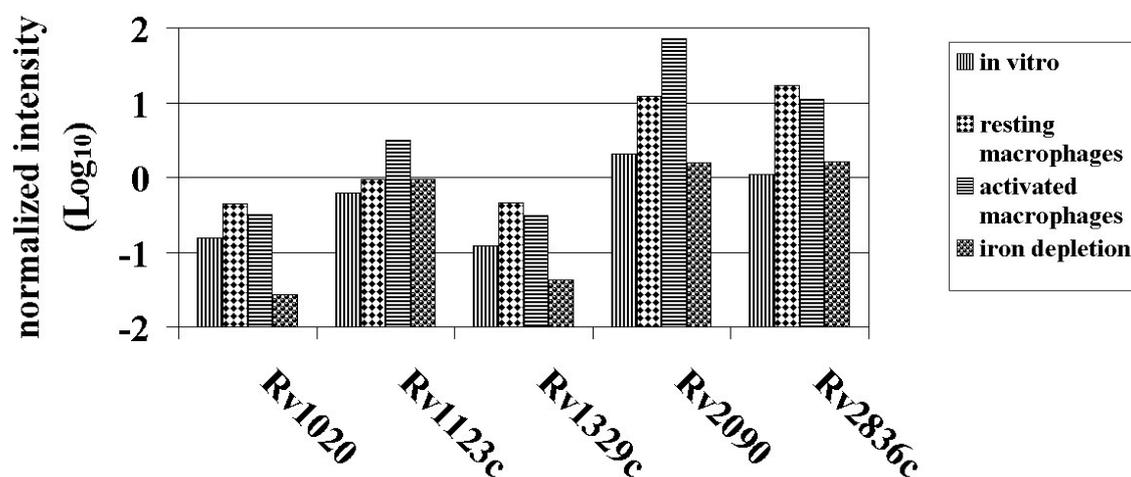

Fig. 23 Relative transcription levels of genes, whose products are involved in DNA metabolism and repair under 4 growth conditions. The relative transcription levels are represented by their corresponding normalised intensities in logarithmic scale.

3.4.8 Resuscitation-promoting factor (Rpf)

Of the five *rpf* genes in the genome of *M. tuberculosis* H37Rv, only *rpfE* (Rv2450) was strongly induced 3 days after infection of activated macrophages.

3.4.9 Ion and drug transport regulation

Some genes which are associated with transport of ions and unknown substances such as Rv1458c, Rv2691 and Rv3270 were upregulated during infection of resting (Fig. 24).
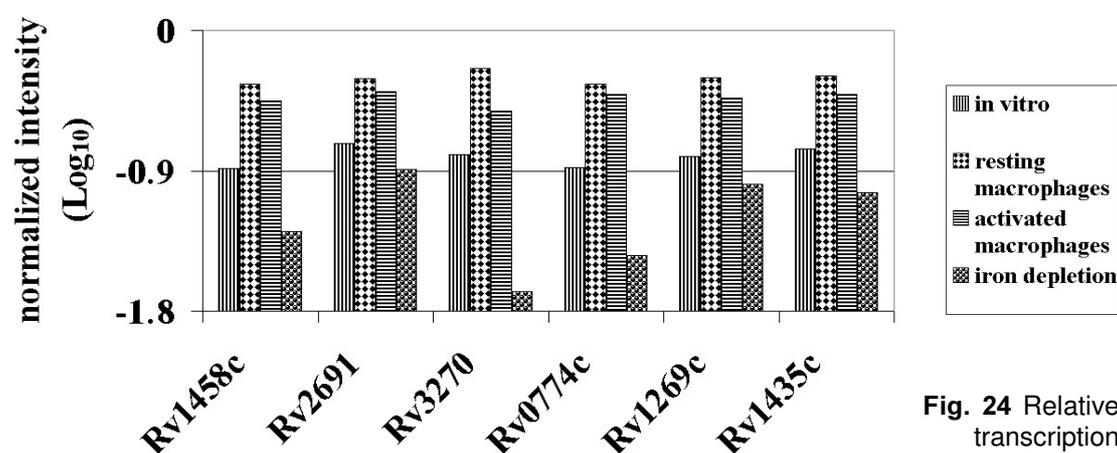
Fig. 24 Relative transcription levels of genes, whose products are involved in the molecule transport across the cell membrane under 4 growth conditions. The relative transcription levels are represented by their corresponding normalised intensities in logarithmic scale.

3.4.10 Regulatory proteins

Some genes that encode different groups of regulatory proteins such as protein kinases and transcriptional regulatory protein ArsR-family were upregulated during infection of both resting and activated macrophages (Rv0014c, Rv1534, Rv2640c, Rv2642, Rv3246, Rv3681, Rv0485, Rv0758, Rv0903c) (Fig. 25).
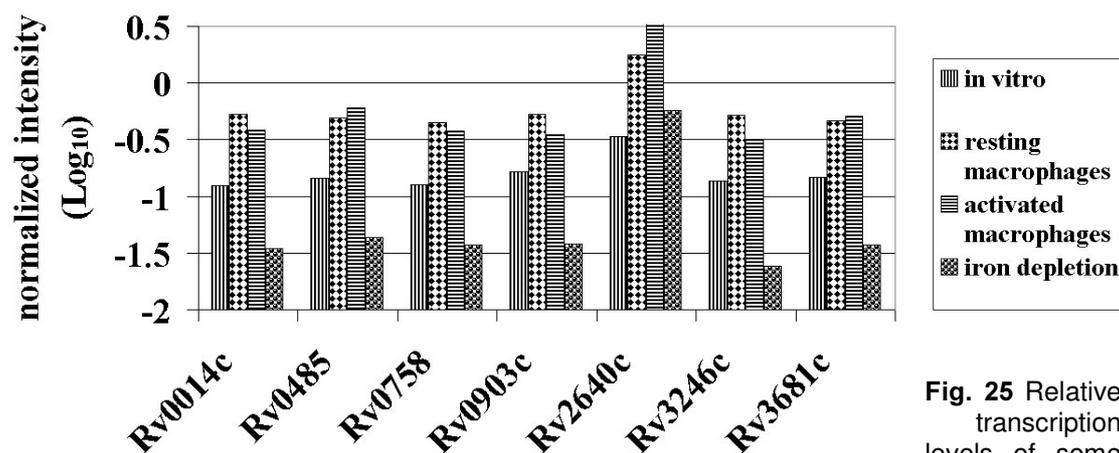
Fig. 25 Relative transcription levels of some genes, whose products are regulatory proteins belonging to different regulatory protein families under 4 growth conditions. The relative transcription levels are represented by their corresponding normalised intensities in logarithmic scale.

3.5 Gene expression profile analysis of *M. tuberculosis* during host infection in human lung

3.5.1 DNA array hybridisation

Total RNA samples were isolated from *M. tuberculosis* inhabiting different sites on the lung of tuberculosis patients: granulomatous lesions, pericavity, and distant lung. Since RNA isolation was performed immediately after removal of lung tissues containing tubercle bacilli, the total RNA preparations contained contaminating total RNA from patient lung tissues. Preliminary hybridisation experiments, applying ~75% total RNA isolated from lung tissue at a given site of infection, delivered only ~12% positive signals on DNA arrays in spite of highly sensitive detection system with radioactive-labelled cDNA targets. Because of the solid nature of the lung tissues at the sites of infection, we assume that only a small part of the total RNA from *M. tuberculosis* could be extracted from infected tissue. Since a DNA array system with radioactive labelling of cDNA targets was used which allows more sensitive signal detection, 0.5–1 µg pure *M. tuberculosis* total RNA was usually sufficient for one hybridisation experiment to deliver more than 70% positive signals on DNA arrays. From these facts it can be inferred that *M.*

_____

tuberculosis total RNA only comprised less than 10% total RNA isolated from infected human lung. To increase the detection sensitivity of the DNA array system, an RNA amplification method was developed to enrich total RNA of *M. tuberculosis.* The establishment and assessment of this amplification method is described in section 4.4. Using this amplification method, the number of positive signals was able to be increased to more than 70%. As negative control the same amount of total RNA isolated from lung cancer was applied. Hybridisation of DNA arrays with this sample resulted in less than 3% positive signals.

To gain information about the genes which play important roles during infection *in vivo*, the gene expression profile of *in vivo* growth was compared with that *in vitro*. For this purpose *M. tuberculosis* cells from the center of caseous granuloma were cultured into 7H9 medium. To obtain the global correlation of *M. tuberculosis* transcriptomes from the 4 different growth conditions: *in vitro*, granuloma, pericavity, and distant lung, average linkage hierarchical clustering of the normalised DNA array data was performed. The gene expression profiles of *M. tuberculosis* in pericavity and distant lung appeared very similar with correlation coefficient more than 90% (Fig. 26). Most of the genes which were differentially regulated between infection of *M. tuberculosis* in pericavity and distant lung are either involved in cell membrane and cell wall synthesis or encode conserved hypothetical proteins (see website). Comparison of gene expression profiles of *M. tuberculosis* residing pericavity and granuloma resulted in much more differentially expressed genes as can be inferred from the correlation coefficients < 80% and from the result of hierarchical clustering in Figure 26.

Due to the limited amounts of available RNA samples, it was impossible to repeat the hybridisation experiment using the same samples. Instead, this problem was overcome by performing hybridisation experiments using RNA samples from the same sites of infection isolated for each infection site at least from 3 different tuberculosis patients.

_____
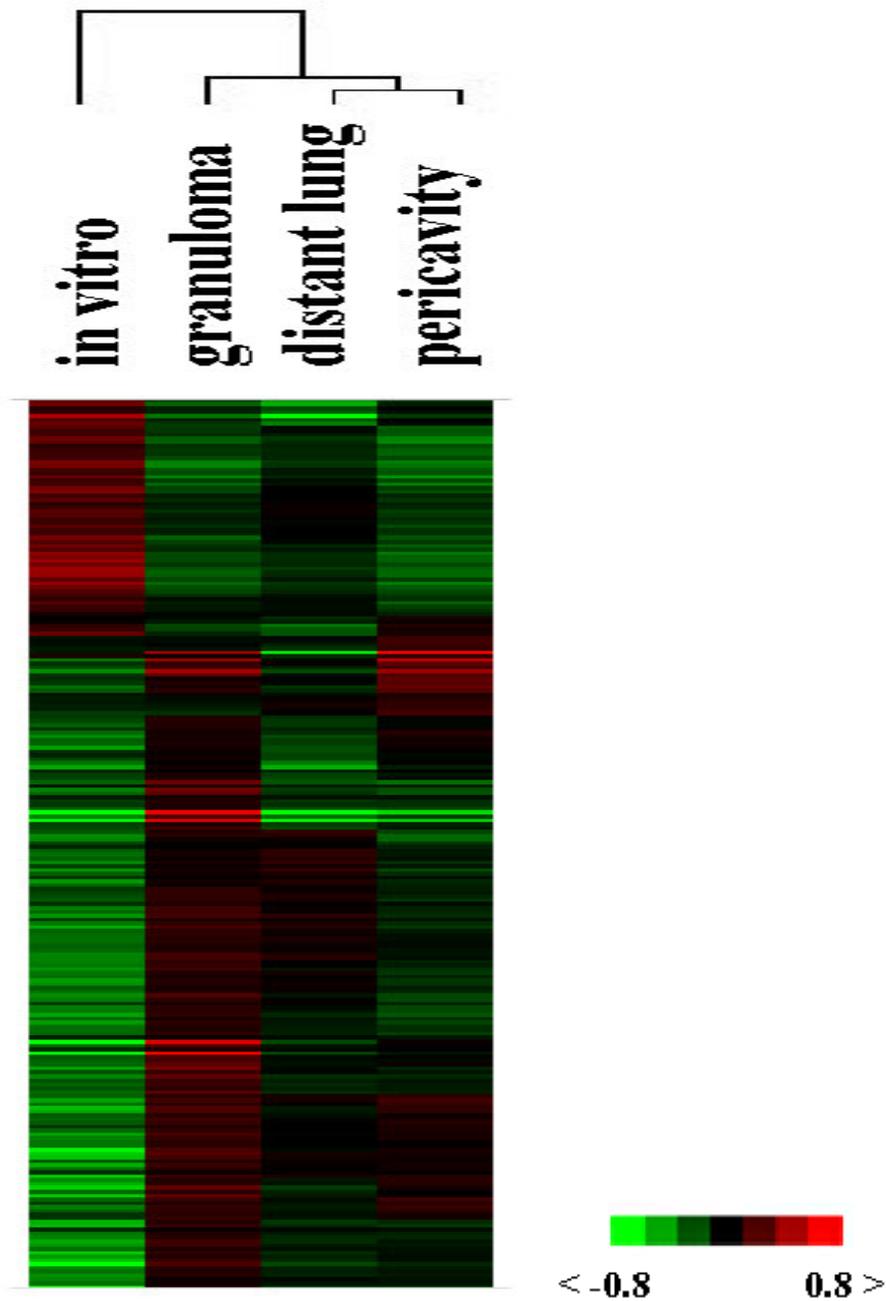
Fig. 26 Dendrogram and matrix displaying the hierarchical clustering of all genes of *M. tuberculosis* gene expression data generated from 4 different growth environments *in vitro* and *in vivo* as indicated in dendrogram using algorithm based on the average linkage method. The matrix below the dendrogram depicts the relative gene expression levels of individual samples and represents a part of the whole matrix chosen arbitrarily to show area with great differences in the 4 growth environments. Columns represent individual growth conditions and rows represent individual genes ordered according to hierarchical clustering. The colour scale represents relative gene expression levels.
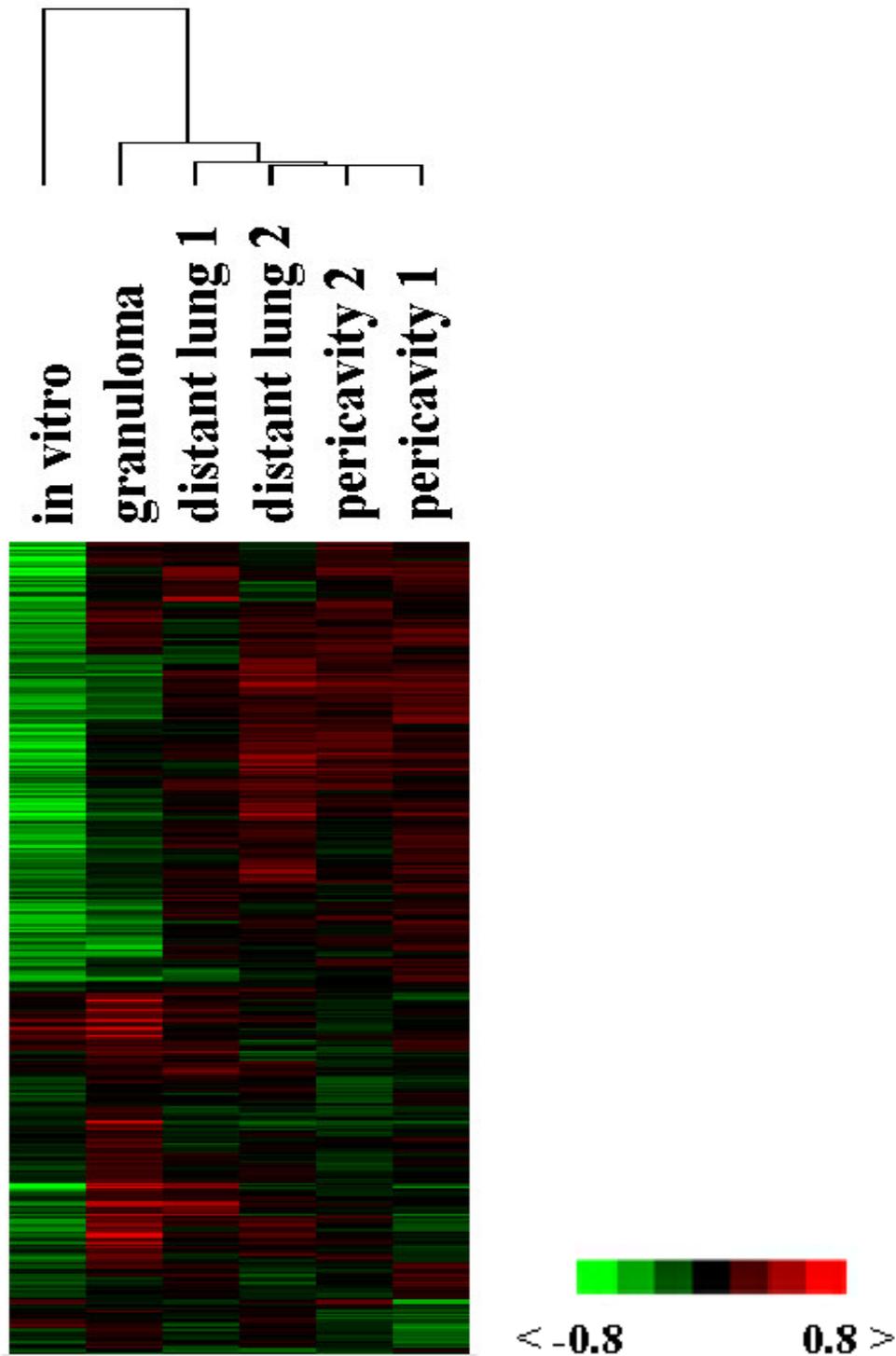
Fig 27 Dendogram and matrix displaying the hierarchical clustering of all genes of *M. tuberculosis* gene expression data generated from six data sets representing four different growth environments *in vitro* and *in vivo* as indicated in dendogram using algorithm based on the average linkage method. The matrix below the dendrogram depicts the relative gene expression levels of individual samples and represents a part of the whole matrix chosen arbitrarily to show area with great differences in the six data sets. Columns represent individual growth conditions and rows represent individual genes ordered according to hierarchical clustering. The colour scale represents relative gene expression levels.

_____

The correlation coefficients for each group of data sets of each infection site from different patients varied between 0.92 to 0.95. These results emphasise that gene expression profiles of *M. tuberculosis* from the same site of infection from different patients are very similar and can be reliably treated as experimental duplicates. In addition, some variations in the degree of up- or downregulation of some genes from different patients in each site of infection were observed which may reflect the differences in the environmental conditions encountered by tubercle bacilli partly due to variations in host immune responses.

Hierarchical clustering of six DNA array data sets comprising one *in vitro* data set, one granuloma data set, two pericavity data sets, and two distant lung data sets delivered a dendrogram (Fig. 27) which would highly resemble the one in Figure 26 with the two data sets from pericavity and distant lung being interchangeable.

3.5.2 Fatty acid metabolism

Some genes whose products are thought to oxidise fatty acids were induced in three sites of infection in human lung (Fig. 28 and website). A series of fatty-acid-CoA synthetases was also highly induced. This observation strongly supports the use of fatty acids as carbon and energy source. However, DNA array results in this study did not identify induction of *icl* and two other genes (*AceAa* and *AceAb*) whose products also function as isocitrate lyase, in all three sites of infection in human lung with chronic tuberculosis. On the contrary, *citA* (Rv0889c, citrate synthase II) and succinate dehydrogenase subunits (Rv0248c, Rv0249c) were upregulated *in vivo* (Fig. 29 and website). In addition, the transcript level of Rv1617 (*pykA*), whose product represents the key enzyme linking the fatty acid degradation to gluconeogenesis and thus enabling the conversion of fatty acids to the components of cell wall, was also elevated *in vivo*.
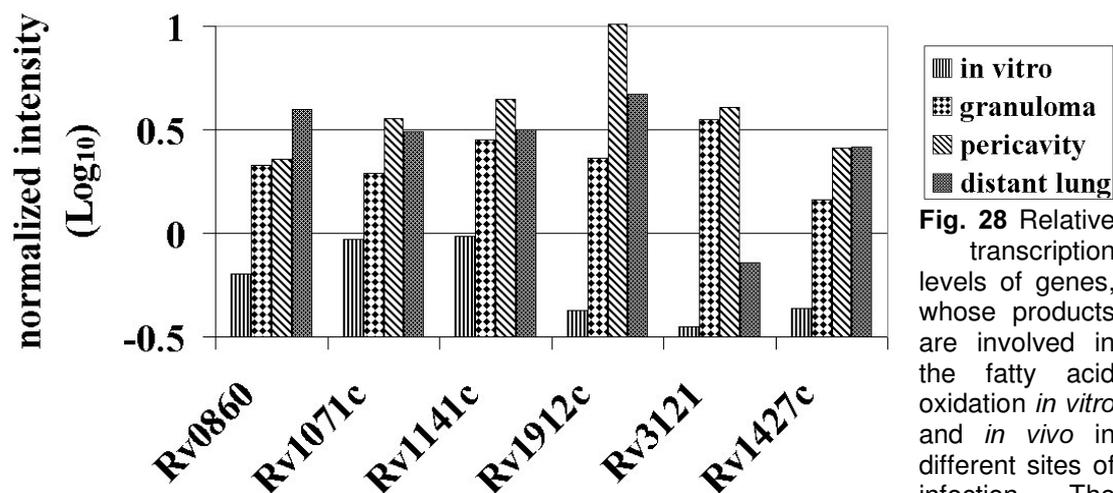
Fig. 28 Relative transcription levels of genes, whose products are involved in the fatty acid oxidation *in vitro* and *in vivo* in different sites of infection. The relative transcription levels are represented by their corresponding normalised intensities in logarithmic scale.

3.5.3 Energy metabolism

3.5.3.1 Anaerobic respiration
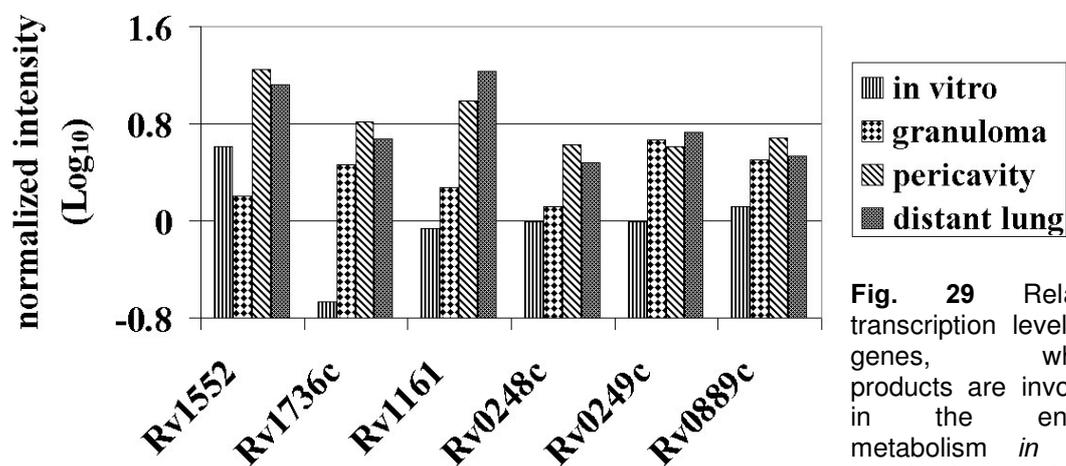
Fig. 29 Relative transcription levels of genes, whose products are involved in the energy metabolism *in vitro* and *in vivo* in different sites of infection. The relative transcription levels are represented by their corresponding normalised intensities in logarithmic scale.

The expression of *narG* (Rv1161) and *narX* (Rv1736c) were stimulated *in vivo* (Fig. 29). Both genes encode nitrate reductase which allows *M. tuberculosis* to use nitrate instead of oxygen as terminal electron acceptor. The high

transcription level of Rv1552 encoding flavosubunit of fumarate reductase, an enzyme complex which enables bacteria to utilise fumarate as terminal electron acceptor in anaerobic respiration was also observed in pericavity and distant lung but not in granuloma (Fig. 29).

3.5.3.2 Aerobic respiration

Rv1620c, Rv1623c, and *glbO* (Rv2470) whose products are involved in aerobic respiratory chain were highly expressed *in vivo*. To boost the electron transfer in the respiratory chain some NADH dehydrogenases were also induced. In addition, the induction *in vivo* of a panel of genes which are known to be highly expressed during *in vitro* growth of *M. tuberculosis* under hypoxia were also observed (see website).
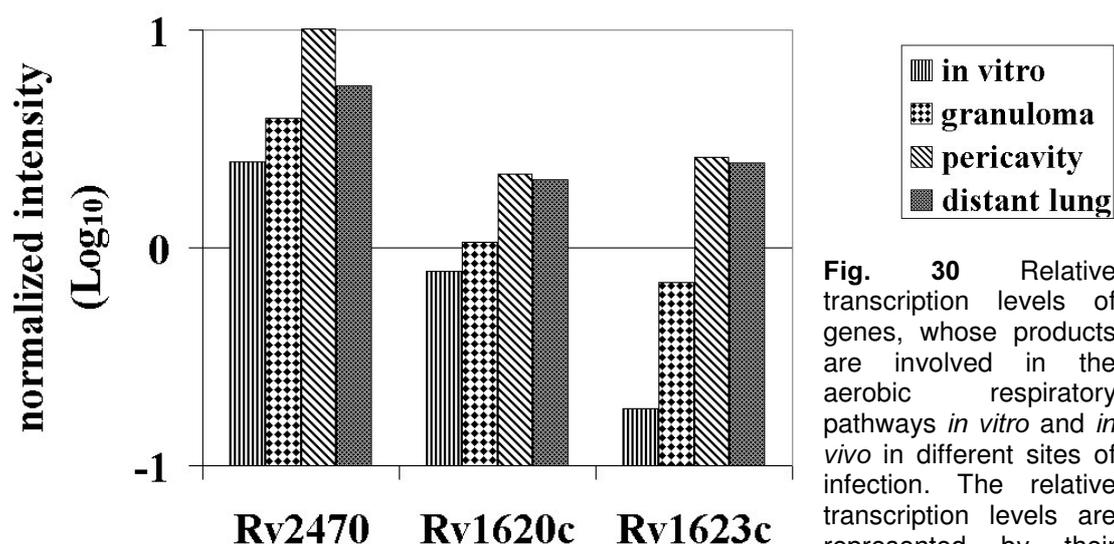
Fig. 30 Relative transcription levels of genes, whose products are involved in the aerobic respiratory pathways *in vitro* and *in vivo* in different sites of infection. The relative transcription levels are represented by their corresponding normalised intensities in logarithmic scale. Legend see Figure 31.

3.5.4 Nutrient (proteins, amino acids, iron and vitamines) metabolism

Some genes encoding protein or peptide hydrolysing enzymes were induced suggesting that *M. tuberculosis* may match its amino acid requirements *in vivo*

by hydrolysing proteins and peptides present in its surrounding in addition to biosynthesis of some amino acids as can be inferred from the strong activation of their biosynthesis pathways. Some genes involved in siderophore mycobactin synthesis were also upregulated implying the condition of iron deprivation in the sites of infection. DNA array data showed that the extent of oxygen and nutrient limitation to tubercle bacilli in human lung was greater in pericavity and distant than that in granuloma.

3.5.5 Cell wall and cell membrane synthesis
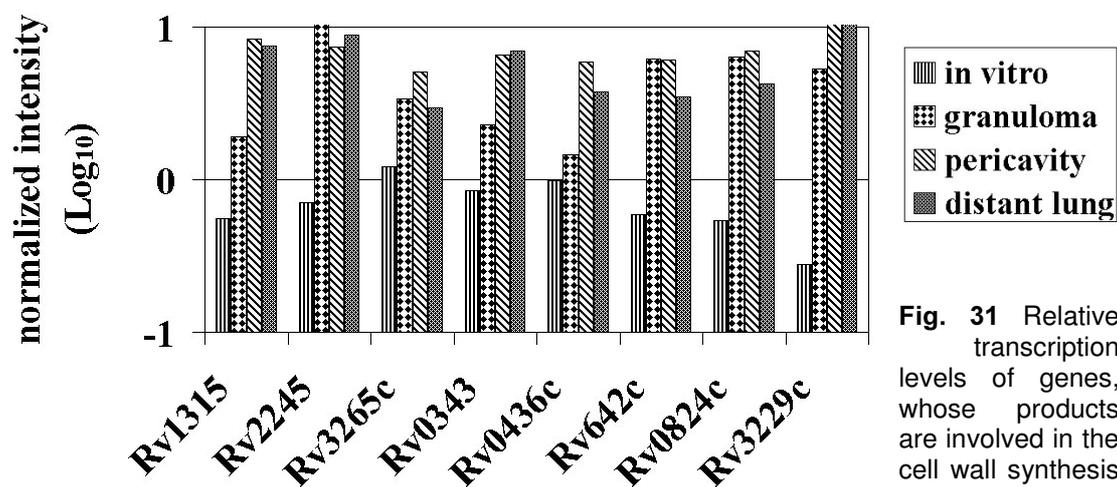
Fig. 31 Relative transcription levels of genes, whose products are involved in the cell wall synthesis *in vitro* and *in vivo* in different sites of infection. The relative transcription levels are represented by their corresponding normalised intensities in logarithmic scale.

DNA array results in this study showed that all major constituents of mycobacterial cell wall: mycolic acid, arabinogalactan, peptidoglycan, and lipoarabinomannan and those of cell membrane especially membrane proteins including transport proteins were actively synthesised (Fig. 31). In addition, the transcription induction of inositol monophosphatase gene *suhB* (Rv2701c) was also observed *in vivo*.

3.5.6 Drug resistance
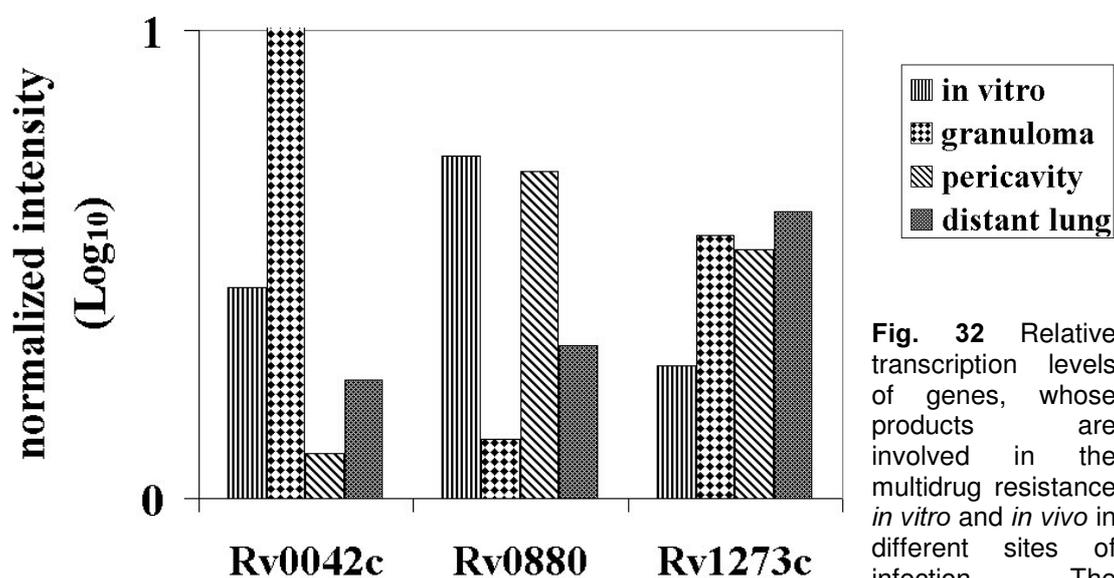
Fig. 32 Relative transcription levels of genes, whose products are involved in the multidrug resistance *in vitro* and *in vivo* in different sites of infection. The relative transcription levels are represented by their corresponding normalised intensities in logarithmic scale.

Two genes whose products belonging to MarR family, Rv0042c and Rv0880, were subject to strong differential regulation *in vivo* (Fig. 32). Rv0042c was downregulated in pericavity and distant lung, whereas Rv0880 was downregulated in granuloma and distant lung.

In addition to *marRAB* system, a high expression of some genes whose products are involved in multidrug resistance via active transport of drugs across the membrane was also observed (see website).

3.5.7 Tetronasin transport system

Two genes, Rv1217c and Rv1218c, which encode integral membrane proteins thought to be involved in active transport of tetronasin across the membrane (export) and thus conferring tetronasin resistance, were upregulated *in vivo* (see website).

3.5.8 DNA repair, radical neutralisation, and detoxification
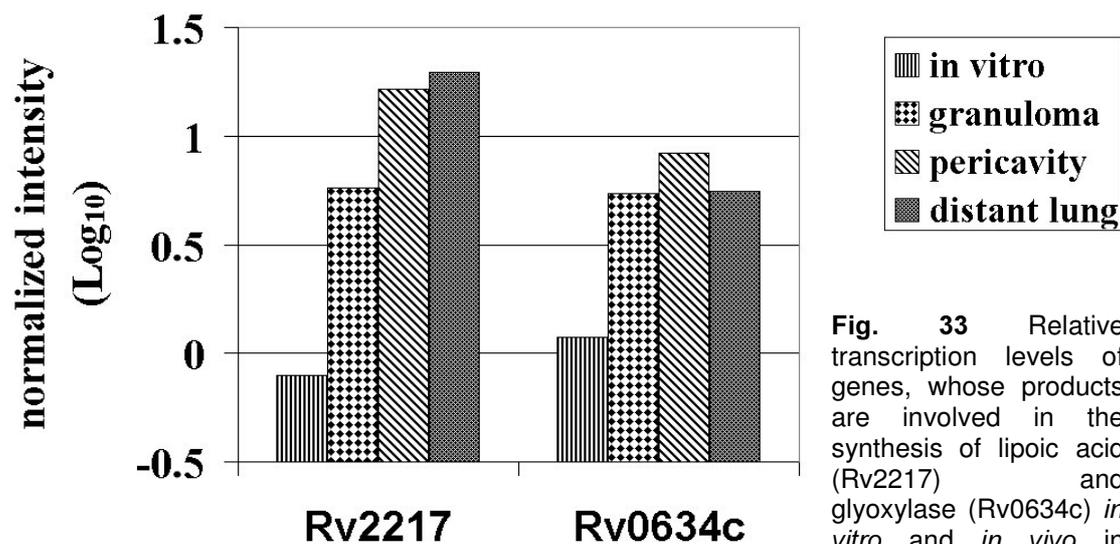
Fig. 33 Relative transcription levels of genes, whose products are involved in the synthesis of lipoic acid (Rv2217) and glyoxylase (Rv0634c) *in vitro* and *in vivo* in different sites of infection. The relative transcription levels are represented by their corresponding normalised intensities in logarithmic scale.

The upregulation of a panel of genes involved in repair systems of macromolecules, neutralisation of radicals and toxic substances in addition to transport systems to pump out the harmful molecules was observed *in vivo* (Fig. 33 and see website). In addition, the high induction of detoxification system glyoxylase and the antioxidant lipoic acid was also observed.

3.5.9 Urease

DNA array data in this study revealed that some genes which are involved in urease biosynthesis were upregulated *in vivo*.

3.5.10 Gene with unknown functions

In addition to the genes which have been described to be induced *in vivo* by other groups, DNA array results in this study also revealed the upregulation of a great number of genes which have not been reported previously to be highly expressed during infection. Many of these genes have not been attributed to any known function.

3.6 Development of linear RNA amplification system for mycobacteria

3.6.1 RNA amplification

The original and amplified RNA was quantified using Bioanalyzer^{TM}. From 200 ng total RNA, an amount of up to 20 µg amplified RNA was obtained, corresponding to an amplification fold of about 100. The size distribution of amplified RNA culminates at the position directly to the left of 16S ribosomal RNA peak (fig. 34A and B).

A
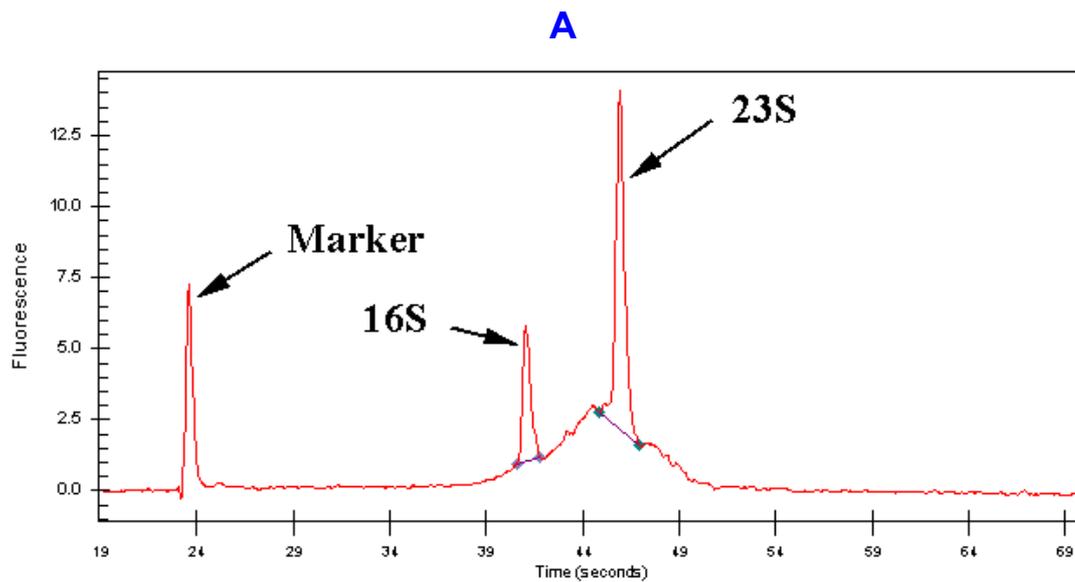
B
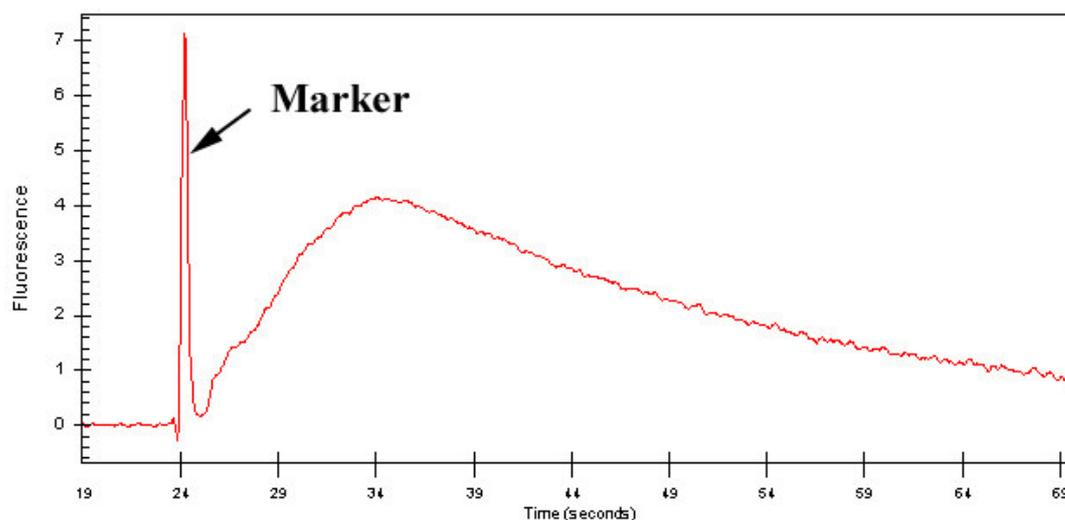
Fig. 34 Bioanalyzer^{TM} result for total RNA of *M. bovis* BCG Copenhagen grown *in vitro* culture: **(A)** before amplification **(B)** after amplification . The two major peaks in Figure 34A represent the two ribosomal RNAs: 16S and 23S rRNA.

3.6.2 Comparison of DNA array hybridisation results with unamplified and amplified RNA

After data normalization, the correlation coefficients (CCs) of the data sets resulting from hybridization of DNA arrays were calculated. For pure mycobacterial total RNA the CCs between unamplified and amplified total RNA ranged from 0.9 to 0.95 for mtGDPs and 0.88 to 0.92 for random primers. For spiked mycobacterial total RNA similar ranges of CCs were obtained. When the array data from mycobacterial RNA spiked with human lung total RNA were compared with those from pure mycobacterial total RNA, the CCs varied between 0.89 and 0.95 for unamplified RNA and 0.87 and 0.94 for amplified RNA when mtGDPs were applied for synthesizing dscDNA as template for RNA amplification and for generating labeled cDNA probes. For random primers these CCs range from 0.86 to 0.9 and 0.84 to 0.88, respectively.
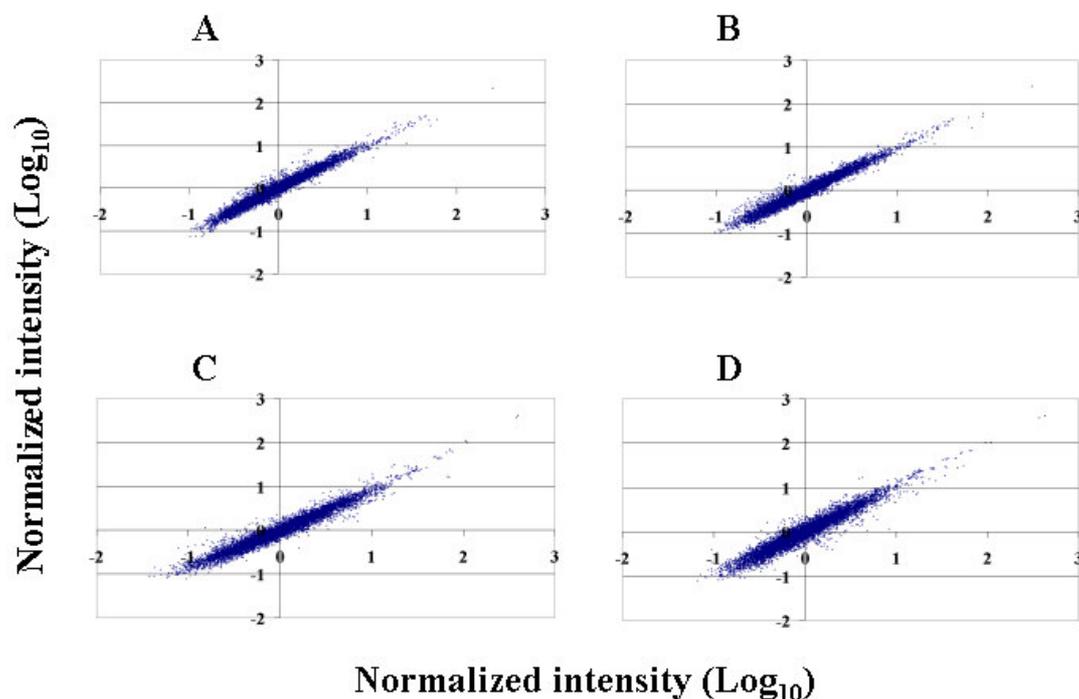
Figure 35 Scatter plots generated from the data of filter-based DNA array hybridizations with *M. tuberculosis* RNA. **(A)** mtGDPs: unamplified (X axis) vs amplified pure RNA (Y axis). **(B)** random decamers: unamplified (X axis) vs amplified (Y axis) pure RNA. **(C)** mtGDPs: unamplified (X axis) vs amplified (Y axis) spiked RNA. **(D)** random decamers: unamplified (X axis) vs amplified (Y axis) spiked RNA.

In general, the use of mtGDPs resulted in higher CCs between unamplified and amplified RNA samples than that of random primers, especially for mycobacterial total RNA which was spiked with human total RNA. It can be inferred from the CCs that in general massive excess of contaminating RNA may cause false signal detection to a greater or lesser extent. However, the amplification of diluted RNA only slightly increased unspecific signals when mtGDPs was employed. The use of mtGDPs for the synthesis of dscDNA in the spiked mycobacterial RNA favoured the reverse transcription of mycobacterial RNA. The application of the same primers for the labeling reaction of cDNA targets from the amplified RNA provided further selection toward mycobacterial RNA.
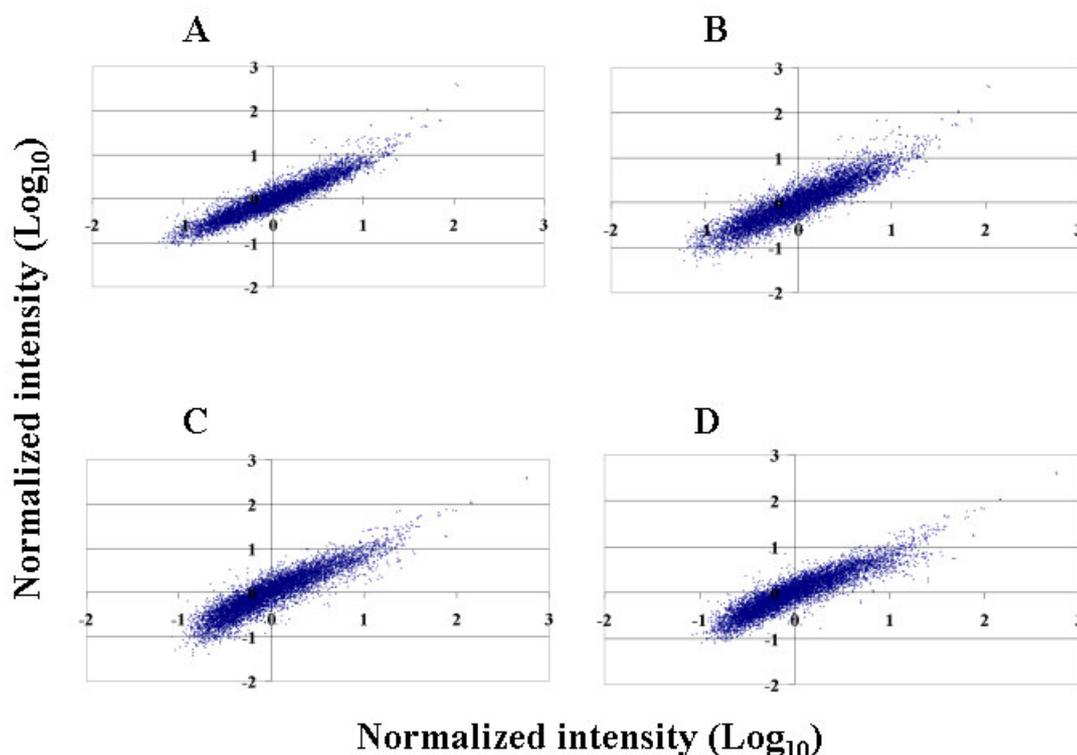
Figure 36 Scatter plots generated from data of filter-based DNA array hybridizations with *M. tuberculosis* RNA. **(A)** mtGDPs and unamplified RNA: pure (X axis) vs spiked RNA (Y axis). **(B)** mtGDPs and amplified RNA: pure (X axis) vs spiked RNA (Y axis). **(C)** random decamers and unamplified RNA: pure (X axis) vs spiked RNA (Y axis). **(D)** random decamers and amplified RNA: pure (X axis) vs spiked RNA (Y axis).

This may explain the relatively similar range of CCs for pure and spiked RNA when the array data sets of unamplified and amplified RNA using mtGDPs were compared. Each RNA amplification and the corresponding hybridization experiment was done in triplicates. The CCs between the repeats ranged from 0.95 to 0.98.

3.6.3 Comparison of DNA array hybridisation results from total RNA of *M. tuberculosis* H37Rv and *M. bovis* BCG Copenhagen

The data analysis for the comparison of array data from total RNA of *M. tuberculosis* H37Rv and *M. bovis* BCG Copenhagen focused on the first place on the known deleted ORFs in the genome of *M. bovis* BCG Copenhagen relative to the genome content of *M. tuberculosis* H37Rv.

The normalised signal intensities for these genes in *M. tuberculosis* H37Rv array data showed 15 to 30 times higher than those in *M. bovis* BCG

Copenhagen. The range of intensity ratios was fairly maintained when pure RNA was amplified. Contamination of total human lung RNA caused shift of these ratios to lower values when random decamers were used (14 to 27 times higher in *M. tuberculosis* H37Rv than in *M. bovis* BCG Copenhagen), whereas the use of mtGDPs delivered similar range of ratios to that of pure total RNA.

4. Discussion

4.1 Comparative Genomic of *M. tuberculosis* complex strains

The increasing availability of complete genome sequences of various microorganisms including microbial pathogens (http://www.tigr.org/) in combination with DNA array technology permits the detailed inspection into their evolutionary scenario, pathogenicity, immunogenicity, and also offer the possibility to find potential drug targets in much greater pace.

DNA array technology was developed and applied to studies on *M. tuberculosis* within the scope of this doctoral thesis.

The majority of the experiments applying DNA array technology use glass slide-based microarrays with fluorescent-labelled DNA targets in the standard dual-channel system. Validation studies have demonstrated that this system can reliably detect about 1:100,000 gene transcript sequences starting from 200 ng PolyA$^+$ RNA sample and can provide accurate and precise differential expression ratios over a 2.5-3 $\log_{10}$ dynamic range (111). The detection threshold of high density array methods using fluorescent-labelled targets is of the order of 2 x 10^7 molecules with a minimum quantity of starting material required of 200 ng to 10 µg mRNA (112).

Nylon microarrays in combination with radioactive detection system permit gene expression analysis using minute amounts of cells or tissues. Such a technique allows large-scale gene expression monitoring of probes generated from 100 ng of total RNA (~10^4 cells) (192-194). In comparison to fluorescence-based DNA microarrays it can provide up to 100-fold higher sensitivity.

Moreover, filter-based array systems in combination with radioactive-labelled targets have been applied to the comparative genomic studies to identify SNPs (single nucleotide polymorphisms), in the differential screening of clone libraries, and in the gene expression profiling, especially in research areas where sample amounts are limited (195-198).

One method to screen for DNA sequence variations within a given species is to compare the genome sequences of different strains belonging to the same

species. This approach requires the availability of the genome sequence, and in case of global screening, the whole genome sequence. Although DNA sequencing methods are developing rapidly and the current methods enable the sequencing of Megabases-long DNA with increasing fidelity and reliability, genome sequencing still represents a formidable task. Usually genome sequencing projects are highly laborious and time-consuming. Accordingly, such projects demand enormous financial expenses. Nevertheless, without doubt genome sequence comparisons are of utmost importance since they allow the detection of sequence variations in refined details (199).

Alternative methods such as restriction length polymorphisms, screening for differences in intergenic sequences including the repetitive sequences (direct or indirect repeats) can detect sequence variations which can be used as markers to differentiate strains belonging to one species or genus (200-203). However, these sequence variations can hardly be related to phenotypic differences due to the lack of knowledge about the functions of the sequences. On the other hand, sequence variations within ORFs may more likely be connected with phenotypic variations.

To assess the quality of DNA arrays used in the studies described in this doctoral thesis in terms of reliability and sensitivity, hybridisation experiments were conducted applying radioactive-labelled targets generated from gDNA of *M. tuberculosis* H37Rv, *M. bovis*, and the vaccine strain *M. bovis* BCG Copenhagen on the DNA arrays. Recently, comparative genomic study of *M. tuberculosis* complex strains using glass slide-based microarrays with dual-channel system has successfully revealed 16 deletion regions comprising 129 ORFs in *M. bovis* and the vaccine strains *M. bovis* BCG. These microarrays contain PCR products with the average length of 500 bp representing ORFs of *M. tuberculosis* H37Rv. This method can detect only deletion of DNA sequence longer than 2 kb and fails to detect deletions of DNA smaller than 2 kb, SNPs and point mutations.

To investigate genome variations between the laboratory strain *M. tuberculosis* H37Rv and the clinical strain *M. tuberculosis* CDC1551, a single hybridisation experiment has been performed previously using high-density oligo arrays from Affymetrix. In these oligo arrays each ORF is represented by a set of 25-mer oligos whose total sequence covers the whole sequence of

_____

the corresponding ORF. This system was able to identify three additional regions of deletion in *M. bovis* and *M. bovis* BCG. This method is able to detect deletions or sequence variations longer than 350 bp (204).

In comparative genomic studies based on DNA array technology, determining a threshold for data analysis is somewhat an intricate matter. In various comparative studies using DNA array technology twofold standard deviation of a set of normalised data is used as threshold to determine the presence/absence of a gene in one strain as compared to the other strain (205-208). Another way to classify genes as absent or present in such studies is by establishing empirical metric (209). The results from these analyses require verification by performing PCR and/or Southern analysis.

Since it is known that more than one hundred ORFs are missing in *M. bovis* BCG Copenhagen as compared to *M. tuberculosis* H37Rv, the first data analysis was performed by choosing the first one hundred ORFs with the lowest values of difference of normalised intensity. By increasing the lower limit of difference of normalised intensity, in addition to some IS6110, some ORFs were identified that have not been known previously to show sequence variation between *M. tuberculosis* H37Rv and *M. bovis*. The gene of IS6110, whose product is putative transposase for insertion sequence element IS6110, is present in 20 copies in the genome of *M. tuberculosis* H37Rv and only one copy in *M. bovis* and *M. bovis* BCG Copenhagen. BLAST search of the DNA sequence of these ORFs from *M. tuberculosis* H37Rv in *M. bovis* AF2122/97:

(http://www.sanger.ac.uk/Projects/M_bovis/blast_server.shtml)

revealed sequence variations in all five ORFs. These sequence variations include base mismatches and deletions of small DNA regions. Two genes, Rv0278c and Rv0279c, belong to PE-PGRS family. It has been reported that genes of PE/PPE and PE-PGRS family exhibit high sequence variations among mycobacterial strains. This observation has led to the postulate that these genes play an important role in modulating host immune responses. Three other genes belong to different functional groups with Rv0050 (*ponA1)* involved in peptidogylcan biosynthesis, Rv2090 in DNA metabolism whereas the gene product of Rv3281 belongs to conserved hypothetical proteins with unknown function. Based on the result of BLAST search, primer pairs were

_____

designed to amplify the region on the three chosen ORFs (Rv0050, Rv2090, and Rv3281) containing putative sequence variations. The primer pairs were used to amplify the corresponding region in *M. tuberculosis* H37Rv, *M. bovis* and *M. bovis* BCG Copenhagen. DNA sequencing confirmed the presence of sequence variations within the investigated region in the 3 mycobacterial strains. Rv2090 and Rv3281 in *M. bovis* and *M. bovis* BCG Copenhagen exhibited partial deletion as compared to *M. tuberculosis* H37Rv. On the contrary, Rv0050 in *M. tuberculosis* H37Rv showed small deletion of 9 bp as compared to *M. bovis* and *M. bovis* BCG Copenhagen. These results show that DNA arrays used in this study detected relatively small deletions. For the deletion in Rv0050 which only encompasses several base pairs it is still inscrutable whether the low value of normalised intensity difference was relevant to the small deletion observed in this experiment.

The three primer pairs were then applied to gDNA of clinical isolates belonging to *M. tuberculosis* Beijing, *M. canettii*, *M. tuberculosis* Haarlem, and *M. africanum*. The PCR products from these gDNAs for Rv2090 and Rv3281 showed various lengths when run on agarose gel. The DNA sequencing of the PCR products confirmed the sequence variations in the 3 genes for these clinical isolates.

Interestingly, Rv3281 showed higher sequence variation among the *M. tuberculosis* complex strains tested than Rv2090. Almost all strains exhibited different lengths in Rv3281, whereas the sequence variation in Rv2090 was observed between species in *M. tuberculosis* complex. The amplification of gDNA of *M. africanum* yielded no PCR product. The reason is that one or the two primers used for amplification did not bind to the gDNA of this strain due to lack of sequence homology. *M. tuberculosis* H37Rv, *M. tuberculosis* Beijing, and *M. tuberculosis* Haarlem which belong to the same species exhibited the same DNA sequence in the region examined in Rv2090. On the contrary, the length of this region was variable when the comparison was performed between *M. tuberculosis* H37Rv, *M. bovis*, *M. bovis* BCG, *M. canettii* and *M. africanum* which represent different species in the genus *Mycobacterium.* Therefore, the sequence variation in the examined region of Rv2090 represents a potential marker to identify mycobacterial strains belonging to the *M. tuberculosis* complex. The sequence variation of Rv0050

_____

was more randomly observed within the strains investigated. Interestingly, Rv0050 of *M. tuberculosis* Beijing showed a 9-bp insertion in the investigated region as compared to *M. tuberculosis* H37Rv and Haarlem. This 9-bp insertion thus represents a potential marker to differentiate *M. tuberculosis* Beijing from H37Rv and Haarlem. To exploit this potential, further experiments using much more number of clinical isolates are required.

In addition to their potential as genotypic marker to differentiate various strains within *M. tuberculosis* complex, the DNA sequence polymorphisms observed here could also contribute to the phenotypic diversity, especially in terms of immunologic and virulence factors among clinical isolates of *M. tuberculosis.* Moreover, DNA sequence variations encompassing small region in ORFs may greatly contribute to phenotypic differences. Most SNPs in the genome of *M. tuberculosis* are related to the antibiotics resistance characteristics. However, it has been shown recently that a point mutation in the *mma3* gene impairs the methoxymycolic acid production in certain *M. bovis* BCG strains (210). It has also been suggested that the lack of the ability to produce mycolic acid may have direct influence on the nature of survival of BCG vaccines in macrophages. It is therefore important to consider mutations or sequence variations comprising small DNA sequences, e.g. point mutations and deletions of several bases as the causes of differences in phenotypes, especially those which are related to virulence and immunogenicity.

To address the question as to whether DNA sequence polymorphism observed in certain genes may contribute the differences in phenotypes, the first step to be taken is to investigate the expression of these genes. One step RT-PCR reactions were performed using the primer pairs for Rv0050, Rv0278c, Rv0279c, Rv2090, and Rv3281 with total RNA isolated from *in vitro* culture of the mycobacterial strains which were used in the genotyping experiments described above. The RNA transcripts of the five genes could be detected in all mycobacterial strains investigated. This observation also indicates that none of the sequence variations has led to deactivation of the corresponding genes, at least at the transcriptional level.

Furthermore, to address the possible roles of the genes with DNA sequence polymorphism in virulence and immunogenicity, the first step to be taken is to monitor their expression *in vivo*. Real-time RT-PCR reactions were performed

_____

using the primer pairs for Rv0050, Rv2090, and Rv3281 with total RNA isolated from human lung granulomas. The RNA transcripts of the five genes could also be detected in granulomas. The data analysis of DNA array results using RNA samples isolated from *M. tuberculosis* infecting *in vitro* cultured murine bone marrow-derived macrophages and human lung (the experiments will be discussed in details in section 4.2) showed that Rv2090 was differentially regulated *in vitro* and *in vivo* (see website).

4.2 Gene expression profile analysis of *M. tuberculosis* H37Rv infecting *in vitro* grown murine bone marrow-derived macrophages

4.2.1 Infection model and RNA isolation

In order to identify putative intracellular survival genes for *M. tuberculosis*, comparative gene expression analysis was performed using DNA array technology of *in vitro* grown mycobacteria versus those isolated from resting or IFNγ-activated murine bone marrow-derived macrophages. Total RNA was isolated from *M. tuberculosis* H37Rv isolated at day 1 and 3 after infection. The use of DNA arrays and the radioactive-labelled target cDNA in monitoring the whole-genome gene expressions in these experiments allowed sensitive detection of transcript levels thereby applying less material than using fluorescent-labelled target cDNA. The generation of large amounts of murine bone marrow-derived macrophages and the laborious purification of intracellular mycobacteria could therefore be extenuated. The result of DNA array hybridisation with an RNA sample isolated from day 1 after infection of activated macrophages suggests that *M. tuberculosis* H37Rv entered the condition of growth cessation or even started to stall one day after infection of activated macrophages. It is also possible that the majority of engulfed mycobacteria were killed by activated macrophages but residual organisms could start growing after a lag phase. It has been reported that the number of viable *M. tuberculosis* decreased in activated murine bone marrow-derived macrophages at day 1 to 4 after infection but some *M. tuberculosis* strains

started growing already at day 4 after infection (211). The transitory decrease in metabolic activity probably belongs to the strategy of the cells to adapt to their new environment within activated macrophages. The hybridisation result with total RNA from day 1 after infection served also as negative control for the contaminating effect of total RNA from infected macrophages. Assuming that all positive signals observed on the DNA arrays from day 1, which accounted for less than 4% of the whole spots on DNA arrays, resulted from the contaminating macrophage total RNA, then it can be inferred that hybridisation system used in this study only allowed cross hybridisation on maximum of 4%.

4.2.2. Iron metabolism

Both the host and microbial pathogen require iron for growth, thus constantly competing for iron during infection (212). Iron is required for several cellular functions, including respiration, energy metabolism, and DNA synthesis. In addition, iron participates in redox reactions that generate free radicals, which may activate signalling pathways for cell proliferation.

Iron is an obligate cofactor for a number of proteins encoded by genes in the *M. tuberculosis* genome, such as cytochromes and oxidoreductases (213;214). Therefore, one host defense strategy against intracellular bacteria is limiting the iron access by chelating extracellular iron by host proteins, such as transferrin (TF) and lactoferrin, and/or storage of iron intracellularly by ferritin (215). It has been observed that pulmonary tuberculosis patients are often anaemic, suggesting sequestration of iron by the host (216).

M. tuberculosis and other mycobacterial pathogens survive and multiply within phagosomes of macrophages (217;218). These phagosomes are retarded in their maturation towards the phagolysosomal stage and have therefore access to iron-loaded transferrin. Interruption of transferrin/iron delivery to intracellular mycobacteria by treatment with anti-transferrin-receptor antibodies or lactoferrin or by IFN-γ activation inhibits growth of mycobacteria (219). Thus, iron availability or sequestration and the efforts of tubercle bacilli to circumvent this restriction are critical determinants of the outcome of infection with *M. tuberculosi*s.

_____

M. tuberculosis produces siderophores of the mycobactin class containing a salicylic acid-derived moiety (220-222). The core hydroxyphenyloxazoline structure of mycobactins is thought to be derived from salicylic acid after condensation with a serine residue, analogous to the biosynthetic pathway for both yersiniabactin and pyochelin, which contain hydroxyphenylthiazoline cores.

The *M. tuberculosis* H37Rv genome contains genes (*mbtA-mbtJ*) which are homologous to those involved in hydroxyphenylthiazoline biosynthesis in other bacteria (223). Functionally, siderophore molecules have been classified in two classes thought to act in concert: (i) secreted molecules with the major assignment to catch iron molecules in the environment, (ii) cell-associated molecules, which are more hydrophobic and act as an ionophore to facilitate transport of iron or iron complexes across the cell wall.

The induction of these genes after IFN-γ activation may indicate the condition of iron starvation in activated macrophages. Indeed, recent evidence also revealed that in activated macrophages ferritin, TF receptor (TFR) expression, and iron acquisition from TF is decreased (224-226). However, although IFN-γ activation reduced the total iron content of macrophages, the ability of *M. tuberculosis* to retrieve intracellular iron was only slightly impaired in these cells (227). The fact that the mycobactin synthesis machinery was increased in activated macrophages according to DNA array results in this study suggests that transferrin and mycobactin compete for iron. An excess of siderophore may tip the iron equilibrium in favour of *M. tuberculosis*. It has been shown that the deletion of *mbtB* (Rv2383c) gene strongly impaired the growth of *M. tuberculosis* in culture medium depleted of iron. In addition, siderophore production appears to enhance growth of *M. tuberculosis* in macrophages. In the study of iron-dependent regulator (IdeR), mRNA level of *mbtI* was induced in cultures of *M. tuberculosis* deprived of iron (228). This gene was also induced during *M. tuberculosis* infection of human THP-1 macrophages.

Siderophores serve basically as transient reservoirs of ferric iron for transport into the cytoplasm. On the other hand, bacterioferritin acts as a long-term iron storage protein. The *M. tuberculosis* genome contains two bacterioferritin

homologues, *bfrA* (Rv1876) and *bfrB* (Rv3841) (229). Under the condition of iron deprivation, synthesis of iron-storing proteins is excrescent for the pathogen and limiting the amount of bacterioferritin allows release of iron for metabolic purpose. The decreased expression of these genes may be the response to the iron deprivation in the environment of mycobacterial cells. This indicates a direct influence of iron deprivation on the transcription of iron depending genes. The DNA array results in this study and results from previous studies underscore the importance of the gene products involved in the iron metabolism as potential drug targets. For instance, p-aminosalicylic acid that belongs to second-line antitubercular drugs is thought to interfere with mycobactin synthesis (230-232).

4.2.3 Cell membrane and cell wall synthesis

M. tuberculosis has a unique cell wall consisting of an outer layer composed of mycolic acids and an inner layer of peptidoglycan (6). Both layers are covalently linked via the polysaccharide arabinogalactan. Noncovalently interspersed within mycolic acid residues are lipoarabinomannan (LAM), lipomannan (LM), and various proteins (9). Many of these cell wall components have been associated with virulence of this pathogen and modulation of the host immune response (11;12).

The analysis of ultrastructural morphology using transmission electron microscopy (TEM) of *M. tuberculosis* H37Rv growing in microaerobic and anaerobic *in vitro* cultures revealed that the cells developed a remarkably thickened cell wall outer layer. Moreover, microscopic analysis of *M. tuberculosis* cells isolated from macrophages showed noticeable changes in size and form as compared with those grown *in vitro* (233).

The gene product of *galU* (Rv0993) has glucose-1-phosphate uridylyltransferase activity regulating the supply of UDP-galactose and UDP-glucose (234;235). Both UDP complexes serve as major precursors for the biosynthesis of capsule polysaccharides (CPS) and lipopolysaccharides (LPS). UDP-glucose is also implicated as a potential intracellular signal molecule in *E. coli* (236). Moreover, the mutants lacking *galU* in *Klebsiella pneumoniae* and *Streptococcus pneumoniae* show a loss of virulence

_____

(237;238). Since eukaryotic UDP-Glc pyrophosphorylases appear to be completely unrelated to their prokaryotic counterparts, it has been postulated that GalU may be an appropriate target for the search of new drugs to control the pathogenicity of bacteria like pneumococcus and *S. pyogenes*.

Although the function of lipoproteins in *M. tuberculosis* has not been well characterised, some lipoproteins including phospholipase C have been suggested to modulate the host immune response and may play an important role in virulence (9). Similar functions have also been attributed to some exported proteins (239-243). The high induction of the genes encoding exported proteins, conserved membrane proteins, and proteins that are involved in signal peptide cleavage may also be the cause for changes of cell wall characteristics of *M. tuberculosis* during macrophage infection, although further studies such as knockout mutant analysis will be required.

Rv2962c shows high homology to a glycosyltransferase which catalyses the transfer of sugar subunits to nascent compounds such as glycoproteins and polysaccharides. The expression of this gene in *M. smegmatis* increased survival rates of this avirulent strain in THP-1 macrophages (244). Glycoproteins and glycolipids are important components of the mycobacterial cell surface and are involved in interaction with host cells during infection (6;11;16;17). It has been observed recently that the synthesis of trehalose in some mycobacterial strains including *M. tuberculosis* is catalysed by trehalose-phosphate synthase (TPS) which possesses glycosyltransferase activity (16;17). Trehalose is a nonreducing disaccharide which plays a major role in many organisms, most notably in survival and stress responses. In *M. tuberculosis*, it plays a central role as the carbohydrate core of numerous immunomodulating glycolipids including "cord factor" (trehalose 6,6'-dimycolate). In an effort to elucidate the pathway involved in biosynthesis of LAM in mycobcaterial strains, amino acid sequence used to identify putative mannosyltransferases is also found in glycosyltransferases (245). Based on these considerations, the gene product of Rv2962c could be any pyranosyl- or furanosyltransferase such as mannosyltransferase or galactosyltransferase which may be involved in the biosynthesis of LAM or arabinogalactan, the major components of the mycobacterial cell wall. The gene products of *embA*, *embB* and *embC* in mycobacteria exhibit arabinosyltransferase activity

involved in the biosynthesis of arabinan containing mycobacterial cell wall compounds (246). The EmbB protein is the target of tuberculostatic ethambutol, which inhibits the synthesis of arabinogalactan (AG). Contemplating the roles of glycosyltransferases and the mycobacteriocidal activity of ethambutol, such proteins represent valuable targets for chemotherapeutic intervention in tuberculosis, especially the one that is induced *in vivo*.

For *Legionella pneumophila*, the agent of Legionnaires' disease, a uridine diphospho (UDP)-glucosyltransferase has recently been described as virulence factor (247). This glucosyltransferase can modify a 45-kDa host protein which may influence host cell function and promote *L. pneumophila* proliferation.

CTP synthase encoded by *pyrG* (Rv1699) catalyses the ATP-dependent formation of CTP from UTP using either NH_3 or L-glutamine as the nitrogen source. CTP is required for various metabolic pathways, including RNA, cell wall and cell membrane synthesis. The synthesis of phospholipids also requires CTP for the formation of activated components such as CDP-diacylglycerol, CDP-ethanolamine and CDP-choline. The transfer of the major building blocks, such as N-acetylneuraminic acid (NeuAc, or sialic acid) in group B streptococci, *Haemophilus* and *Neisseria* spp. or CDP-ribitol in *Haemophilus influenzae* types a and b to nascent cell wall polysaccharides or capsular polysaccharides, one of the major virulence factors of these microorganisms, is initiated by activation of these components (248-251). The corresponding synthases catalyse the formation of the intermediate compounds with elevated energy levels in the presence of CTP as energy source. The increasing level of CTP synthase (Rv1669) in activated macrophages may support the upregulation of cell wall synthesis in *M. tuberculosis* under these conditions. CTP synthase is also highly induced in *M. marinum* from granulomas (252). The importance of the CTP synthase was demonstrated by the growth retardation of *Lactococcus lactis* spp. mutants lacking the CTP synthase gene and by the existence of drugs such as acivicin, 3-deazauridine (3-DU), and cyclopentenyl cytosine (CPEC) inhibiting this enzyme (253-257).

_____

Considering this fact, the high expression of CTP synthase of *M. tuberculosis* several days after activated macrophage infection could represent one of the ways employed by this pathogen to vanquish the severe damaging impacts inside activated macrophages on its effort to resuscitate and contingently to proliferate.

4.2.4 Biosynthesis of amino acids

In resting macrophages *M. tuberculosis* resides and multiplies in phagosomes requiring synthesis of a large number of macromolecules. The induction of some genes involved in amino acid biosynthesis suggests that the phagosomal environment is restricted in amino acids. Nutrient deprivation may also contribute to the host defense through restriction of the growth of tubercle bacilli. Auxotrophic mutants of mycobacterial strains carrying disruptions of genes involved in amino acid biosynthesis exhibit reduced virulence. These genes were also induced in *M. tuberculosis* grown *in vitro* under iron depletion suggesting the existence of a co-regulation of iron and amino acid deprivation

Moreover, it has been shown that amino acids by themselves can modify the expression of target genes (258-261). Amino acids can also regulate gene expression at the level of transcription, messenger RNA stability and translation. The notion that intraphagosomal mycobacteria encounter an amino acid deprived condition is further supported by the finding that Rv0346c thought to be involved in transport of L-asparagine across the membrane was upregulated during infection of macrophages. Comparison of the deduced amino acid sequence with sequence databases showed its significant homology with a family of basic and aromatic amino acid permeases (Rv0346c has also been designated *aroP2*). This indicates an additional function of this gene for transport of aromatic amino acids tyrosine, phenylalanine and tryptophan. The concentration of L-tryptophan has been suggested to influence the intracellular survival of *Bordetella pertussis* and the IFN-γ mediated induction of tryptophan degrading enzymes may be an additional defense mechanism of this cytokine against *B. pertussis* (262). The involvement of asparagine synthase (Rv2201) and permease (Rv0346c) in

_____

the response to amino acid limitation may also be supported by the finding that asparigine can serve as the only nitrogen source in minimal medium for mycobacteria (24).

4.2.5 Pyrophosphatase

In the course of biosynthesis of macromolecules such as DNA, RNA, protein and polysaccharide as well as tRNA charging, pyrophosphate is usually produced in the cells as a by-product. The pyrophosphate is released from ATP or GTP during phosphorylation. Pyrophosphatases catalyse the reversible transfer of the phosphoryl group from pyrophosphate to water, causing the dissociation of one molecule pyrophosphate into two molecules inorganic phosphate thereby releasing energy. In this context, the hydrolysis of the pyrophosphate provides additional energy for biosynthesis. The *ppa* gene is ubiquitous and the sequence is relatively conserved among different organisms. The genome sequence of *M. tuberculosis* H37Rv exhibits a 486-bp long ORF (Rv3628) with 44% sequence identity to the *Legionella pneumophila* Ppase (263). It has been reported that Ppase is crucial for the growth in *E. coli* and yeast (264;265). While the expression of *ppa* is constitutive in *E. coli*, it has been observed that the expression of this gene is elevated in *L. pneumophila* growing in resting macrophages (266). However, the expression of the homologous gene in *M. tuberculosis* inside resting murine macrophages is not increased. Some stress stimuli such as exposure to H_2O_2 (oxidative stress), high NaCl concentrations (osmotic shock), deprivation of iron by 2,2'-dipridyl or a reduction in pH (acid shock) also failed to induce *ppa* of *M. tuberculosis* (263).

However, *ppa* transcription levels were significantly increased during 3 day infection of activated macrophages in this study. This re-emphasises the notion that after a temporary stationary state in activated macrophages *M. tuberculosis* resumed growth.

4.2.6 Lipid metabolism

It has been postulated that *M. tuberculosis* nourishes from lipids as a major carbon source inside caseous granulomas (69). The amount of isocitrate

lyase transcript (Rv0467) was observed to be only slightly increased during infection of resting macrophages, whilst activation of macrophages drove the infecting *M. tuberculosis* to produce more isocitrate lyase transcript. The elevated expression of isocitrate lyase during infection was reported for several pathogenic microorganisms, including *M. tuberculosis* (69), *Candida albicans* and the rice blast fungus *Magnaporthe grisea* (267;268). Moreover, mutant strain of *M. tuberculosis* lacking *icl* gene is attenuated in IFN-γ activated macrophages (69). The expression of this gene is especially associated with the utilisation of fatty acids as major carbon source which requires the activation of glyoxylate cycle to bypass the tricarboxylic acid (TCA) cycle resulting in the net incorporation of carbon during microbial growth. The glyoxylate shunt requires the degradation of fatty acids into highly activated acetyl-CoA to enter the pathway. It has been reported that *M. tuberculosis* grown in culture with acetate as the only carbon source also induces the isocitrate lyase (269). It is still not clear whether acetate is also available to *M. tuberculosis* as carbon source in macrophages. Alternatively, Rv0409 and Rv0467 may belong to the same regulon which is stimulated by the presence of fatty acids as a major carbon source.

4.2.7 DNA damage repair

Activation of macrophages with IFN-γ induces the expression of iNOS (inducable nitric oxide synthase) resulting in the generation of the microbiocidal compound nitric oxide (61;64). *bpoB* is thought to be involved in detoxifying reactions. BpoB requires no haem group for its catalytic activity. The choice of this non-haem protein by *M. tuberculosis* in activated macrophages for detoxification may reflect the iron deprived situation in this environment. Recent finding showed that *sodC* is essential for survival of *M. tuberculosis* in macrophages (270).

4.2.8 Resuscitation-promoting factor (rpf)

Rpf (resuscitation-promoting factor), which was isolated for the first time in the supernatant of actively growing cultures of *Micrococcus luteus*, has autocrine and paracrine signalling functions and is required for the resuscitation of

_____

dormant cells (271). This protein is able to stimulate bacterial growth at picomolar concentrations, the property which is very similar to that of cytokines in eukaryotic organisms. Genes similar to rpf are widely distributed among Gram-positive bacteria with high G+C content including streptomycetes, corynebacteria and mycobacteria (272).

Considering the characters of Rpf known so far, the elevated production of RpfE (Rv2450) in this study indicates that this protein is required to resuscitate the mycobacterial cells in activated macrophages. It has been reported that the cells of *M. tuberculosis* isolated from murine macrophages several days after infection exhibited decreased viability when cultured *in vitro* and that Rpf was able to augment the number of viable cells (273). The hostile milieu in macrophages may count for the decreased viability and the need for Rpf to rescue growth. Although, upon activation of macrophages the hostility of intracellular environment increases, mycobacteria were able to resume growth after several days of dormant state in activated macrophages, which is accompanied by the increased expression of *rpfE*. The fact that *rpfE* was strongly induced 3 days after activated macrophage infection mimics the resuscitation effect of Rpf added to *in vitro* cultures of *ex vivo* isolated mycobacteria. The expression of other Rpf proteins in *M. tuberculosis* was relatively constant in three other growth conditions.

4.2.9 Ion and drug transport regulation

In addition to iron, *M. tuberculosis* requires mono- and divalent metal cations such as K^+, Na^+, Ca^{2+} and Mg^{2+} for growth (274). *ceoB* (Rv2691) encodes protein which shows activity of the potassium uptake regulatory protein TrkA. Generally, regulation of the potassium content of the cytoplasm is the primary response of bacterial cells to osmotic stress. In *E. coli* and *Clostridium acetobutylicum* the potassium transporter belonging to the uptake system Kdp is induced by potassium limitation and changes in medium osmolarity (275-277). The disruption of major genes for K^+ transporters in *B. subtilis* caused growth defect in high osmolarity (278). Recently, it has been reported that the expression of Rv3237c encoding a possible potassium channel protein was strongly stimulated during *M. tuberculosis* infection of human macrophages

(279). This fact and the observation in this study may implicate the low potassium concentration and/or the existence of osmotic shock conditions in phagosomes. The induction of another transport protein encoded by Rv3270 may also be related to restricted concentration of unknown metal ion, whereas the induction of Rv1458c may play a role in pumping toxic substances out of the cells. The three genes showed similar expression levels in activated macrophages and were downregulated in iron deprived *M. tuberculosis* culture. Limited concentration of some ions such as Mg^{2+} and inorganic phosphate as indicated by high expression of genes involved in transport system for both ions has also been suggested in *Salmonella*-containing vacuole in human macrophages (280).

In the study on the genetic basis of isoniazid (INH) resistance, it was found that *ceoA, B* and *C* from *M. tuberculosis* which was transformed to *E. coli* conferred INH resistance in otherwise INH sensitive *E. coli*, probably by sequestering or detoxifying INH (281). Moreover, K^+ transport system has been shown to be an indirect target of some novel drugs with antimycobacterial properties such as B4128 (tetramethylpiperidyl (TMP)-substituted phenazine) (282), riminophenazines (283), B669 and clofazimine (284).

4.2.10 Drug activation

Some antitubercular drugs require activation by pathogen derived enzymes before they can efficiently interfere with cellular metabolism leading to growth retardation or killing of tubercle bacilli. For instance, the first line antimycobacterial drug isoniazid (INH) requires the mycobacterial *katG* gene which codes for a catalase-peroxidase enzyme to carry out conversion into its bioactive form (285). Moreover, the expression of *katG* is required for growth and persistence of *M. tuberculosis* in animal models (286). A series of compounds containing a nitroimidazopyran nucleus has been reported recently to exhibit antitubercular activity against both replicating and static *M. tuberculosis* (287). These compounds require activation through a mechanism that depends on F420 (the 5-deazaflavin coenzyme). F420 is found to serve as electron acceptor of glucose-6-phosphate dehydrogenase in *M. smegmatis*

(288). In addition, there is a panel of oxidoreductases which utilise F420 as their cofactor (289). The increased expression in resting macrophages of *fbiB* (Rv3262) encoding protein involved in the biosynthesis of F420 was observed in this study. This gene was further induced in activated macrophages. The fact that the production of F420 is enhanced in macrophages may support the application of nitroimidazopyrans as antitubercular drug.

4.2.11 Regulatory proteins

Genes coding for regulatory proteins play vital roles in metabolic processes, including transcription, cell development and interactions with host cells. It was observed in this study that the changes in expression profile during macrophage infection were accompanied by the stimulation of a panel of genes encoding regulatory proteins.

Most of the bacterial eukaryote-like protein kinases that have been characterised so far have been shown to be involved in the regulation of different developmental states of bacteria. For instance, the Ser/Thr kinase *pkn1* of *Myxococcus xanthus* is expressed exclusively during sporulation and its inactivation inhibits spore formation in this bacterium (290). The cell wall thickening of mycobacterial cells growing in prolonged stationary phase has been suggested as a condition analogous to sporulation (291). The expression of *M. tuberculosis* Ser/Thr kinase *pknB* (Rv0014c) which belongs to the protein serine/threonine kinase (PSTK) family has been detected *in vitro* and *in vivo* (292). It has been proposed that *pknB* plays a role during infection. DNA array results in this study support this notion. Another member of PSTK family, Ser/Thr kinase *pknL* (Rv2176), and a transcriptional regulatory protein belonging to LuxR-family, Rv0195, were specifically induced during infection of activated macrophages. Little is known about the function of these genes. The protein kinase inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) was found to inhibit the activity of *pknB* and consequently the growth of *M. tuberculosis* (293).

Rv0758 (*phorR*) is a sensor part of a two component regulatory system PhoP/PhoR. In *B. subtilis* the induction of this regulon is stimulated by phosphate starvation (294). The study of this regulon in *M. tuberculosis* has

just started. The *phoP* mutant strain of *M. tuberculosis* shows impaired growth but is still able to survive when cultured in murine bone marrow-derived macrophages (295). The finding from this study that the expression of Rv0758 was augmented in macrophages may implicate the condition of phosphate deprivation and might be consistent with the induction of pyrophosphatase gene *ppa*. The hydrolysis of pyrophosphate will be able to provide the cells with more inorganic phosphate. The condition of phosphate limitation was also suggested in *Salmonella*-containing vacuoles during infection of this pathogen in macrophages. Since inorganic phosphate availability is closely related to the energy level in the cells, the restriction of inorganic phosphate in phagosomes may constitute another host strategy to inhibit pathogen growth. The induction of Rv0903c (*prrA*) has also been observed previously during the initial state of macrophage infection, which is confirmed by the results in this study (296). Morever, the *prrA* mutant showed a defect in intracellular growth during the early phase of infection.

4.2.12 Genes with unknown functions

The complete genome sequence of *M. tuberculosis* H37Rv revealed a large proportion of open reading frames with unknown functions. Many genes which were strongly induced in macrophages according to the DNA array data in this study have unknown functions but can be claimed to be involved in virulence, intracellular survival and host cell interactions. Unravelling the function of these genes will deliver more exact pattern of host-pathogen interactions and survival strategy of *M. tuberculosis* within the host macrophages. Moreover, the products of these genes will offer novel candidates for drug targets.

4.2.13 Gene cluster

Many genes in the *M. tuberculosis* genome have been suggested to be organised in operons. Some of these putative operons have been identified and confirmed by various studies. These include operons for mammalian cell-entry (mce) genes, the operon involved in mycolic acid synthesis (Rv2243-Rv2247) (297), the operon consisting of four genes (*aroCKBQ*) of shikimate pathway (298), DNA gyrase operon with *gyrA* and *gyrB* (299), the operon

related to ethambutol resistance with *embA, embB,* and *embC*, the operon encompassing Rv3803c-Rv3805c of secreted immunogenic proteins with mostly unknown functions (300), the DNA damage inducable operon of *ruvA, ruvB,* and *ruvC* (301), the operon of two-component response regulator pair Rv3133c/Rv3132c (302), the operon of *esat-6* and *lhp* (303) and many others. To confirm the existence of an operon, molecular methods such Northern blot and RT-PCR are usually applied which reveal the presence of transcripts with the length corresponding to the whole operon due to polycistronic nature of the genes in an operon (304-307). The expression of an operon is regulated by one or several common promoters upstream of the ORFs belonging to the same operon. Based on these characteristics, it can be expected that the genes in the same operon will show similar expression patterns under different growth conditions. This proposition can be used to predict an operon. Focusing on the genes that are up- and down regulated *in vitro* versus *in vivo* in this study, the expression pattern of the genes, which are found next to each other in *M. tuberculosis* genome were analysed. DNA array results in this study revealed groups of genes that showed similar expression pattern (see website). In these groups some genes have unknown function whilst the other are already assigned a putative function. It can be proposed that these groups of genes may represent operons. However, molecular studies are required to confirm their operon organisation.

4.3 Gene expression profile analysis of *M. tuberculosis* infecting human lung

Increasing incidences of MDR strains of *M. tuberculosis* in several parts of Russia render surgical lung resection an unavoidable measure of tuberculosis control (308;309). For determining the gene expression profiles of the pathogen in different infection sites on the lung, surgical resection of highly affected pulmonary tissue from MDR tuberculosis patients was used in this study.

The high similarity of the gene expression profiles of *M. tuberculosis* in pericavity and distant lung may implicate similar environmental conditions

encountered and most likely also similar host responses faced by *M. tuberculosis* in both sites of infection.

The fact that the transcriptome of *in vitro* grown *M. tuberculosis* is closer to that of granuloma than pericavity and distant lung may imply that the tubercle bacilli in the center of caseous granuloma grow more actively than in other sites of infection which is consistent with the condition of active disease.

4.3.1 Fatty acid metabolism

It has been proposed that fatty acids may be the major carbon source for *M. tuberculosis* during persistence in the host. The use of fatty acids requires β-oxidation cycle which oxidises fatty acids to the final activated compound acetyl-CoA (69). DNA array results in this study strongly supports the use of fatty acids as carbon and energy source. Furthermore, it has been suggested that the produced acetyl-CoA is then metabolised via glyoxylate cycle, the preposition which is supported by the induction of *icl* (isocitrate lyase) during infection of activated macrophages and the requirement of *icl* for *M. tuberculosis* persistence in immunocompetent mice (69). However, DNA array results in this study did not identify induction of *icl* and two other genes (*AceAa* and *AceAb*) whose products also function as isocitrate lyase, in all three sites of infection in human lung with chronic tuberculosis. The induction of *icl* was proposed to be directly linked to the activation of macrophages harbouring tubercle bacilli with IFN-γ (69). In the lung of IFN-$\gamma^{-/-}$ mice *M. tuberculosis* lacking *icl* gene can proliferate progressively, implying the presence of another possible pathway for utilisation of fatty acids as major carbon source. It has been described in section 4.2.6 that gene expression profiling of *M. tuberculosis* H37Rv infecting activated murine bone marrow-derived macrophages using the same DNA arrays in this study confirmed the induction of *icl* suggesting that the DNA arrays used in this study were able to detect the transcript of *icl* if it was present in the investigated RNA pool. *citA* (Rv0889c, citrate synthase II) and succinate dehydrogenase subunits (Rv0248c, Rv0249c) are directly involved in tricarboxylic acid (TCA) cycle. However, citrate synthase is also involved in glyoxylate cycle and succinate dehydrogenase can oxidise succinate produced in this cycle to fumarate.

4.3.2 Energy metabolism

4.3.2.1 Anaerobic respiration

Mycobacterial persistence *in vivo* is thought to depend on anaerobic metabolism, an apparent paradox since all mycobacteria are obligate aerobes. *M. tuberculosis* and *M. bovis* are thought to face an anaerobic environment during infection, notably inside abscesses and granulomas. In such condition bacteria usually utilise organic or inorganic compounds as the terminal electron acceptor in an effort to regenerate the reducing agents NADH and $FADH_2$ and thereby to gain energy rich molecule ATP (310-312). *narG* (Rv1161) and *narX* (Rv1736c) encode nitrate reductase which allows *M. tuberculosis* to use nitrate instead of oxygen as terminal electron acceptor.

The genome of *M. tuberculosis* contains genes whose products are homologous to an anaerobic nitrate reductase (NarGHJI), an enzyme allowing nitrate respiration when oxygen is absent. It has been shown that the *narGHJI* cluster of *M. tuberculosis* is functional as it conferred anaerobic nitrate reductase activity to *Mycobacterium smegmatis*. A *narG* mutant of *M. bovis* BCG was generated by targeted gene deletion (313). The mutant lacked the ability to reduce nitrate under anaerobic conditions. Both mutant and *M. bovis* BCG wild type grew equally well under aerobic conditions *in vitro*. Immunodeficient mice (SCID) infected with *M. bovis* BCG wild type revealed large granulomas and succumbed after 80 days. In contrast, mice infected with the mutant had smaller granulomas containing fewer bacteria and showed no signs of clinical disease after more than 200 days. Thus, it seems that nitrate respiration contributes significantly to virulence of *M. bovis* BCG in immunodeficient SCID mice.

In another infection study it was also shown that anaerobic nitrate reduction is essential for metabolism of *M. bovis* BCG in immunocompetent but not immunodeficient mice. *M. bovis* BCG lacking anaerobic nitrate reductase (NarGHJI), an enzyme essential for nitrate respiration, failed to persist in the lungs, liver and kidneys of immunocompetent (BALB/c) mice. In immunodeficient (SCID) mice, however, bacilli caused chronic infection despite disruption of *narG*, even if growth of the mutant was severely impaired in lungs, liver, and kidneys. The role of anaerobic nitrate reduction in

_____

mycobacterial disease is therefore tissue specific (314). All these observations suggest close association of anaerobic nitrate reduction with the pathogenesis of *M. bovis* BCG. The nitrate reductase has also been implicated as an interesting target for the vaccine development.

Periplasmic nitrate reductase (NapABC enzyme) has been characterised in a variety of proteobacteria, especially *Paracoccus pantotrophus* (315;316). Like fumarate reductase, it can function in anaerobic respiration but does not constitute a site for generating proton motive force.

The high transcription level of Rv1552 encoding flavosubunit of fumarate reductase, an enzyme complex which enables bacteria to utilise fumarate as terminal electron acceptor in anaerobic respiration indicates that either the intracellular or/and extracellular environment of tubercle bacilli in pericavity and distant lung is rich in fumarate and structurally and functionally related compounds or the oxygen tension in these sites of infection is so low that *M. tuberculosis* is forced to use fumarate for energy metabolism in addition to nitrate to generate the proper amount of energy for its survival and persistence. It has been found that, under anaerobic growth conditions in which fumarate is utilised as the terminal electron acceptor, succinate reductase would function to support anaerobic growth of *E. coli* (317).

Fumarate reductase (FRD) catalyses reduction of fumarate to succinate in the Krebs cycle and is also a key enzyme in anaerobic respiration with fumarate as the terminal electron acceptor (fumarate respiration) for many facultative intracellular bacteria. It couples the reduction of fumarate to succinate to the oxidation of quinol to quinone, in a reaction opposite to that catalysed by the related complex II of the respiratory chain (succinate dehydrogenase) (318;319). The expression of FRD in *Klebsiella pneumoniae* and *Escherichia coli* is induced by anaerobic growth of bacteria, which is regulated by the transcription factor Fnr (fumarate nitrate reductase regulator), which globally regulates gene expression in response to oxygen deprivation (320).

Fumarate reductase and succinate dehydrogenase transcription levels are increased in *Mycobacterium phlei* grown under low oxygen tension (321). This is consistent with the results in this study in which both enzymes were upregulated in pericavity and distant lung implying low oxygen tension in these sites of infection. Thus, upregulation of these enzymes in *M.*

_____

tuberculosis in pericavity and distant lung indicates that these enzymes function together in energy metabolism.

FRD plays an important role in the energy metabolism of *Helicobacter pylori* and is essential for its colonisation in the acidic stomach as demonstrated in the mouse model of infection. This enzyme appears to be constitutively expressed in *H. pylori* under microaerobic conditions and is not essential for its survival *in vitro*. The menaquinone and fumarate reduction activity is also observed during anaerobic fumarate respiration of the rumen bacterium *Wolinella succinogenes* as well as of *Sulfurospirillum deleyianum, Campylobacter fetus, Campylobacter rectus*, *Dehalospirillum multivorans,* and *Campylobacter jejuni* (322). Cell growth of *Photobacterium leiognathi*, *Vibrio fischeri,* and *Vibrio harveyi* under microaerophilic growth conditions resulted in elevated levels of nitrate reductase and fumarate reductase activity in each strain, whereas growth in the presence of the respective substrate for each enzyme further elevated enzyme activity (323).

Three FRD inhibitors usually used to cure helminthic infection in animals and humans, morantel, oxantel and thiabendazole, have both inhibitory and bacteriocidal effects on *H. pylori* grown *in vitro*. In addition, metronidazole and nizatidine are able to inhibit the activity of fumarate reductase of *H. pylori* in a dose-dependent way. Based on these evidences, it has been suggested that FRD may be a promising novel chemotherapeutic target in *H. pylori* as well as in several pathogenic and epsilon-proteobacteria infection. Futhermore, FrdA (fumarate reductase A) is strongly immunogenic in the sera from *H. pylori*-positive patients, suggesting its potential as a candidate for the development of an anti-*H. pylori* vaccine.

4.3.2.2 Aerobic respiration

Rv1623c has been reported to be especially induced at low aeration (324). In addition, the expression of *glbO* was also stimulated *in vivo*. GlbO is membrane-associated protein of *M. tuberculosis* and enhances its aerobic respiration (325). Expression of *glbO* in *M. bovis* is observed throughout its aerobic growth (326). HbO, the gene product of *glbO*, interacts with the component of aerobic electron transport chain and may facilitate oxygen

transfer during aerobic metabolism of *M. tuberculosis*. Membrane-associated HbO has been suggested to play an essential role in sequestering oxygen and in facilitating its availability to internalised *M. tuberculosis* under the hypoxic conditions of its intracellular milieu.

The concurrent expression of the genes involved in aerobic and anaerobic metabolisms also suggests that the environment in the infection sites in human lung is microaerophilic rather than anaerobic with granuloma having higher oxygen tension than pericavity and distant lung. This observation is not really surprising since granuloma has more direct contact to alveoli. Moreover, lung is rich in oxygen and the small size of oxygen molecules allow them to penetrate to some extent the barriers in lung tissues making total exclusion of oxygen in most part of this respiratory organ unlikely. To boost the electron transfer in the respiratory chain some NADH dehydrogenases were also induced. In addition, the induction *in vivo* of a panel of genes which are known to be highly expressed during *in vitro* growth of *M. tuberculosis* under hypoxia were also observed (see website).

4.3.3 Nutrient (proteins, amino acids, iron and vitamines) metabolism

The induction of genes encoding protein or peptide hydrolysing enzymes suggests that *M. tuberculosis* matches its amino acid requirements *in vivo* by hydrolysing proteins and peptides present in its surrounding in addition to biosynthesis of some amino acids as can be inferred from the strong activation of their biosynthesis pathways. Some genes involved in siderophore mycobactin synthesis were also upregulated implying the condition of iron deprivation in the sites of infection. The extent of oxygen and nutrient limitation was similar in the three sites of infection in human lung with tubercle bacilli in pericavity and distant lung experiencing more severe deprivation than those in granuloma.

4.3.3.1 Riboflavin

One interesting gene that was induced *in vivo* in this study is *ribA2* which is involved in the biosynthesis of riboflavin. This gene was especially induced in

pericavity and distant lung. In the *M. tuberculosis* genome there are two *ribA* genes designated *ribA1* and *ribA2. ribA1* was also induced to a lower extent. An auxotrophic mutant of *Rhodococcus equi* lacking the ability to synthesise riboflavin cannot grow in minimal medium in the absence of riboflavin supplementation (327). This microorganism is a facultative intracellular opportunistic pathogen in immunocompromised individuals and a major cause of pneumonia in young horses. Murine infection studies revealed that the riboflavin-requiring mutant is attenuated because it is unable to replicate *in vivo* implicating the role of riboflavin in virulence of *R. equi*. An effective live attenuated vaccine would be extremely useful in the prevention of *R. equi* disease in horses and the use of such mutant as live vaccine has been proposed. *Actinobacillus pleuropneumoniae* riboflavin auxotrophs are avirulent and fail to multiply *in vivo*, indicating a role of riboflavin in its virulence. *A. pleuropneumoniae* is a gram negative pleiomorphic rod that is the causative agent of a severe, highly infectious and often fatal pleuropneumonia in swine. Immunisation with live avirulent riboflavin auxotrophs can elicit significant protective immunity against experimental challenge with both homologous and heterologous virulent serotypes of *A. pleuropneumoniae* (328;329).

Various strains of *H. pylori* lyse erythrocytes from sheep, horse, and human when grown on blood agar (330). This property is attributed to the expression of *ribA* gene encoding the enzyme GTP-cyclohydrolase II that catalyses the initial step in the synthesis of riboflavin. The production of streptolysin S (SLS) by *Streptococcus pyogenes* is also genetically linked to the biosynthetic pathway of riboflavin (331). It is tempting to investigate whether *M. tuberculosis ribA* genes contribute to the lysis of alveolar macrophage.

4.3.4 Cell wall and cell membrane synthesis

DNA array results in this study suggested an active production of ATP as an energy source inside *M. tuberculosis* cells *in vivo*. The questions arise concerning the utilisation of this energy. As already described in section 4.2.3, microaerobically and anaerobically cultured *M. tuberculosis* developed a conspicuously thickened cell wall outer layer. The thick cell wall may provide

the tubercle bacilli with more robust fortification against host defense mechanisms and the transport proteins may pump toxic substances out of the cells. In addition, the cell wall components of *M. tuberculosis* have been shown to modulate host immune responses (16;17). The enhanced production of phosphatidylinositol, which was marked by the transcription induction of inositol monophosphatase gene *suhB* (Rv2701c), observed *in vivo* in this study may closely correlate with the active granuloma formation. Recent reports suggested that phosphatidylinositolmannosides (PIM) are responsible for the recruitment of murine natural killer (NK) T cells which play active roles in granuloma formation. The biosynthesis of PIM was found in this study to peak in pericavity. This underscores the roles of pericavity as the center of granuloma formation by focusing active interactions between immune cells and tubercle bacilli.

4.3.5 Drug resistance

The active synthesis of cell wall components could also contribute to drug resistance observed for instance in vancomycin-intermediate resistant *Staphylococcus aureus* strains (VISA) which produce a markedly thickened cell wall (332). It has been observed that some genes which are associated with cell wall synthesis are induced upon treatment of *M. tuberculosis* with certain drugs. The tuberculosis patients who served as donors of RNA samples in DNA array experiments described here are intensively treated with a series of antitubercular drugs. The drug treatment may influence the gene expression profiles observed in this study. In fact, DNA array results were able to confirm the induction of genes reported recently to be highly expressed upon treatment of *M. tuberculosis* with the drugs applied for patient treatment (see website). In *E. coli* an operon termed *marRAB* (Multiple Antibiotic Resistance RAB) confers multidrug resistance via activation of membrane-associated efflux system (333-335). It has been hypothesised that the relatively high frequency of multidrug resistance in mycobacteria and the suggested relationship of inadequate treatment to the emergence of resistance fit with the selection of *E. coli* Mar mutants (336). Furthermore, the expression of *E. coli marA* gene in *M. smegmatis* caused this microorganism

resistant to various drugs, suggesting the existence of a *mar*-like regulatory drug resistance response in *M. smegmatis*. On the other hand, lacking of MarR acitivity led to the constitutive expression of *marA* rendering permanent multidrug resistance. The differential regulation of some *marR* genes *in vivo* may have great contribution to the multidrug resistance observed in the strains analysed in this study. Since *marRAB* operon regulates many other genes, further study of this operon and the corresponding regulon may help to understand the emergence of multidrug resistance in mycobacteria and to find better solution to handle the world health problem arising from it. In addition to *marRAB* system, a high expression of some genes whose products are involved in multidrug resistance via active transport of drugs across the membrane was also observed (see website).

4.3.6 Tetronasin transport system

Tetronasin is a divalent antiporter that binds preferentially with Ca^{2+} or Mg^{2+}. Ruminal bacteria (e.g. *Megasphaera elsdenii*, *Selenomonas ruminantium*, *Streptococcus bovis*, *Lactobacillus* sp., and *Bacteroides ruminicola*), protozoa, anaerobic fungi (e.g. *Neocallimastix* sp.) and bacteria with a gram-positive ultrastructure are generally sensitive to the ionophore tetronasin (formerly ICI 139603) and unable to adapt to grow in its presence (337-339). Tetronasin-transport integral membrane proteins in *M. tuberculosis* may also be able to export other compounds with similar molecular size and physiological characters to those of tetronasin. Considering the high growth inhibiting effect of tetronasin on anaerobic microorganisms, tetronasin-transport integral membrane proteins may especially export compounds with toxic affect on *M. tuberculosis* during growth in microaerophilic environments which are produced either as by-products of *M. tuberculosis* metabolisms or generated by host immune cells as part of host defense mechanisms. The expression enhancement of Rv1217c and Rv1218c *in vivo* may indicate that such compounds are present in high concentration in the site of infections. These transport proteins may also be able to export some drugs and other antibiotics and thus contribute to multidrug resistance *in vivo*.

4.3.7 DNA repair, radical neutralisation, and detoxification

Intracellular bacteria are exposed to various host defense mechanisms with bacteriocidal or bacteriostatic effects such as reactive nitrogen intermediate (RNI), reactive oxygen intermediate (ROI), and toxic substances. One interesting detoxification system stimulated *in vivo* according to DNA array results in this study is the glyoxalase system which is involved in the detoxification of methylglyoxal and other alpha-oxoaldehydes. These substances exhibit very high toxicity and are able to induce rapid cell death. Physiological substrates of the glyoxalase system in intracellular *M. tuberculosis* are among others glyoxal which is formed from lipid peroxidation emerging mostly from the action of reactive radicals and methylglyoxal (MG) which may have several possible sources. The production of MG by tubercle bacilli can be attributed to an important role in adaptation to conditions of nutrient imbalance as also observed in some other microorganisms (340-342). Alternatively, MG may be present in great amount in extracellular environment of infecting *M. tuberculosis.* The granuloma formation is accompanied by apoptosis of immune cells. It has been found that tumor necrosis factor (TNF) induces a substantial increase in intracellular levels of MG which plays an essential role in apoptosis (343-345). The lysis of immune cells during apoptosis may thus release high amount of MG to the sites of infection. Microarray data of *Salmonella enterica* infection of macrophages have also suggested the accumulation of methylglyoxal in *Salmonella*-containing vacuoles.

In addition, DNA array result showed the enhanced synthesis *in vivo* of a lipophilic antioxidant, lipoate. Lipoate, or its reduced form, dihydrolipoate, reacts with reactive oxygen species such as superoxide radicals, hydroxyl radicals, hypochlorous acid, peroxyl radicals, and singlet oxygen (346;347). Although lipoic acid is an essential cofactor for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (348), the expression of these enzymes were downregulated *in vivo*, implying another role for lipoic acid rather then cofactor of these enzymes. Moreover, recent study found that lipoic acid is able to inhibit nitric oxide (NO) production in RAW 264.7 macrophages activated by IFN-γ and LPS (349). All these facts underscore the importance of glyoxalase

_____

system and lipoate biosynthesis pathway for survival of *M. tuberculosis in vivo* and thus as novel target for antitubercular drug development.

4.3.8 Urease

DNA array data in this study revealed that some genes which are involved in urease biosynthesis were upregulated *in vivo*.

Urease is a multisubunit metalloenzyme which catalyses the hydrolysis of urea to yield ammonia and carbamate. The latter compound spontaneously decomposes to yield another molecule of ammonia and carbonic acid. Under physiological conditions, this reaction can result in an increase in pH. In many microorganisms it has been found that the urease expression is regulated by a urease regulator protein. Urease activity is observed in many mycobacterial strains (350;351). Urease is identified as virulence factor in a series of microorganisms such as *Helicobacter pylori* (352), *Proteus mirabilis* (353), *Providencia stuartii* (354), *Bordetella parapertussis* (355), *Bordetella bronchiseptica* (356), *Klebsiella aerogenes* (357), *Yersinia enterocolitica* (358), *Morganella morganii* (359), *Vibrio parahaemolyticus* (360), *Proteus vulgaris*, and *Proteus penneri* (361) and in many fungal pathogens such as *Cryptococcus neoformans* (Cn) var. gattii and *Coccidioides immitis* (362).

It has been postulated that the hydrolysis of urea and the resulting increase in pH allow *Helicobacter pylori* to survive within the gastric mucosa (363-365). Cytoplasmic ammonia generated by the action of urease has been proposed to be protonated by H^+ ions leaking in from the acidic medium and that the NH_4^+ formed is extruded from the cytoplasm via an unidentified transport system and may induce much of the tissue damage by this pathogen. *Yersinia enterocolitica* and *Morganella morganii* can survive very low pH (1–1.5) for long periods of time in the presence of urea. The urease of these bacteria is strongly activated in low-pH conditions, which has been attributed to their acid tolerance. In *Bordetella bronchiseptica* urease expression has been proposed to be involved in protection from phagolysosomal damage (366). The role of urease in maintaining alkaline microenvironment due to the release of ammonia and ammonium ions has also been proposed for *Coccidioides immitis* (367).

The urease of *Helicobacter pylori* serves as the major antigen and diagnostic marker for gastritis and peptic ulcer disease in humans (368). It has been found that ammonia is able to accelerate cytokine (TNF-α-induced apoptosis in gastric epithelial cells (369).

The *Actinobacillus pleuropneumoniae* urease mutant strain fails to persist in healthy pig lung tissue (370). The role of urease activity in this microorganism has been implicated to cause sufficient impairment of the local immune response as marked by improved persistence of the urease-positive *Actinobacillus pleuropneumoniae* strain. The subunit of urease in *Yersinia enterocolitica* is found to be an immunodominant antigen both for proliferative CD4$^+$ T cells and for synovial CD8$^+$ T-cell clones. Furthermore, the urease subunit was shown to be a target for the synovial T-cell response of patients with *Yersinia enterocolitica*-caused ReA (reactive arthritis).

Currently, urease inhibitors and urease vaccines are being developed for clinical use for the treatment of ulcer disease (371).

4.3.9 Influence of drug treatment on gene expression profiles of *M. tuberculosis*

Since the tuberculosis patients in this study are treated with antitubercular drugs, the influences of these drugs on the gene expression profiles of *M. tuberculosis in vivo* can not be excluded. The differential expression of some genes *in vivo* as compared to *in vitro* without drug addition may result from the drug influences. However, the presence of drugs in the growth environment of bacteria frequently elicits responses similar to those induced by other shock factors including host defense mechanisms. Therefore, it may be able to be expected that drug treatment affords relatively minor contribution to the overall transcriptomes of *M. tuberculosis in vivo*. If the drugs exert the same impacts on *M. tuberculosis* expression profile in all infection sites in the lung, then it can be assumed that the differential expressions of the genes observed between these infection sites result from factors other than drugs.

4.3.10 Gene with unknown functions

The *in vivo* upregulated genes with unknown functions may constitute novel pathways which are essential for the virulence, persistence and pathogenesis of *M. tuberculosis* and may serve as novel drug targets. The observations that certain genes of *M. tuberculosis* are expressed differentially in different sites of infection in human lung may contribute greatly to the strategy of novel drug development. The design of novel antitubercular drugs should be specifically directed to the target genes which are highly expressed in all sites of infection to obtain most effective clearance of the pathogen.

4.4 The development of RNA amplification system for mycobacteria

4.4.1 Comparison of hybridisation results with unamplified and amplified RNA

More recently, there has been increasing interest in examining clinical samples using DNA array technology. Such clinical samples include core needle or fine needle aspiration (FNA) biopsies, laser capture microdissection, breast ductal or bronchial lavage, or microendoscopy (145-147). In the field of infection biology, it is of utmost interest to elucidate and interpret the mechanistic roles of genes in the pathogenesis of infectious disease, to identify genes from pathogens that may be involved in pathogenicity. This will require the analysis of gene expression profile of the pathogens isolated directly from infected cells or tissues. In all these cases, the biological samples usually collected from patients deliver only limited amount of RNA for DNA array analysis. The most commonly used dual channel DNA array system employing glass slide as solid support for DNA array fabrication and fluorescent-labelled targets necessitates between 5 µg and 200 µg total RNA or between 200 ng and 2 µg mRNA (110;111). Another solid support for DNA array production is positively charged nylon membranes with radioactive-labelled targets allowing the use of much less sample RNA to obtain similar achievements to the glass slide-based DNA arrays. Frequently,

the amount of materials is so limited that the RNA amplification is inevitable to obtain DNA array data able to be evaluated properly.

The most commonly used method for linear amplification of RNA is based on T7 amplification system. In this system T7 promoter sequence for T7 RNA polymerase is introduced into the cDNA during the first strand cDNA synthesis in which a synthetic oligo consisting of oligo(dT) and T7 promoter sequence at its 5´-end anneals to the poly(A) tail of the mRNA and serves as primer in the first-strand cDNA synthesis (372). Since this method relies on the the polyadenylation of mRNA, it applies very well to eukaryotic mRNA. It has been reported that mRNA of some microrganisms, especially mycobacterial RNA are polyadenylated to a greater or lesser extent (373-375). However, it is anticipated that the polyadenylation in prokaryotic RNA is less extensive than in eukaryotic RNA. Recent study demonstrated that priming mycobacterial RNA with oligo(dT) in cDNA labelling reaction for DNA array experiments did not augment the DNA array achievement (376).

To obviate the need for RNA polyadenylation, RNA amplification system was developed in which the promoter sequence of T7 RNA polymerase is introduced into first-strand cDNA in polyadenylation-independent manner. It has been observed that Moloney murine leukemia virus reverse transcriptase (MMLV-RT) is able to add a few non-template nucleotides (mostly deoxycytidine) to the 3´-end of a newly-synthesised cDNA strand upon reaching the 5´-end of the RNA template (377). When an oligonucleotide having oligo(G) sequence on its 3´-end (so called 'template switch' (TS) oligo) is present in the reverse transcription reaction, it anneals with the attached deoxycytidine stretch (378-380). Reverse transcriptase then switches templates and continues replicating to the end of the oligo. Thus the complementary TS-oligo sequence becomes attached to the 3´-end of the cDNA. All these reactions are possible due to the intrinsic activities of MMLV-RT: (i) terminal transferase (ii) template switching (iii) DNA polymerase. If reverse transcriptase pauses before the end of the template, the addition of deoxycytidine nucleotides is much less efficient than with full-length cDNA-RNA hybrids. These intrinsic characteristics of MMLV-RT were used in this study to introduce T7 promoter sequence to the 3´-end of the newly-synthesised first-strand cDNA. An oligo with the sequence 5´-

GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGGGGGG-3´ was designed. This oligo contains T7 promoter sequence and a short stretch of deoxyguanidine nucleotides at its 3´-end, so that it can serve as a switching template for MMLV-RT. The second-strand synthesis was initiated with RNase H treatment of the RNA-DNA hybrid to produce RNA fragments binding on the first-strand cDNA. DNA polymerase utilises these fragments as primers for the formation of the second strand. T4 ligase joins the second-strand cDNA together and T4 DNA polymerase fills the gap at the 3´-end of the second-strand cDNA.

To prime the first-strand cDNA formation, two different set of primers were tested in this study, mtGPDs (mycobacterial genome-directed primers) and random decamers. For priming of mycobacterial RNA with genome-directed primers, the 37 heptamers and octamers published recently were used (190). The whole process of RNA amplification is depicted and summarised below.

_____

━━━━━━GGG = T7 primer

━━━━━━━━━━= RNA

━━━━━━ = mtGDPs or random primers

━━━━━━━━━━ = 1. strand cDNA

━━━━━━━━━━ = 2. strand cDNA

━━━━━━━━ = amplified RNA
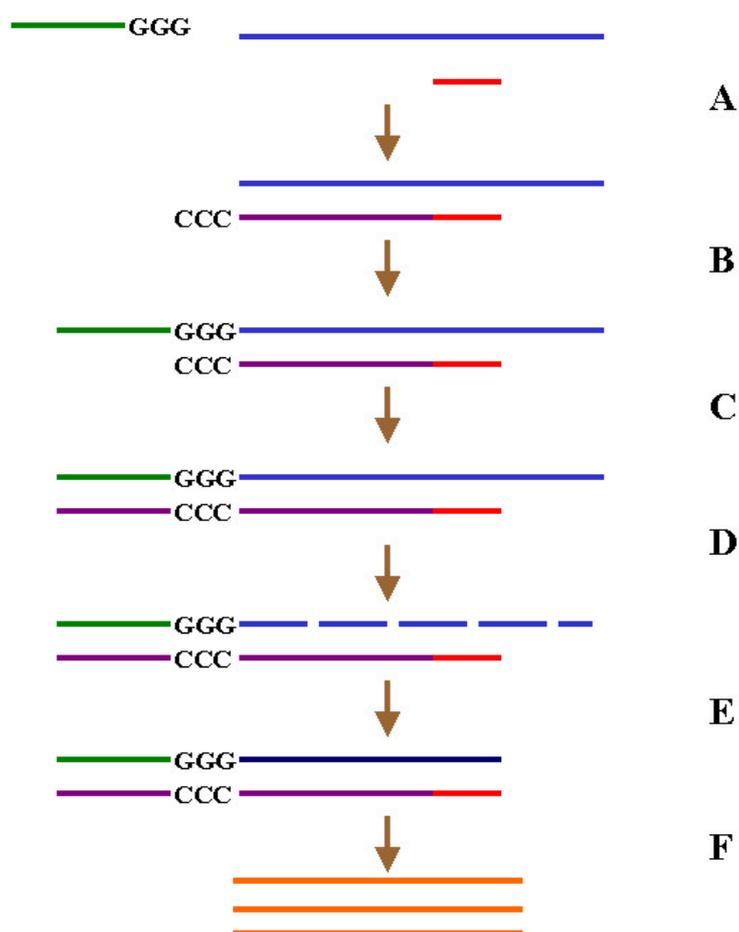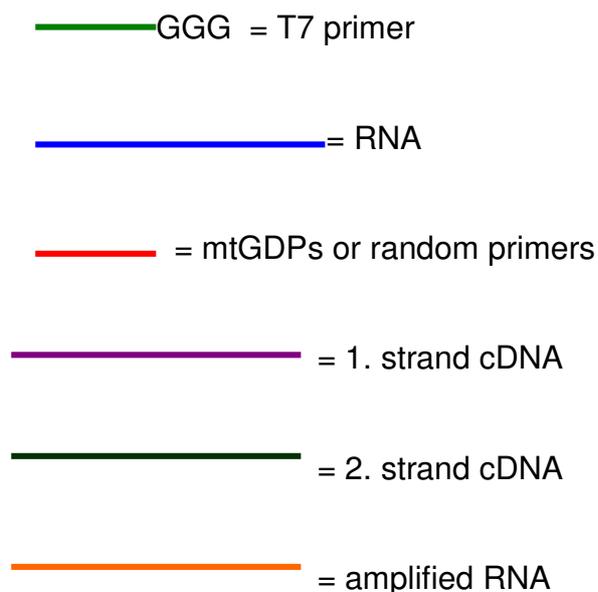
A = annealing of mtGDPs or random primers on RNA and reverse transcription of the RNA by MMLV-RT. At the 3´-end of the 1. strand cDNA several deoxycytidine nucleotides are added by MMLV-RT.

B = the stretch of deoxyguanidine nucleotides at the 3´-end of T7 primer anneals to the oligo(dC) of the 1. strand cDNA.

C = MMLV-RT switches the template and extends the 1. strand cDNA by copying the rest of the T7 primer sequence.

D = RNase H introduces nicks into template RNA.

E = DNA polymerase synthesises the 2. strand cDNA using the digested template RNA as primers. The synthesis of dscDNA is thus completed.

F = Synthesis of amplified RNA using T7 RNA polymerase and dscDNA as template.

Since the terminal transferase activity of MMLV-RT is most efficient when this enzyme reaches 5´-end of the reverse transcribed RNA, it is notable that the sparse use of primers to initiate cDNA synthesis may enhance the incorporation of T7 promoter sequence into the synthesised cDNA.

An optimum design of genome-directed primers requires knowledge of the whole genome sequence of scrutinised organisms (190). To surmount the obstacle of the lack of complete DNA sequence information, the usability of

_____

random octamers for priming RNA in the first-strand cDNA synthesis was also tested in this study.

The quantity and quality of amplified RNA were assessed with Bioanalyzer^{TM}. There are several points that deserve attention. When the RNA distributions before and after amplification were compared, the size distribution of the amplified RNA showed typical statistical Gaussian distribution which culminated at the position close to the 16S ribosomal RNA (rRNA) peak (slightly smaller than 16S rRNA). The two peaks of rRNA (16S and 23S) observed on the original total RNA samples disappear on the pool of the amplified RNA. This should not be interpreted that the ribosomal RNA does not undergo amplification. Either mtGDPs or random octamers bind on several positions along ribosomal RNA. This will result in the formation of ribosomal cDNA fragments of different sizes, just like those of mRNA. Based on this fact, the original total RNA amount was taken into account in calculating the fold of amplification. Up to 100 fold RNA amplification in a single amplification round could be attained.

The T7 promoter sequence for template switching primer was so designed that sense RNA will be produced during *in vitro* transcription of the dscDNA. Such a design consideration is particularly important when the amplified RNA will be applied for experiments using oligo arrays. Unlike DNA arrays with PCR products deposited as probes on the solid support, oligonucleotides are single-stranded and therefore can only represent sense or antisense RNA sequences. In most cases, oligonucleotides for DNA array fabrication represent the sense RNA sequences of the corresponding genes so that the DNA arrays will be able to hybridised with cDNA target generated from direct labelling procedure.

The usefulness of the RNA amplification method was assessed by performing DNA array experiments comparing the array data generated from unamplified and amplified RNA. Two different mycobacterial strains were used for this study, *M. tuberculosis* H37Rv and *M. bovis* BCG Copenhagen.

Since *in vitro* linear RNA amplification using T7 RNA polymerase can skew the original RNA presentation, the bias between transcript ratios was examined by comparing expression profiles of *in vitro* grown *M. tuberculosis* H37Rv and *M. bovis* BCG Copenhagen in late logarithmic phase using pure

and spiked unamplified and amplified total RNA, first within each strain and then between the two strains. In general, the use of mtGDPs resulted in higher CCs between unamplified and amplified RNA samples than that of random primers, especially for mycobacterial total RNA which was spiked with human total RNA. It can be inferred from the CCs that in general massive excess of contaminating RNA may cause false signal detection to a greater or lesser extent. However, the amplification of diluted RNA only slightly increased unspecific signals. The use of mtGDPs for the synthesis of dscDNA in the spiked mycobacterial RNA favoured the reverse transcription of mycobacterial RNA. The application of the same primers for the labelling reaction of cDNA targets from the amplified RNA provided further selection toward mycobacterial RNA. This may explain the relatively similar range of CCs for pure and spiked RNA when the array data sets of unamplified and amplified RNA were compared in each case.

The lower yield of RNA amplification using random primers can be explained by the assumption that random primers bind in average more frequently on each RNA species than mtGDPs. After all, the algorithm used to design mtGDPs was intended to examine minimal amount of primers to cover the whole genome of *M. tuberculosis* H37Rv. The more number of primers bind to a RNA species the shorter the resulted dscDNA and consequently the amplified RNA will be. In the subsequent labelling reaction even much shorter cDNA target will be obtained from the reverse transcription of short amplified RNA. The very short cDNA will either get lost during its purification after labelling reaction or not bind to the DNA arrays under the applied hybridisation condition.

4.4.2 Comparison of array data from total RNA of *M. tuberculosis* H37Rv and *M. bovis* BCG Copenhagen

Since many ORFs in the genome of *M. bovis* BCG Copenhagen have been known to be deleted as compared to the genome content of *M. tuberculosis* H37Rv (95), the normalised intensity ratios of these ORFs in both strains can serve as sound control to monitor any cross hybridisation, which in particular can emerge from amplification of ribosomal RNA and contaminating host total

_____

RNA. The increase in background signal can be best assessed by monitoring the ratios of normalised signal intensities for the deleted ORFs between *M. tuberculosis* H37Rv and *M. Bovis* BCG Copenhagen. Calculation of these ratios revealed that the amplification of pure and spiked mycobacterial RNA caused a shift of the ratios to insignificantly lower values. From the comparative studies performed to examine the quality of the amplified RNA using the method described here it can be concluded that the transcript levels in overall conditions tested were quite well preserved upon amplification. The use of mtGDPs for dscDNA synthesis and cDNA labelling reaction with the amplified RNA as template facilitated the suppression of the influences of the contaminating RNA. When random primers were employed, higher background signals on DNA array data were observed, especially in the presence of contaminating RNA. This is because random primers anneal with approximately equal efficiency to every RNA species in the reverse transcription reaction. However, if no sufficient genome sequence information is available for the scrutinised organism, random primers offer the only alternative. By optimising the hybridisation and washing conditions in DNA array experiments, problems of cross hybridisation can be subdued. Based on the characteristics of the amplified RNA, it is feasible to carry out second round of amplification using the method developed in this study. In this case, the resulted amplified RNA may show smaller size distribution than that in the first round of amplification.

It is worth noting that the RNA amplification method described here can also be employed to eukaryotic RNA. The use of oligo(dT) for priming RNA in the first-strand synthesis along with the switching template will primarily yield amplified sense RNA in full-length.

5. Summary

Tuberculosis is one of the most perilous infectious diseases in the world causing very high number of morbidity annually. The success of *M. tuberculosis*, the causative agent of tuberculosis, to establish disease depends on its ability to survive and persist inside the host, the mechanisms of which are still ill-defined. Moreover, the increasing incidents of tuberculosis in the recent years, partly due to HIV pandemic, aggravated by the increasing number of multidrug-resistant strains urges the need for more efficient drugs and diagnosis tools.

The ability of the genome sequence of *M. tuberculosis* H37Rv allows the performance of genomic comparison and genome-wide gene expression profile analyses of *M. tuberculosis*.

Based on the genome sequence of *M. tuberculosis* H37Rv, filter-based DNA arrays were produced representing all known ORFs. Since the deleted genes in *M. bovis* and *M. bovis* BCG Copenhagen as compared to *M. tuberculosis* H37Rv have been reported recently, the quality of the DNA arrays in terms of sensitivity and reliability was assessed by comparing the hybridisation results using gDNA from the three mycobacterial strains. In addition to the known deletions, some sequence variations which have not been reported previously could be detected using the DNA arrays and the detection system (including radioactive labelling of the targets) applied in this study. Sequence variations were observed in Rv0050, Rv0278c, Rv0279c, Rv2090, and Rv3281. The sequence variations in Rv0050, Rv2090, and Rv3281 were also investigated in other members of *M. tuberculosis* complex: *M. tuberculosis* Beijing, *M. tuberculosis* Haarlem, *M. canettii*, and *M. africanum*. Different degrees of sequence variations could be observed in these strains for the investigated genes. Most deletions detected here comprised DNA regions less than 200 bp. This suggested that the DNA arrays and the detection system used in this study was more sensitive than the array systems used recently to perform similar study. The sequence polymorphism observed here may serve as potential markers to differentiate the investigated strains mentioned above. The gene expression study revealed that these genes were expressed *in vitro* and *in vivo* indicating that they may still be functional. The genotypic

variations in this case may contribute to the phenotypic differences between the investigated strains.

In the transcriptome study the gene expression profiles of *M. tuberculosis* during host infection were monitored. In the first part of this study murine bone marrow-derived macrophages were prepared and then infected with *M. tuberculosis* H37Rv. In one part of the experiment macrophages were activated with the cytokine IFN-γ before infection. Since iron metabolism plays an important role in host defense mechanisms against pathogens, an additional experiment was performed in which *M. tuberculosis* H37Rv was cultured *in vitro* under iron depletion. Gene expression profiles obtained from four different growth conditions: *in vitro* in rich medium, resting macrophages, activated macrophages, and *in vitro* under iron depletion were compared. The DNA array data revealed that the gene expression profiles of infection in activated macrophages and *in vitro* under iron depletion were similar to a certain extent indicating that the milieu of *M. tuberculosis* in activated macrophage is deprived of iron and this condition has a great impact on its overall gene expression profile in activated macrophages. *M. tuberculosis* experienced more severe conditions in the activated than in resting macrophages. DNA array results revealed that the *in vivo* induced genes represent some signatures which are critical for the survival of *M. tuberculosis* in macrophages such as DNA repair mechanism, detoxification systems, and active synthesis of cell wall and membrane components which may reflect the efforts of the tubercle bacilli to counteract the actions of host defense mechanisms.

In the second part of the transcriptome study the gene expression profiles of *M. tuberculosis* during infection in human lung were investigated. Tubercle bacilli were surgically removed (along with the surrounding lung tissues) from different sites of infection in human lung: granuloma, pericavity and distant lung and then subjected to total RNA isolation. A part of tubercle bacilli from granuloma was cultured *in vitro* in rich medium. Due to the tiny amount of total RNA of *M. tuberculosis* isolated from human lung an RNA amplification method was developed. This method was able to efficiently amplify total RNA of *M. tuberculosis* with very small bias of RNA composition from the original pool. The hierarchical clustering of the array data from the three sites of

_____

infection and *in vitro* growth showed that the expression profiles of pericavity and distant lung were very similar indicating the similar environments encountered by tubercle bacilli in these sites of infection. The DNA array data from human lung infection showed similar signatures to those observed in the macrophage infection study which play pivotal roles for the survival of *M. tuberculosis* inside the host such as DNA repair mechanism, detoxification systems, and the active synthesis of cell wall and membrane components. Some cell wall components are able to modulate the host immune response which can contribute to the disease manifestation. The induction of genes responsible for anaerobic and aerobic respiration at the same time indicates the microaerophilic rather than anaerobic environment in the infection sites. Severe conditions in the infection sites were also indicated by the array results.

Many induced genes observed in the transcriptome studies in the framework of this doctoral thesis have been reported recently by other groups either in the context of infection studies with mycobacterial strains or other intracellular pathogens. This provides means to judge the reliability of the DNA array results as a whole. DNA array data from this doctoral thesis also revealed many genes which have not been described to be induced during infection of *M. tuberculosis* using other methods. In addition, many of the differentially regulated genes have not been attributed to any physiological function. These genes may represent unknown biochemical pathways which may play critical roles in persistence and pathogenicity of *M. tuberculosis.* The *in vivo* induced genes may serve as excellent candidates for drug targets and development of novel vaccines and diagnosis tools.

Zusammenfassung

Tuberkulose ist eine der bedrohlichsten Infektionskrankheiten mit hoher Mortalitätsrate weltweit. Erschwerend kommt die Zunahme an multiresistenten Erregerstämmen und die gefährliche Liaison mit dem AIDS-Erreger HIV. Die Fähigkeit von *Mycobacterium tuberculosis*, dem Erreger der Tuberkulose, eine Erkrankung zu etablieren, wird besonders von seiner Eigenschaft geprägt, innerhalb von Wirtszellen zu überleben. Die zugrundeliegenden Mechanismen werden jedoch noch unvollständig verstanden. Neue effizientere Chemotherapeutika, Diagnostika und ein besser wirksamer Impfstoff werden daher dringend benötigt.

Die Verfügbarkeit der kompletten Genomsequenz von *M. tuberculosis* H37Rv erlaubt den Genomvergleich mit anderen Mykobakterienarten und Stämmen sowie die Erstellung des Genexpressionsprofils von *M. tuberculosis*. Filterbasierte DNA-Arrays wurden hergestellt, die sämtliche offene Leseraster von *M. tuberculosis* repräsentieren. Da die in *M. bovis* und *M. bovis* BCG im Vergleich zu *M. tuberculosis* H37Rv fehlenden Gene bekannt sind, konnte die Qualität der hergestellten DNA-Arrays bezüglich Sensitivität und Zuverlässigkeit verifiziert werden. Zusätzlich zu den bekannten Deletionen wurden weitere, bislang unbekannte, Sequenzvariationen mit Hilfe der DNA-Arraysysteme entdeckt. Sequenzvariationen wurden in den Genen Rv0050, Rv0278c, Rv0279c, Rv2090, und Rv3281 festgestellt. Die Sequenzvariationen in Rv0050, Rv2090, und Rv3281 wurden auch in anderen Mitgliedern des *M. tuberculosis* Komplexes analysiert. Hierbei handelte es sich um *M. tuberculosis* Beijing, *M. tuberculosis* Haarlem, *M. canettii* und *M. africanum*. In den verschiedenen Stämmen variierten die Sequenzunterschiede für die untersuchten Gene, wobei die meisten Deletionen weniger als 200 Basenpaare umfassten. Dies deutet an, dass die eingesetzten DNA-Arrays und die entsprechenden Nachweissysteme sensitiver als die bislang beschriebenen waren. Der in dieser Arbeit festgestellte Sequenzpolymorphismus kann möglicherweise als Marker zur Differenzierung der untersuchten Stämme dienen. Die durchgeführten Expressionsexperimente zeigen, dass diese Gene sowohl *in vitro* als auch *in vivo* exprimiert werden und demnach funktionsfähig sind. Die phänotypischen

_____

Unterschiede zwischen den untersuchten Stämme sind zumindest teilweise auf diese Sequenzpolymorphismen zurückzuführen.

Die Transkriptionsanalysen der Genexpressionsprofile von *M. tuberculosis* während des Infektionsverlaufs wurden analysiert. Im ersten Teil der Arbeit wurden Makrophagen der Maus mit *M. tuberculosis* H37Rv infiziert und ein Teil der Zellen vor der Infektion mit Interferon-γ aktiviert. Da der Eisenmetabolismus einen wesentlichen Abwehrmechanismus der Wirtszelle darstellt, wurde *M. tuberculosis* H37Rv zusätzlich *in vitro* unter Eisenmangel kultiviert. Die Genexpressionsprofile von *M. tuberculosis* zeigen zwischen aktivierten Makrophagen und *in vitro* Kultur unter Eisenmangel eine bemerkenswerte Ähnlichkeit. Dies unterstützt die Annahme, dass Eisenentzug einen wichtigen Abwehrmechanismus infizierter Makrophagen darstellt und weist auf dessen Auswirkungen auf das globale Expressionsprofil von *M. tuberculosis* hin. Im Vergleich zu nicht aktivierten Makrophagen wurde für aktivierte Makrophagen eine höhere Aktivität von Genen, die an der Zellwand und Zellmembran-Biosynthese beteiligt sind, festgestellt. Dies ist als Abwehr des Erregers gegen Effektormechanismen der Wirtszelle zu deuten.

Im zweiten Teil der Transkriptomexperimente wurden Erreger untersucht, die aus Lungen von Tuberkulosepatienten isoliert worden waren. Das Material wurde aus folgenden Lokalisationen der menschlichen Lunge operativ gewonnen: Granulome, perikavitäre und weiterentfernte Lungenbereiche. Ein Teil der Erreger wurde *in vitro* unter optimalen Bedingungen kultiviert. Da die verfügbaren RNA-Mengen von *M. tuberculosis* aus Lungengewebe äußerst gering waren, wurde eine Methode zur Amplifikation mykobakterieller RNA entwickelt. Auf diese Weise konnte die mykobakterielle RNA mit hoher Effizienz und nur geringer Abweichung vom ursprünglichen RNA-Pool amplifiziert werden. Die hierarchische Anordnung der Arraydaten aus den drei Infektionsstellen und nach *in vitro* Kultur zeigte große Ähnlichkeit zwischen Expressionsprofilen aus perikavitärem und weiterentferntem Lungenbereich auf. Daraus ist zu schließen, dass die körpereigene Abwehr an diesen beiden Infektionsstellen auf den Erreger ähnlich einwirkt. Auch hier fiel besonders die hohe Aktivität von Genen für die Biosynthese von Zellwand- und Zellmembrankomponenten auf. Einige der vermehrt gebildeten

_____

Zellwandkomponenten sind in der Lage, die Immunantwort des Wirts zu modulieren. Dies könnte auf den Krankheitsverlauf Auswirkungen haben. Die gleichwertige Induktion von Genen für die anaerobe und aerobe Atmungskette deutet auf mikroaerophile Bedingungen an den Infektionsherden hin. Schließlich wurde gezeigt, dass am Ort der Infektion Gene für DNA-Reparatur und Detoxifizierung erhöht exprimiert werden.

Mehrere Gene, die in dieser Arbeit als hochaktiv identifiziert wurden, waren bereits von anderen Arbeitsgruppen in Untersuchungen mit Mykobakterien und anderen intrazellulären Erregern beschrieben worden. Daneben wurde aber auch die Induktion zahlreicher bislang nicht beschriebener Gene beobachtet. Mehrere dieser differentiell regulierten Gene haben bislang unbekannte Funktionen. Ihre Charakterisierung kann neue Mechanismen, die für das Überleben des Erregers im Wirt essentiell sind, aufklären. Diese Genprodukte stellen damit wichtige Kandidaten für Impfantigene und Zielstrukturen für neue Chemotherapeutika gegen *M. tuberculosis* dar.

6. Reference List

1. Lipsky BA, Gates J, Tenover FC, Plorde JJ. Factors affecting the clinical value of microscopy for acid-fast bacilli. Rev.Infect.Dis. 1984;6(2):214-22.

2. Curione CJ, Jr., Kaneko GS, Voss JL, Hesse F, Smith RF. Gram stain evaluation of the quality of sputum specimens for mycobacterial culture. J.Clin.Microbiol. 1977;5(3):381-2.

3. Rastogi N, Legrand E, Sola C. The mycobacteria: an introduction to nomenclature and pathogenesis. Rev.Sci.Tech. 2001;20(1):21-54.

4. Steingrube VA, Murphy D, McMahon S, Chapman JS, Nash DR. The effect of metal ions on the atypical mycobacteria: growth and colony coloration. Zentralbl.Bakteriol.[Orig.A] 1975;230(2):223-36.

5. McCarter YS, Ratkiewicz IN, Robinson A. Cord formation in BACTEC medium is a reliable, rapid method for presumptive identification of Mycobacterium tuberculosis complex. J.Clin.Microbiol. 1998;36(9):2769-71.

6. Brennan PJ, Nikaido H. The envelope of mycobacteria. Annu.Rev.Biochem. 1995;64:29-63.

7. Barry CE, III, Mdluli K. Drug sensitivity and environmental adaptation of mycobacterial cell wall components. Trends Microbiol. 1996;4(7):275-81.

8. Lambert PA. Cellular impermeability and uptake of biocides and antibiotics in Gram-positive bacteria and mycobacteria. J.Appl.Microbiol. 2002;92 Suppl:46S-54S.

9. Jarlier V, Nikaido H. Mycobacterial cell wall: structure and role in natural resistance to antibiotics. FEMS Microbiol.Lett. 1994;123(1-2):11-8.

10. Chatterjee D. The mycobacterial cell wall: structure, biosynthesis and sites of drug action. Curr.Opin.Chem.Biol. 1997;1(4):579-88.

11. Barry CE, III. Interpreting cell wall 'virulence factors' of Mycobacterium tuberculosis. Trends Microbiol. 2001;9(5):237-41.

12. Barry CE, III, Lee RE, Mdluli K, Sampson AE, Schroeder BG, Slayden RA et al. Mycolic acids: structure, biosynthesis and physiological functions. Prog.Lipid Res. 1998;37(2-3):143-79.

13. Asselineau C, Asselineau J, Laneelle G, Laneelle MA. The biosynthesis of mycolic acids by Mycobacteria: current and alternative hypotheses. Prog.Lipid Res. 2002;41(6):501-23.

14. Butler WR, Guthertz LS. Mycolic acid analysis by high-performance liquid chromatography for identification of Mycobacterium species. Clin.Microbiol.Rev. 2001;14(4):704-26, table.

15. Minnikin DE, Kremer L, Dover LG, Besra GS. The methyl-branched fortifications of Mycobacterium tuberculosis. Chem.Biol. 2002;9(5):545-53.

16. Ryll R, Kumazawa Y, Yano I. Immunological properties of trehalose dimycolate (cord factor) and other mycolic acid-containing glycolipids--a review. Microbiol.Immunol. 2001;45(12):801-11.

17. Daffe M, Draper P. The envelope layers of mycobacteria with reference to their pathogenicity. Adv.Microb.Physiol 1998;39:131-203.

18. Couture M, Yeh SR, Wittenberg BA, Wittenberg JB, Ouellet Y, Rousseau DL et al. A cooperative oxygen-binding hemoglobin from Mycobacterium tuberculosis. Proc.Natl.Acad.Sci.U.S.A 1999;96(20):11223-8.

19. Pathania R, Navani NK, Rajamohan G, Dikshit KL. Mycobacterium tuberculosis hemoglobin HbO associates with membranes and stimulates cellular respiration of recombinant Escherichia coli. J.Biol.Chem. 2002;277(18):15293-302.

20. Kana BD, Weinstein EA, Avarbock D, Dawes SS, Rubin H, Mizrahi V. Characterization of the cydAB-encoded cytochrome bd oxidase from Mycobacterium smegmatis. J.Bacteriol. 2001;183(24):7076-86.

21. Hirsch P. Microbial life at extremely low nutrient levels. Adv.Space Res. 1986;6(12):287-98.

22. Winder FG, Rooney SA. Effects of nitrogenous components of the medium on the carbohydrate and nucleic acid content of Mycobacterium tuberculosis BCG. J.Gen.Microbiol. 1970;63(1):29-39.

23. Kilburn JO, Stottmeier KD, Kubica GP. Asparatic acid as a precursor for niacin synthesis by tubercle bacilli grown on 7H-10 agar medium. Am.J.Clin.Pathol. 1968;50(5):582-6.

24. Yabu K. On the role of culture medium asparagine in tuberculin production. Tubercle. 1970;51(4):437-45.

25. Corner LA, Nicolacopoulos C. Comparison of media used for the primary isolation of Mycobacterium bovis by veterinary and medical diagnostic laboratories. Aust.Vet.J. 1988;65(7):202-5.

26. Kanetsuna F. Bactericidal effect of fatty acids on mycobacteria, with particular reference to the suggested mechanism of intracellular killing. Microbiol.Immunol. 1985;29(2):127-41.

27. Gallagher J, Horwill DM. A selective oleic acid albumin agar medium for the cultivation of Mycobacterium bovis. J.Hyg.(Lond) 1977;79(1):155-60.

28. Kaufmann SH. Is the development of a new tuberculosis vaccine possible? Nat.Med. 2000;6(9):955-60.

29. Kaufmann SH. Protection against tuberculosis: cytokines, T cells, and macrophages. Ann.Rheum.Dis. 2002;61 Suppl 2:ii54-ii58.

30. Kaufmann SH. How can immunology contribute to the control of tuberculosis? Nat.Rev.Immunol. 2001;1(1):20-30.

31. de Boer AS, Borgdorff MW, Vynnycky E, Sebek MM, van Soolingen D. Exogenous re-infection as a cause of recurrent tuberculosis in a low-incidence area. Int.J.Tuberc.Lung Dis. 2003;7(2):145-52.

32. Arend SM, van Dissel JT. Evidence of endogenous reactivation of tuberculosis after a long period of latency. J.Infect.Dis. 2002;186(6):876-7.

33. Lambert ML, Hasker E, Van Deun A, Roberfroid D, Boelaert M, Van der SP. Recurrence in tuberculosis: relapse or reinfection? Lancet Infect.Dis. 2003;3(5):282-7.

34. Gatell JM, Marrades R, el Ebiary M, Torres A. Severe pulmonary infections in AIDS patients. Semin.Respir.Infect. 1996;11(2):119-28.

35. Dunlap NE, Briles DE. Immunology of tuberculosis. Med.Clin.North Am. 1993;77(6):1235-51.

36. Dunn PL, North RJ. Virulence ranking of some Mycobacterium tuberculosis and Mycobacterium bovis strains according to their ability to multiply in the lungs, induce lung pathology, and cause mortality in mice. Infect.Immun. 1995;63(9):3428-37.

37. Masotti A, Rodella L, Inaspettato G, Foccoli P, Morandini GC. Clinical and bronchoscopic features of endobronchial tuberculosis. Monaldi Arch.Chest Dis. 1995;50(2):89-92.

38. Kim YH, Kim HT, Lee KS, Uh ST, Cung YT, Park CS. Serial fiberoptic bronchoscopic observations of endobronchial tuberculosis before and early after antituberculosis chemotherapy. Chest 1993;103(3):673-7.

39. Fenhalls G, Stevens-Muller L, Warren R, Carroll N, Bezuidenhout J, Van Helden P et al. Localisation of mycobacterial DNA and mRNA in human tuberculous granulomas. J.Microbiol.Methods 2002;51(2):197-208.

40. Sugawara I, Yamada H, Kazumi Y, Doi N, Otomo K, Aoki T et al. Induction of granulomas in interferon-gamma gene-disrupted mice by avirulent but not by virulent strains of Mycobacterium tuberculosis. J.Med.Microbiol. 1998;47(10):871-7.

41. du Plessis DG, Warren R, Richardson M, Joubert JJ, van Helden PD. Demonstration of reinfection and reactivation in HIV-negative autopsied cases of secondary tuberculosis: multilesional genotyping of Mycobacterium tuberculosis utilizing IS 6110 and other repetitive element-based DNA fingerprinting. Tuberculosis.(Edinb.) 2001;81(3):211-20.

42. Fayyazi A, Eichmeyer B, Soruri A, Schweyer S, Herms J, Schwarz P et al. Apoptosis of macrophages and T cells in tuberculosis associated caseous necrosis. J.Pathol. 2000;191(4):417-25.

43. Dannenberg AM, Jr. Roles of cytotoxic delayed-type hypersensitivity and macrophage-activating cell-mediated immunity in the pathogenesis of tuberculosis. Immunobiology 1994;191(4-5):461-73.

44. Im JG, Itoh H, Lee KS, Han MC. CT-pathology correlation of pulmonary tuberculosis. Crit Rev.Diagn.Imaging 1995;36(3):227-85.

45. Fanning A. Tuberculosis: 6. Extrapulmonary disease. CMAJ. 1999;160(11):1597-603.

46. Watterson SA, Drobniewski FA. Modern laboratory diagnosis of mycobacterial infections. J.Clin.Pathol. 2000;53(10):727-32.

47. Foulds J, O'Brien R. New tools for the diagnosis of tuberculosis: the perspective of developing countries. Int.J.Tuberc.Lung Dis. 1998;2(10):778-83.

48. Werneck-Barroso E. Innate resistance to tuberculosis: revisiting Max Lurie genetic experiments in rabbits. Int.J.Tuberc.Lung Dis. 1999;3(2):166-8.

49. Malik S, Schurr E. Genetic susceptibility to tuberculosis. Clin.Chem.Lab Med. 2002;40(9):863-8.

50. van Lettow M, Fawzi WW, Semba RD. Triple trouble: the role of malnutrition in tuberculosis and human immunodeficiency virus co-infection. Nutr.Rev. 2003;61(3):81-90.

51. Schwenk A, Macallan DC. Tuberculosis, malnutrition and wasting. Curr.Opin.Clin.Nutr.Metab Care 2000;3(4):285-91.

52. Hawker JI, Bakhshi SS, Ali S, Farrington CP. Ecological analysis of ethnic differences in relation between tuberculosis and poverty. BMJ 1999;319(7216):1031-4.

53. Elender F, Bentham G, Langford I. Tuberculosis mortality in England and Wales during 1982-1992: its association with poverty, ethnicity and AIDS. Soc.Sci.Med. 1998;46(6):673-81.

54. Ulrichs T, Kaufmann SH. Mycobacterial persistence and immunity. Front Biosci. 2002;7:d458-d469.

55. Collins HL, Kaufmann SH. The many faces of host responses to tuberculosis. Immunology 2001;103(1):1-9.

56. Pieters J. Entry and survival of pathogenic mycobacteria in macrophages. Microbes.Infect. 2001;3(3):249-55.

57. Deretic V, Fratti RA. Mycobacterium tuberculosis phagosome. Mol.Microbiol. 1999;31(6):1603-9.

58. Deretic V, Via LE, Fratti RA, Deretic D. Mycobacterial phagosome maturation, rab proteins, and intracellular trafficking. Electrophoresis 1997;18(14):2542-7.

59. Fratti RA, Vergne I, Chua J, Skidmore J, Deretic V. Regulators of membrane trafficking and Mycobacterium tuberculosis phagosome maturation block. Electrophoresis 2000;21(16):3378-85.

60. Schaible UE, Collins HL, Priem F, Kaufmann SH. Correction of the iron overload defect in beta-2-microglobulin knockout mice by lactoferrin abolishes their increased susceptibility to tuberculosis. J.Exp.Med. 2002;196(11):1507-13.

61. Clemens DL. Characterization of the Mycobacterium tuberculosis phagosome. Trends Microbiol. 1996;4(3):113-8.

62. Flynn JL, Chan J. Immunology of tuberculosis. Annu.Rev.Immunol. 2001;19:93-129.

63. Flynn JL, Chan J. Tuberculosis: latency and reactivation. Infect.Immun. 2001;69(7):4195-201.

64. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. J.Exp.Med. 1993;178(6):2249-54.

65. Cooper AM, Flynn JL. The protective immune response to Mycobacterium tuberculosis. Curr.Opin.Immunol. 1995;7(4):512-6.

66. Clemens DL, Horwitz MA. The Mycobacterium tuberculosis phagosome interacts with early endosomes and is accessible to exogenously administered transferrin. J.Exp.Med. 1996;184(4):1349-55.

67. Olakanmi O, Schlesinger LS, Ahmed A, Britigan BE. Intraphagosomal Mycobacterium tuberculosis acquires iron from both extracellular transferrin and intracellular iron pools. Impact of interferon-gamma and hemochromatosis. J.Biol.Chem. 2002;277(51):49727-34.

68. Lewis KN, Liao R, Guinn KM, Hickey MJ, Smith S, Behr MA et al. Deletion of RD1 from Mycobacterium tuberculosis mimics bacille Calmette-Guerin attenuation. J.Infect.Dis. 2003;187(1):117-23.

69. McKinney JD, Honer zu BK, Munoz-Elias EJ, Miczak A, Chen B, Chan WT et al. Persistence of Mycobacterium tuberculosis in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. Nature 2000;406(6797):735-8.

70. Reyrat JM, Berthet FX, Gicquel B. The urease locus of Mycobacterium tuberculosis and its utilization for the demonstration of allelic exchange in Mycobacterium bovis bacillus Calmette-Guerin. Proc.Natl.Acad.Sci.U.S.A 1995;92(19):8768-72.

71. Sirakova TD, Dubey VS, Cynamon MH, Kolattukudy PE. Attenuation of Mycobacterium tuberculosis by disruption of a mas-like gene or a chalcone synthase-like gene, which causes deficiency in dimycocerosyl phthiocerol synthesis. J.Bacteriol. 2003;185(10):2999-3008.

72. Bahammam A, Choudhri S, Long R. The validity of acid-fast smears of gastric aspirates as an indicator of pulmonary tuberculosis. Int.J.Tuberc.Lung Dis. 1999;3(1):62-7.

73. Al Moamary MS, Black W, Bessuille E, Elwood RK, Vedal S. The significance of the persistent presence of acid-fast bacilli in sputum smears in pulmonary tuberculosis. Chest 1999;116(3):726-31.

74. Mitchison DA. Bacteriological aspects of mycobacterial infections. Br.Med.J. 1972;1(797):424-5.

75. Singh NP, Parija SC. The value of fluorescence microscopy of auramine stained sputum smears for the diagnosis of pulmonary tuberculosis. Southeast Asian J.Trop.Med.Public Health 1998;29(4):860-3.

76. Christie JD, Callihan DR. The laboratory diagnosis of mycobacterial diseases. Challenges and common sense. Clin.Lab Med. 1995;15(2):279-306.

77. Frei R. [Current methods in rapid diagnosis of tuberculosis]. Schweiz.Med.Wochenschr. 1993;123(5):147-52.

78. Menzies D. What does tuberculin reactivity after bacille Calmette-Guerin vaccination tell us? Clin.Infect.Dis. 2000;31 Suppl 3:S71-S74.

79. Rajajee S. Tuberculin and BCG tests. Indian J.Pediatr. 2000;67(2 Suppl):S9-13.

80. Shinnick TM. Diagnostic test needs for evaluating antituberculosis vaccines. Clin.Infect.Dis. 2000;30 Suppl 3:S276-S278.

81. Chan ED, Heifets L, Iseman MD. Immunologic diagnosis of tuberculosis: a review. Tuber.Lung Dis. 2000;80(3):131-40.

82. Gennaro ML. Immunologic diagnosis of tuberculosis. Clin.Infect.Dis. 2000;30 Suppl 3:S243-S246.

83. Gupta S, Bhatia R, Datta KK. Serodiagnosis of tuberculosis. J.Commun.Dis. 1995;27(4):208-14.

84. Brewer TF, Wilson ME, Nardell EA. BCG immunization: review of past experience, current use, and future prospects. Curr.Clin.Top.Infect.Dis. 1995;15:253-70.

85. Murthy NK, Dutt AK. Tuberculin skin testing: present status. Semin.Respir.Infect. 1994;9(2):78-83.

86. Huebner RE, Schein MF, Bass JB, Jr. The tuberculin skin test. Clin.Infect.Dis. 1993;17(6):968-75.

87. Toossi Z, Ellner JJ. Mechanisms of anergy in tuberculosis. Curr.Top.Microbiol.Immunol. 1996;215:221-38.

88. Hegde HR. Anergy, AIDS and tuberculosis. Med.Hypotheses 1995;45(5):433-40.

89. Iseman MD. Tuberculosis therapy: past, present and future. Eur.Respir.J.Suppl 2002;36:87s-94s.

90. Iseman MD. Treatment and implications of multidrug-resistant tuberculosis for the 21st century. Chemotherapy 1999;45 Suppl 2:34-40.

91. Grosset J. Current problems with tuberculosis treatment. Res.Microbiol. 1996;147(1-2):10-6.

92. Heifets LB. Antimycobacterial drugs. Semin.Respir.Infect. 1994;9(2):84-103.

93. Fox W. The chemotherapy of pulmonary tuberculosis: a review. Chest 1979;76(6 Suppl):785-96.

94. Mostowy S, Behr MA. Comparative genomics in the fight against tuberculosis: diagnostics, epidemiology, and BCG vaccination. Am.J.Pharmacogenomics. 2002;2(3):189-96.

95. Behr MA, Wilson MA, Gill WP, Salamon H, Schoolnik GK, Rane S et al. Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science 1999;284(5419):1520-3.

96. Collins HL, Kaufmann SH. Prospects for better tuberculosis vaccines. Lancet Infect.Dis. 2001;1(1):21-8.

97. Agger EM, Andersen P. A novel TB vaccine; towards a strategy based on our understanding of BCG failure. Vaccine 2002;21(1-2):7-14.

98. Agger EM, Andersen P. Tuberculosis subunit vaccine development: on the role of interferon-gamma. Vaccine 2001;19(17-19):2298-302.

99. Louise R, Skjot V, Agger EM, Andersen P. Antigen discovery and tuberculosis vaccine development in the post-genomic era. Scand.J.Infect.Dis. 2001;33(9):643-7.

100. Hess J, Kaufmann SH. Development of live recombinant vaccine candidates against tuberculosis. Scand.J.Infect.Dis. 2001;33(10):723-4.

101. Hess J, Grode L, Hellwig J, Conradt P, Gentschev I, Goebel W et al. Protection against murine tuberculosis by an attenuated recombinant Salmonella typhimurium vaccine strain that secretes the 30-kDa antigen of Mycobacterium bovis BCG. FEMS Immunol.Med.Microbiol. 2000;27(4):283-9.

102. Hess J, Szalay G, Miko D, Gentschev I, Goebel W, Ladel CH et al. Vaccination strategies using viable carrier systems against intracellular pathogens. Behring Inst.Mitt. 1994(95):67-79.

103. Brandt L, Orme I. Prospects for new vaccines against tuberculosis. Biotechniques 2002;33(5):1098, 1100, 1102.

104. Young DB, Stewart GR. Tuberculosis vaccines. Br.Med.Bull. 2002;62:73-86.

105. Doherty TM, Andersen P. Tuberculosis vaccine development. Curr.Opin.Pulm.Med. 2002;8(3):183-7.

106. Doherty TM, Olsen AW, van Pinxteren L, Andersen P. Oral vaccination with subunit vaccines protects animals against aerosol infection with Mycobacterium tuberculosis. Infect.Immun. 2002;70(6):3111-21.

107. Bloom BR, Flynn J, McDonough K, Kress Y, Chan J. Experimental approaches to mechanisms of protection and pathogenesis in M. tuberculosis infection. Immunobiology 1994;191(4-5):526-36.

108. Kaech SM, Hemby S, Kersh E, Ahmed R. Molecular and functional profiling of memory CD8 T cell differentiation. Cell 2002;111(6):837-51.

109. Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 1995;270(5235):467-70.

110. Shalon D, Smith SJ, Brown PO. A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization. Genome Res. 1996;6(7):639-45.

111. Lockhart DJ, Winzeler EA. Genomics, gene expression and DNA arrays. Nature 2000;405(6788):827-36.

112. Duggan DJ, Bittner M, Chen Y, Meltzer P, Trent JM. Expression profiling using cDNA microarrays. Nat.Genet. 1999;21(1 Suppl):10-4.

113. Kane MD, Jatkoe TA, Stumpf CR, Lu J, Thomas JD, Madore SJ. Assessment of the sensitivity and specificity of oligonucleotide (50mer) microarrays. Nucleic Acids Res. 2000;28(22):4552-7.

114. Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS et al. Expression monitoring by hybridization to high-density oligonucleotide arrays. Nat.Biotechnol. 1996;14(13):1675-80.

115. Southern EM. DNA chips: analysing sequence by hybridization to oligonucleotides on a large scale. Trends Genet. 1996;12(3):110-5.

116. Lipshutz RJ, Fodor SP, Gingeras TR, Lockhart DJ. High density synthetic oligonucleotide arrays. Nat.Genet. 1999;21(1 Suppl):20-4.

117. Fodor SP, Read JL, Pirrung MC, Stryer L, Lu AT, Solas D. Light-directed, spatially addressable parallel chemical synthesis. Science 1991;251(4995):767-73.

118. Gress TM, Hoheisel JD, Lennon GG, Zehetner G, Lehrach H. Hybridization fingerprinting of high-density cDNA-library arrays with cDNA pools derived from whole tissues. Mamm.Genome 1992;3(11):609-19.

119. Park GH, Choe J, Choo HJ, Park YG, Sohn J, Kim MK. Genome-wide expression profiling of 8-chloroadenosine- and 8-chloro-cAMP-treated human neuroblastoma cells using radioactive human cDNA microarray. Exp.Mol.Med. 2002;34(3):184-93.

120. Salin H, Vujasinovic T, Mazurie A, Maitrejean S, Menini C, Mallet J et al. A novel sensitive microarray approach for differential screening using probes labelled with two different radioelements. Nucleic Acids Res. 2002;30(4):e17.

121. Dodson JM, Charles PT, Stenger DA, Pancrazio JJ. Quantitative assessment of filter-based cDNA microarrays: gene expression profiles of human T-lymphoma cell lines. Bioinformatics. 2002;18(7):953-60.

122. Donovan DM, Becker KG. Double round hybridization of membrane based cDNA arrays: improved background reduction and data replication. J.Neurosci.Methods 2002;118(1):59-62.

123. Eickhoff H, Schuchhardt J, Ivanov I, Meier-Ewert S, O'Brien J, Malik A et al. Tissue gene expression analysis using arrayed normalized cDNA libraries. Genome Res. 2000;10(8):1230-40.

124. Salama N, Guillemin K, McDaniel TK, Sherlock G, Tompkins L, Falkow S. A whole-genome microarray reveals genetic diversity among Helicobacter pylori strains. Proc.Natl.Acad.Sci.U.S.A 2000;97(26):14668-73.

125. Kozal MJ, Shah N, Shen N, Yang R, Fucini R, Merigan TC et al. Extensive polymorphisms observed in HIV-1 clade B protease gene using high-density oligonucleotide arrays. Nat.Med. 1996;2(7):753-9.

126. Wilson JW, Bean P, Robins T, Graziano F, Persing DH. Comparative evaluation of three human immunodeficiency virus genotyping systems: the HIV-GenotypR method, the HIV PRT GeneChip assay, and the HIV-1 RT line probe assay. J.Clin.Microbiol. 2000;38(8):3022-8.

127. Gingeras TR, Ghandour G, Wang E, Berno A, Small PM, Drobniewski F et al. Simultaneous genotyping and species identification using hybridization pattern recognition analysis of generic Mycobacterium DNA arrays. Genome Res. 1998;8(5):435-48.

128. Troesch A, Nguyen H, Miyada CG, Desvarenne S, Gingeras TR, Kaplan PM et al. Mycobacterium species identification and rifampin resistance testing with high-density DNA probe arrays. J.Clin.Microbiol. 1999;37(1):49-55.

129. Hakenbeck R, Balmelle N, Weber B, Gardes C, Keck W, de Saizieu A. Mosaic genes and mosaic chromosomes: intra- and interspecies genomic variation of Streptococcus pneumoniae. Infect.Immun. 2001;69(4):2477-86.

130. Kato-Maeda M, Rhee JT, Gingeras TR, Salamon H, Drenkow J, Smittipat N et al. Comparing genomes within the species Mycobacterium tuberculosis. Genome Res. 2001;11(4):547-54.

131. Kato-Maeda M, Bifani PJ, Kreiswirth BN, Small PM. The nature and consequence of genetic variability within Mycobacterium tuberculosis. J.Clin.Invest 2001;107(5):533-7.

132. Cummings CA, Relman DA. Genomics and microbiology. Microbial forensics--"cross-examining pathogens". Science 2002;296(5575):1976-9.

133. Cummings CA, Relman DA. Using DNA microarrays to study host-microbe interactions. Emerg.Infect.Dis. 2000;6(5):513-25.

134. Manger ID, Relman DA. How the host 'sees' pathogens: global gene expression responses to infection. Curr.Opin.Immunol. 2000;12(2):215-8.

135. Eckmann L, Smith JR, Housley MP, Dwinell MB, Kagnoff MF. Analysis by high density cDNA arrays of altered gene expression in human intestinal epithelial cells in response to infection with the invasive enteric bacteria Salmonella. J.Biol.Chem. 2000;275(19):14084-94.

136. Belcher CE, Drenkow J, Kehoe B, Gingeras TR, McNamara N, Lemjabbar H et al. The transcriptional responses of respiratory epithelial cells to Bordetella pertussis reveal host defensive and pathogen counter-defensive strategies. Proc.Natl.Acad.Sci.U.S.A 2000;97(25):13847-52.

137. Detweiler CS, Cunanan DB, Falkow S. Host microarray analysis reveals a role for the Salmonella response regulator phoP in human macrophage cell death. Proc.Natl.Acad.Sci.U.S.A 2001;98(10):5850-5.

138. Guiney DG. Regulation of bacterial virulence gene expression by the host environment. J.Clin.Invest 1997;99(4):565-9.

139. Eckmann L, Smith JR, Housley MP, Dwinell MB, Kagnoff MF. Analysis by high density cDNA arrays of altered gene expression in human intestinal epithelial cells in response to infection with the invasive enteric bacteria Salmonella. J.Biol.Chem. 2000;275(19):14084-94.

140. de Saizieu A, Gardes C, Flint N, Wagner C, Kamber M, Mitchell TJ et al. Microarray-based identification of a novel Streptococcus pneumoniae regulon controlled by an autoinduced peptide. J.Bacteriol. 2000;182(17):4696-703.

141. Debouck C, Goodfellow PN. DNA microarrays in drug discovery and development. Nat.Genet. 1999;21(1 Suppl):48-50.

142. Wilson M, DeRisi J, Kristensen HH, Imboden P, Rane S, Brown PO et al. Exploring drug-induced alterations in gene expression in Mycobacterium tuberculosis by microarray hybridization. Proc.Natl.Acad.Sci.U.S.A 1999;96(22):12833-8.

143. Braxton S, Bedilion T. The integration of microarray information in the drug development process. Curr.Opin.Biotechnol. 1998;9(6):643-9.

144. Gygi SP, Rochon Y, Franza BR, Aebersold R. Correlation between protein and mRNA abundance in yeast. Mol.Cell Biol. 1999;19(3):1720-30.

145. Schutze K, Lahr G. Identification of expressed genes by laser-mediated manipulation of single cells. Nat.Biotechnol. 1998;16(8):737-42.

146. Emmert-Buck MR, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR et al. Laser capture microdissection. Science 1996;274(5289):998-1001.

147. Cole KA, Krizman DB, Emmert-Buck MR. The genetics of cancer--a 3D model. Nat.Genet. 1999;21(1 Suppl):38-41.

148. Stears RL, Getts RC, Gullans SR. A novel, sensitive detection system for high-density microarrays using dendrimer technology. Physiol Genomics 2000;3(2):93-9.

149. Luo L, Salunga RC, Guo H, Bittner A, Joy KC, Galindo JE et al. Gene expression profiles of laser-captured adjacent neuronal subtypes. Nat.Med. 1999;5(1):117-22.

150. Brady G. Expression profiling of single mammalian cells--small is beautiful. Yeast 2000;17(3):211-7.

151. Stears RL, Getts RC, Gullans SR. A novel, sensitive detection system for high-density microarrays using dendrimer technology. Physiol Genomics 2000;3(2):93-9.

152. Perou CM, Sorlie T, Eisen MB, van de RM, Jeffrey SS, Rees CA et al. Molecular portraits of human breast tumours. Nature 2000;406(6797):747-52.

153. Fink JL, Drewes S, Patel H, Welsh JB, Masys DR, Corbeil J et al. 2HAPI: a microarray data analysis system. Bioinformatics. 2003;19(11):1443-5.

154. Draghici S, Kulaeva O, Hoff B, Petrov A, Shams S, Tainsky MA. Noise sampling method: an ANOVA approach allowing robust selection of differentially regulated genes measured by DNA microarrays. Bioinformatics. 2003;19(11):1348-59.

155. Broberg P. Statistical methods for ranking differentially expressed genes. Genome Biol. 2003;4(6):R41.

156. Segal E, Shapira M, Regev A, Pe'er D, Botstein D, Koller D et al. Module networks: identifying regulatory modules and their condition-specific regulators from gene expression data. Nat.Genet. 2003;34(2):166-76.

157. Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C et al. Minimum information about a microarray experiment

(MIAME)-toward standards for microarray data. Nat.Genet. 2001;29(4):365-71.

158. He YD, Dai H, Schadt EE, Cavet G, Edwards SW, Stepaniants SB et al. Microarray standard data set and figures of merit for comparing data processing methods and experiment designs. Bioinformatics. 2003;19(8):956-65.

159. Chuaqui RF, Bonner RF, Best CJ, Gillespie JW, Flaig MJ, Hewitt SM et al. Post-analysis follow-up and validation of microarray experiments. Nat.Genet. 2002;32 Suppl:509-14.

160. Slonim DK. From patterns to pathways: gene expression data analysis comes of age. Nat.Genet. 2002;32 Suppl:502-8.

161. Quackenbush J. Microarray data normalization and transformation. Nat.Genet. 2002;32 Suppl:496-501.

162. Churchill GA. Fundamentals of experimental design for cDNA microarrays. Nat.Genet. 2002;32 Suppl:490-5.

163. Holloway AJ, van Laar RK, Tothill RW, Bowtell DD. Options available-- from start to finish--for obtaining data from DNA microarrays II. Nat.Genet. 2002;32 Suppl:481-9.

164. Rosenkrands I, King A, Weldingh K, Moniatte M, Moertz E, Andersen P. Towards the proteome of Mycobacterium tuberculosis. Electrophoresis 2000;21(17):3740-56.

165. Wilkins MR. What do we want from proteomics in the detection and avoidance of adverse drug reactions. Toxicol.Lett. 2002;127(1-3):245-9.

166. Saleh MT, Fillon M, Brennan PJ, Belisle JT. Identification of putative exported/secreted proteins in prokaryotic proteomes. Gene 2001;269(1-2):195-204.

167. De Groot AS, Bosma A, Chinai N, Frost J, Jesdale BM, Gonzalez MA et al. From genome to vaccine: in silico predictions, ex vivo verification. Vaccine 2001;19(31):4385-95.

168. Covert BA, Spencer JS, Orme IM, Belisle JT. The application of proteomics in defining the T cell antigens of Mycobacterium tuberculosis. Proteomics. 2001;1(4):574-86.

169. Betts JC. Transcriptomics and proteomics: tools for the identification of novel drug targets and vaccine candidates for tuberculosis. IUBMB.Life 2002;53(4-5):239-42.

170. Anderson NL, Anderson NG. Proteome and proteomics: new technologies, new concepts, and new words. Electrophoresis 1998;19(11):1853-61.

171. Kamath AT, Groat NL, Bean AG, Britton WJ. Protective effect of DNA immunization against mycobacterial infection is associated with the early emergence of interferon-gamma (IFN-gamma)-secreting lymphocytes. Clin.Exp.Immunol. 2000;120(3):476-82.

172. Daugelat S, Ladel CH, Kaufmann SH. Influence of mouse strain and vaccine viability on T-cell responses induced by Mycobacterium bovis bacillus Calmette-Guerin. Infect.Immun. 1995;63(5):2033-40.

173. Daugelat S, Gulle H, Schoel B, Kaufmann SH. Secreted antigens of Mycobacterium tuberculosis: characterization with T lymphocytes from patients and contacts after two-dimensional separation. J.Infect.Dis. 1992;166(1):186-90.

174. Mahairas GG, Sabo PJ, Hickey MJ, Singh DC, Stover CK. Molecular analysis of genetic differences between Mycobacterium bovis BCG and virulent M. bovis. J.Bacteriol. 1996;178(5):1274-82.

175. Clemens DL, Lee BY, Horwitz MA. Purification, characterization, and genetic analysis of Mycobacterium tuberculosis urease, a potentially critical determinant of host-pathogen interaction. J.Bacteriol. 1995;177(19):5644-52.

176. Urquhart BL, Atsalos TE, Roach D, Basseal DJ, Bjellqvist B, Britton WL et al. 'Proteomic contigs' of Mycobacterium tuberculosis and Mycobacterium bovis (BCG) using novel immobilised pH gradients. Electrophoresis 1997;18(8):1384-92.

177. Sonnenberg MG, Belisle JT. Definition of Mycobacterium tuberculosis culture filtrate proteins by two-dimensional polyacrylamide gel electrophoresis, N-terminal amino acid sequencing, and electrospray mass spectrometry. Infect.Immun. 1997;65(11):4515-24.

178. Mattow J, Jungblut PR, Schaible UE, Mollenkopf HJ, Lamer S, Zimny-Arndt U et al. Identification of proteins from Mycobacterium tuberculosis missing in attenuated Mycobacterium bovis BCG strains. Electrophoresis 2001;22(14):2936-46.

179. Jungblut PR, Schaible UE, Mollenkopf HJ, Zimny-Arndt U, Raupach B, Mattow J et al. Comparative proteome analysis of Mycobacterium tuberculosis and Mycobacterium bovis BCG strains: towards functional genomics of microbial pathogens. Mol.Microbiol. 1999;33(6):1103-17.

180. Marcotte EM, Pellegrini M, Ng HL, Rice DW, Yeates TO, Eisenberg D. Detecting protein function and protein-protein interactions from genome sequences. Science 1999;285(5428):751-3.

181. Pellegrini M, Marcotte EM, Thompson MJ, Eisenberg D, Yeates TO. Assigning protein functions by comparative genome analysis: protein phylogenetic profiles. Proc.Natl.Acad.Sci.U.S.A 1999;96(8):4285-8.

182. Overbeek R, Fonstein M, D'Souza M, Pusch GD, Maltsev N. The use of gene clusters to infer functional coupling. Proc.Natl.Acad.Sci.U.S.A 1999;96(6):2896-901.

183. Dandekar T, Snel B, Huynen M, Bork P. Conservation of gene order: a fingerprint of proteins that physically interact. Trends Biochem.Sci. 1998;23(9):324-8.

184. Salgado H, Moreno-Hagelsieb G, Smith TF, Collado-Vides J. Operons in Escherichia coli: genomic analyses and predictions. Proc.Natl.Acad.Sci.U.S.A 2000;97(12):6652-7.

185. Imai T, Ohta K, Kigawa H, Kanoh H, Taniguchi T, Tobari J. Preparation of high-molecular-weight DNA: application to mycobacterial cells. Anal.Biochem. 1994;222(2):479-82.

186. Belisle JT, Sonnenberg MG. Isolation of genomic DNA from mycobacteria. Methods Mol.Biol. 1998;101:31-44.

187. Schuchhardt J, Beule D, Malik A, Wolski E, Eickhoff H, Lehrach H et al. Normalization strategies for cDNA microarrays. Nucleic Acids Res. 2000;28(10):E47.

188. Herzel H, Beule D, Kielbasa S, Korbel J, Sers C, Malik A et al. Extracting information from cDNA arrays. Chaos. 2001;11(1):98-107.

189. Dietrich G, Schaible UE, Diehl KD, Mollenkopf H, Wiek S, Hess J et al. Isolation of RNA from mycobacteria grown under in vitro and in vivo conditions. FEMS Microbiol.Lett. 2000;186(2):177-80.

190. Talaat AM, Hunter P, Johnston SA. Genome-directed primers for selective labeling of bacterial transcripts for DNA microarray analysis. Nat.Biotechnol. 2000;18(6):679-82.

191. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc.Natl.Acad.Sci.U.S.A 1998;95(25):14863-8.

192. Bertucci F, Bernard K, Loriod B, Chang YC, Granjeaud S, Birnbaum D et al. Sensitivity issues in DNA array-based expression measurements and performance of nylon microarrays for small samples. Hum.Mol.Genet. 1999;8(9):1715-22.

193. Bernard K, Auphan N, Granjeaud S, Victorero G, Schmitt-Verhulst AM, Jordan BR et al. Multiplex messenger assay: simultaneous, quantitative measurement of expression of many genes in the context of T cell activation. Nucleic Acids Res. 1996;24(8):1435-42.

194. Salin H, Vujasinovic T, Mazurie A, Maitrejean S, Menini C, Mallet J et al. A novel sensitive microarray approach for differential screening using probes labelled with two different radioelements. Nucleic Acids Res. 2002;30(4):e17.

195. Drmanac R, Nizetic D, Lennon GG, Beitverda A, Lehrach H. W (A or T)
      sequences as probes and primers suitable for genomic mapping and
      fingerprinting. Nucleic Acids Res. 1991;19(21):5839-42.

196. Gress TM, Hoheisel JD, Lennon GG, Zehetner G, Lehrach H.
      Hybridization fingerprinting of high-density cDNA-library arrays with cDNA
      pools derived from whole tissues. Mamm.Genome 1992;3(11):609-19.

197. Hoheisel JD, Ross MT, Zehetner G, Lehrach H. Relational genome
      analysis using reference libraries and hybridisation fingerprinting.
      J.Biotechnol. 1994;35(2-3):121-34.

198. Zhu G, Reynolds L, Crnogorac-Jurcevic T, Gillett CE, Dublin EA, Marshall
      JF et al. Combination of microdissection and microarray analysis to
      identify gene expression changes between differentially located tumour
      cells in breast cancer. Oncogene 2003;22(24):3742-8.

199. Fleischmann RD, Alland D, Eisen JA, Carpenter L, White O, Peterson J et
      al. Whole-genome comparison of Mycobacterium tuberculosis clinical and
      laboratory strains. J.Bacteriol. 2002;184(19):5479-90.

200. van der Zanden AG, Kremer K, Schouls LM, Caimi K, Cataldi A, Hulleman
      A et al. Improvement of differentiation and interpretability of spoligotyping
      for Mycobacterium tuberculosis complex isolates by introduction of new
      spacer oligonucleotides. J.Clin.Microbiol. 2002;40(12):4628-39.

201. Costello E, O'Grady D, Flynn O, O'Brien R, Rogers M, Quigley F et al.
      Study of restriction fragment length polymorphism analysis and
      spoligotyping for epidemiological investigation of Mycobacterium bovis
      infection. J.Clin.Microbiol. 1999;37(10):3217-22.

202. Frothingham R, Meeker-O'Connell WA. Genetic diversity in the
      Mycobacterium tuberculosis complex based on variable numbers of
      tandem DNA repeats. Microbiology 1998;144 (Pt 5):1189-96.

203. Goyal M, Saunders NA, van Embden JD, Young DB, Shaw RJ.
      Differentiation of Mycobacterium tuberculosis isolates by spoligotyping
      and IS6110 restriction fragment length polymorphism. J.Clin.Microbiol.
      1997;35(3):647-51.

204. Salamon H, Kato-Maeda M, Small PM, Drenkow J, Gingeras TR.
      Detection of deleted genomic DNA using a semiautomated computational
      analysis of GeneChip data. Genome Res. 2000;10(12):2044-54.

205. Yang MC, Ruan QG, Yang JJ, Eckenrode S, Wu S, McIndoe RA et al. A
      statistical method for flagging weak spots improves normalization and ratio
      estimates in microarrays. Physiol Genomics 2001;7(1):45-53.

206. Baggerly KA, Coombes KR, Hess KR, Stivers DN, Abruzzo LV, Zhang W.
      Identifying differentially expressed genes in cDNA microarray
      experiments. J.Comput.Biol. 2001;8(6):639-59.

207. Hess KR, Zhang W, Baggerly KA, Stivers DN, Coombes KR. Microarrays: handling the deluge of data and extracting reliable information. Trends Biotechnol. 2001;19(11):463-8.

208. Mariani TJ, Budhraja V, Mecham BH, Gu CC, Watson MA, Sadovsky Y. A variable fold change threshold determines significance for expression microarrays. FASEB J. 2003;17(2):321-3.

209. Dziejman M, Balon E, Boyd D, Fraser CM, Heidelberg JF, Mekalanos JJ. Comparative genomic analysis of Vibrio cholerae: genes that correlate with cholera endemic and pandemic disease. Proc.Natl.Acad.Sci.U.S.A 2002;99(3):1556-61.

210. Behr MA, Schroeder BG, Brinkman JN, Slayden RA, Barry CE, III. A point mutation in the mma3 gene is responsible for impaired methoxymycolic acid production in Mycobacterium bovis BCG strains obtained after 1927. J.Bacteriol. 2000;182(12):3394-9.

211. Rhoades ER, Orme IM. Susceptibility of a panel of virulent strains of Mycobacterium tuberculosis to reactive nitrogen intermediates. Infect.Immun. 1997;65(4):1189-95.

212. Finkelstein RA, Sciortino CV, McIntosh MA. Role of iron in microbe-host interactions. Rev.Infect.Dis. 1983;5 Suppl 4:S759-S777.

213. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D et al. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature 1998;393(6685):537-44.

214. McCready KA, Ratledge C. Amounts of iron, haem and related compounds in Mycobacterium smegmatis grown in various concentrations of iron [proceedings]. Biochem.Soc.Trans. 1978;6(2):421-3.

215. Kontoghiorghes GJ, Weinberg ED. Iron: mammalian defense systems, mechanisms of disease, and chelation therapy approaches. Blood Rev. 1995;9(1):33-45.

216. Baynes RD, Flax H, Bothwell TH, Bezwoda WR, MacPhail AP, Atkinson P et al. Haematological and iron-related measurements in active pulmonary tuberculosis. Scand.J.Haematol. 1986;36(3):280-7.

217. Sturgill-Koszycki S, Schlesinger PH, Chakraborty P, Haddix PL, Collins HL, Fok AK et al. Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science 1994;263(5147):678-81.

218. Clemens DL, Horwitz MA. Characterization of the Mycobacterium tuberculosis phagosome and evidence that phagosomal maturation is inhibited. J.Exp.Med. 1995;181(1):257-70.

219. Schaible UE, Collins HL, Priem F, Kaufmann SH. Correction of the iron overload defect in beta-2-microglobulin knockout mice by lactoferrin

abolishes their increased susceptibility to tuberculosis. J.Exp.Med. 2002;196(11):1507-13.

220. Quadri LE. Assembly of aryl-capped siderophores by modular peptide synthetases and polyketide synthases. Mol.Microbiol. 2000;37(1):1-12.

221. De Voss JJ, Rutter K, Schroeder BG, Su H, Zhu Y, Barry CE, III. The salicylate-derived mycobactin siderophores of Mycobacterium tuberculosis are essential for growth in macrophages. Proc.Natl.Acad.Sci.U.S.A 2000;97(3):1252-7.

222. De Voss JJ, Rutter K, Schroeder BG, Barry CE, III. Iron acquisition and metabolism by mycobacteria. J.Bacteriol. 1999;181(15):4443-51.

223. Quadri LE, Sello J, Keating TA, Weinreb PH, Walsh CT. Identification of a Mycobacterium tuberculosis gene cluster encoding the biosynthetic enzymes for assembly of the virulence-conferring siderophore mycobactin. Chem.Biol. 1998;5(11):631-45.

224. Mulero V, Brock JH. Regulation of iron metabolism in murine J774 macrophages: role of nitric oxide-dependent and -independent pathways following activation with gamma interferon and lipopolysaccharide. Blood 1999;94(7):2383-9.

225. Zhong W, Lafuse WP, Zwilling BS. Infection with Mycobacterium avium differentially regulates the expression of iron transport protein mRNA in murine peritoneal macrophages. Infect.Immun. 2001;69(11):6618-24.

226. Wardrop SL, Richardson DR. Interferon-gamma and lipopolysaccharide regulate the expression of Nramp2 and increase the uptake of iron from low relative molecular mass complexes by macrophages. Eur.J.Biochem. 2000;267(22):6586-93.

227. Olakanmi O, Schlesinger LS, Ahmed A, Britigan BE. Intraphagosomal Mycobacterium tuberculosis acquires iron from both extracellular transferrin and intracellular iron pools. Impact of interferon-gamma and hemochromatosis. J.Biol.Chem. 2002;277(51):49727-34.

228. Gold B, Rodriguez GM, Marras SA, Pentecost M, Smith I. The Mycobacterium tuberculosis IdeR is a dual functional regulator that controls transcription of genes involved in iron acquisition, iron storage and survival in macrophages. Mol.Microbiol. 2001;42(3):851-65.

229. Rodriguez GM, Smith I. Mechanisms of iron regulation in mycobacteria: role in physiology and virulence. Mol.Microbiol. 2003;47(6):1485-94.

230. Ratledge C, Brown KA. Inhibition of mycobactin formation in Mycobacterium smegmatis by p-aminosalicylate. A new proposal for the mode of action of p-aminosalicylate. Am.Rev.Respir.Dis. 1972;106(5):774-6.

231. Brown KA, Ratledge C. The effect of p-aminosalicyclic acid on iron transport and assimilation in mycobacteria. Biochim.Biophys.Acta 1975;385(2):207-20.

232. Ratledge C, Macham LP, Brown KA, Marshall BJ. Iron transport in Mycobacterium smegmatis: a restricted role for salicylic acid in the extracellular environment. Biochim.Biophys.Acta 1974;372(1):39-51.

233. Cunningham AF, Spreadbury CL. Mycobacterial stationary phase induced by low oxygen tension: cell wall thickening and localization of the 16-kilodalton alpha-crystallin homolog. J.Bacteriol. 1998;180(4):801-8.

234. Boels IC, Ramos A, Kleerebezem M, de Vos WM. Functional analysis of the Lactococcus lactis galU and galE genes and their impact on sugar nucleotide and exopolysaccharide biosynthesis. Appl.Environ.Microbiol. 2001;67(7):3033-40.

235. Weston A, Stern RJ, Lee RE, Nassau PM, Monsey D, Martin SL et al. Biosynthetic origin of mycobacterial cell wall galactofuranosyl residues. Tuber.Lung Dis. 1997;78(2):123-31.

236. Bohringer J, Fischer D, Mosler G, Hengge-Aronis R. UDP-glucose is a potential intracellular signal molecule in the control of expression of sigma S and sigma S-dependent genes in Escherichia coli. J.Bacteriol. 1995;177(2):413-22.

237. Chang HY, Lee JH, Deng WL, Fu TF, Peng HL. Virulence and outer membrane properties of a galU mutant of Klebsiella pneumoniae CG43. Microb.Pathog. 1996;20(5):255-61.

238. Mollerach M, Lopez R, Garcia E. Characterization of the galU gene of Streptococcus pneumoniae encoding a uridine diphosphoglucose pyrophosphorylase: a gene essential for capsular polysaccharide biosynthesis. J.Exp.Med. 1998;188(11):2047-56.

239. Braunstein M, Griffin TJ, IV, Kriakov JI, Friedman ST, Grindley ND, Jacobs WR, Jr. Identification of genes encoding exported Mycobacterium tuberculosis proteins using a Tn552'phoA in vitro transposition system. J.Bacteriol. 2000;182(10):2732-40.

240. Dahl JL, Kraus CN, Boshoff HI, Doan B, Foley K, Avarbock D et al. The role of RelMtb-mediated adaptation to stationary phase in long-term persistence of Mycobacterium tuberculosis in mice. Proc.Natl.Acad.Sci.U.S.A 2003.

241. Daffe M, Etienne G. The capsule of Mycobacterium tuberculosis and its implications for pathogenicity. Tuber.Lung Dis. 1999;79(3):153-69.

242. Lopez M, Sly LM, Luu Y, Young D, Cooper H, Reiner NE. The 19-kDa Mycobacterium tuberculosis protein induces macrophage apoptosis through Toll-like receptor-2. J.Immunol. 2003;170(5):2409-16.

243. Pallen MJ. The ESAT-6/WXG100 superfamily -- and a new Gram-positive secretion system? Trends Microbiol. 2002;10(5):209-12.

244. Miller BH, Shinnick TM. Evaluation of Mycobacterium tuberculosis genes involved in resistance to killing by human macrophages. Infect.Immun. 2000;68(1):387-90.

245. Schaeffer ML, Khoo KH, Besra GS, Chatterjee D, Brennan PJ, Belisle JT et al. The pimB gene of Mycobacterium tuberculosis encodes a mannosyltransferase involved in lipoarabinomannan biosynthesis. J.Biol.Chem. 1999;274(44):31625-31.

246. Escuyer VE, Lety MA, Torrelles JB, Khoo KH, Tang JB, Rithner CD et al. The role of the embA and embB gene products in the biosynthesis of the terminal hexaarabinofuranosyl motif of Mycobacterium smegmatis arabinogalactan. J.Biol.Chem. 2001;276(52):48854-62.

247. Belyi I, Popoff MR, Cianciotto NP. Purification and characterization of a UDP-glucosyltransferase produced by Legionella pneumophila. Infect.Immun. 2003;71(1):181-6.

248. Haft RF, Wessels MR. Characterization of CMP-N-acetylneuraminic acid synthetase of group B streptococci. J.Bacteriol. 1994;176(23):7372-4.

249. Tullius MV, Munson RS, Jr., Wang J, Gibson BW. Purification, cloning, and expression of a cytidine 5'-monophosphate N-acetylneuraminic acid synthetase from Haemophilus ducreyi. J.Biol.Chem. 1996;271(26):15373-80.

250. Samuels NM, Gibson BW, Miller SM. Investigation of the kinetic mechanism of cytidine 5'-monophosphate N-acetylneuraminic acid synthetase from Haemophilus ducreyi with new insights on rate-limiting steps from product inhibition analysis. Biochemistry 1999;38(19):6195-203.

251. Zolli M, Kobric DJ, Brown ED. Reduction precedes cytidylyl transfer without substrate channeling in distinct active sites of the bifunctional CDP-ribitol synthase from Haemophilus influenzae. Biochemistry 2001;40(16):5041-8.

252. Chan K, Knaak T, Satkamp L, Humbert O, Falkow S, Ramakrishnan L. Complex pattern of Mycobacterium marinum gene expression during long-term granulomatous infection. Proc.Natl.Acad.Sci.U.S.A 2002;99(6):3920-5.

253. Wadskov-Hansen SL, Willemoes M, Martinussen J, Hammer K, Neuhard J, Larsen S. Cloning and verification of the Lactococcus lactis pyrG gene and characterization of the gene product, CTP synthase. J.Biol.Chem. 2001;276(41):38002-9.

254. Willemoes M, Sigurskjold BW. Steady-state kinetics of the glutaminase reaction of CTP synthase from Lactococcus lactis. The role of the

allosteric activator GTP incoupling between glutamine hydrolysis and CTP synthesis. Eur.J.Biochem. 2002;269(19):4772-9.

255. Denton JE, Lui MS, Aoki T, Sebolt J, Weber G. Rapid in vivo inactivation by acivicin of CTP synthetase, carbamoyl-phosphate synthetase II, and amidophosphoribosyltransferase in hepatoma. Life Sci. 1982;30(13):1073-80.

256. Gao WY, Johns DG, Mitsuya H. Potentiation of the anti-HIV activity of zalcitabine and lamivudine by a CTP synthase inhibitor, 3-deazauridine. Nucleosides Nucleotides Nucleic Acids 2000;19(1-2):371-7.

257. De Clercq E. Vaccinia virus inhibitors as a paradigm for the chemotherapy of poxvirus infections. Clin.Microbiol.Rev. 2001;14(2):382-97.

258. Pavelka MS, Jr., Chen B, Kelley CL, Collins FM, Jacobs JW, Jr. Vaccine efficacy of a lysine auxotroph of Mycobacterium tuberculosis. Infect.Immun. 2003;71(7):4190-2.

259. Tullius MV, Harth G, Horwitz MA. Glutamine synthetase GlnA1 is essential for growth of Mycobacterium tuberculosis in human THP-1 macrophages and guinea pigs. Infect.Immun. 2003;71(7):3927-36.

260. Sambandamurthy VK, Wang X, Chen B, Russell RG, Derrick S, Collins FM et al. A pantothenate auxotroph of Mycobacterium tuberculosis is highly attenuated and protects mice against tuberculosis. Nat.Med. 2002;8(10):1171-4.

261. Smith DA, Parish T, Stoker NG, Bancroft GJ. Characterization of auxotrophic mutants of Mycobacterium tuberculosis and their potential as vaccine candidates. Infect.Immun. 2001;69(2):1142-50.

262. Mahon BP, Mills KH. Interferon-gamma mediated immune effector mechanisms against Bordetella pertussis. Immunol.Lett. 1999;68(2-3):213-7.

263. Triccas JA, Gicquel B. Analysis of stress- and host cell-induced expression of the Mycobacterium tuberculosis inorganic pyrophosphatase. BMC.Microbiol. 2001;1(1):3.

264. Chen J, Brevet A, Fromant M, Leveque F, Schmitter JM, Blanquet S et al. Pyrophosphatase is essential for growth of Escherichia coli. J.Bacteriol. 1990;172(10):5686-9.

265. Lundin M, Baltscheffsky H, Ronne H. Yeast PPA2 gene encodes a mitochondrial inorganic pyrophosphatase that is essential for mitochondrial function. J.Biol.Chem. 1991;266(19):12168-72.

266. Abu KY. Induced expression of the Legionella pneumophila gene encoding a 20-kilodalton protein during intracellular infection. Infect.Immun. 1998;66(1):203-12.

267. Lorenz MC, Fink GR. The glyoxylate cycle is required for fungal virulence. Nature 2001;412(6842):83-6.

268. Wang ZY, Thornton CR, Kershaw MJ, Debao L, Talbot NJ. The glyoxylate cycle is required for temporal regulation of virulence by the plant pathogenic fungus Magnaporthe grisea. Mol.Microbiol. 2003;47(6):1601-12.

269. Honer zu BK, Miczak A, Swenson DL, Russell DG. Characterization of activity and expression of isocitrate lyase in Mycobacterium avium and Mycobacterium tuberculosis. J.Bacteriol. 1999;181(23):7161-7.

270. Piddington DL, Fang FC, Laessig T, Cooper AM, Orme IM, Buchmeier NA. Cu,Zn superoxide dismutase of Mycobacterium tuberculosis contributes to survival in activated macrophages that are generating an oxidative burst. Infect.Immun. 2001;69(8):4980-7.

271. Mukamolova GV, Turapov OA, Kazarian K, Telkov M, Kaprelyants AS, Kell DB et al. The rpf gene of Micrococcus luteus encodes an essential secreted growth factor. Mol.Microbiol. 2002;46(3):611-21.

272. Mukamolova GV, Kaprelyants AS, Young DI, Young M, Kell DB. A bacterial cytokine. Proc.Natl.Acad.Sci.U.S.A 1998;95(15):8916-21.

273. Biketov S, Mukamolova GV, Potapov V, Gilenkov E, Vostroknutova G, Kell DB et al. Culturability of Mycobacterium tuberculosis cells isolated from murine macrophages: a bacterial growth factor promotes recovery. FEMS Immunol.Med.Microbiol. 2000;29(4):233-40.

274. Agranoff DD, Krishna S. Metal ion homeostasis and intracellular parasitism. Mol.Microbiol. 1998;28(3):403-12.

275. Sugiura A, Nakashima K, Tanaka K, Mizuno T. Clarification of the structural and functional features of the osmoregulated kdp operon of Escherichia coli. Mol.Microbiol. 1992;6(13):1769-76.

276. Jung K, Tjaden B, Altendorf K. Purification, reconstitution, and characterization of KdpD, the turgor sensor of Escherichia coli. J.Biol.Chem. 1997;272(16):10847-52.

277. Treuner-Lange A, Kuhn A, Durre P. The kdp system of Clostridium acetobutylicum: cloning, sequencing, and transcriptional regulation in response to potassium concentration. J.Bacteriol. 1997;179(14):4501-12.

278. Holtmann G, Bakker EP, Uozumi N, Bremer E. KtrAB and KtrCD: two K+ uptake systems in Bacillus subtilis and their role in adaptation to hypertonicity. J.Bacteriol. 2003;185(4):1289-98.

279. Dubnau E, Fontan P, Manganelli R, Soares-Appel S, Smith I. Mycobacterium tuberculosis genes induced during infection of human macrophages. Infect.Immun. 2002;70(6):2787-95.

280. Eriksson S, Lucchini S, Thompson A, Rhen M, Hinton JC. Unravelling the biology of macrophage infection by gene expression profiling of intracellular Salmonella enterica. Mol.Microbiol. 2003;47(1):103-18.

281. Chen P, Bishai WR. Novel selection for isoniazid (INH) resistance genes supports a role for NAD+-binding proteins in mycobacterial INH resistance. Infect.Immun. 1998;66(11):5099-106.

282. Matlola NM, Steel HC, Anderson R. Antimycobacterial action of B4128, a novel tetramethylpiperidyl-substituted phenazine. J.Antimicrob.Chemother. 2001;47(2):199-202.

283. Reddy VM, O'Sullivan JF, Gangadharam PR. Antimycobacterial activities of riminophenazines. J.Antimicrob.Chemother. 1999;43(5):615-23.

284. Steel HC, Matlola NM, Anderson R. Inhibition of potassium transport and growth of mycobacteria exposed to clofazimine and B669 is associated with a calcium-independent increase in microbial phospholipase A2 activity. J.Antimicrob.Chemother. 1999;44(2):209-16.

285. Marcinkeviciene JA, Magliozzo RS, Blanchard JS. Purification and characterization of the Mycobacterium smegmatis catalase-peroxidase involved in isoniazid activation. J.Biol.Chem. 1995;270(38):22290-5.

286. Li Z, Kelley C, Collins F, Rouse D, Morris S. Expression of katG in Mycobacterium tuberculosis is associated with its growth and persistence in mice and guinea pigs. J.Infect.Dis. 1998;177(4):1030-5.

287. Stover CK, Warrener P, VanDevanter DR, Sherman DR, Arain TM, Langhorne MH et al. A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. Nature 2000;405(6789):962-6.

288. Purwantini E, Daniels L. Molecular analysis of the gene encoding F420-dependent glucose-6-phosphate dehydrogenase from Mycobacterium smegmatis. J.Bacteriol. 1998;180(8):2212-9.

289. Purwantini E, Daniels L. Purification of a novel coenzyme F420-dependent glucose-6-phosphate dehydrogenase from Mycobacterium smegmatis. J.Bacteriol. 1996;178(10):2861-6.

290. Udo H, Munoz-Dorado J, Inouye M, Inouye S. Myxococcus xanthus, a gram-negative bacterium, contains a transmembrane protein serine/threonine kinase that blocks the secretion of beta-lactamase by phosphorylation. Genes Dev. 1995;9(8):972-83.

291. DeMaio J, Zhang Y, Ko C, Young DB, Bishai WR. A stationary-phase stress-response sigma factor from Mycobacterium tuberculosis. Proc.Natl.Acad.Sci.U.S.A 1996;93(7):2790-4.

292. Av-Gay Y, Jamil S, Drews SJ. Expression and characterization of the Mycobacterium tuberculosis serine/threonine protein kinase PknB. Infect.Immun. 1999;67(11):5676-82.

293. Drews SJ, Hung F, Av-Gay Y. A protein kinase inhibitor as an antimycobacterial agent. FEMS Microbiol.Lett. 2001;205(2):369-74.

294. Hulett FM, Lee J, Shi L, Sun G, Chesnut R, Sharkova E et al. Sequential action of two-component genetic switches regulates the PHO regulon in Bacillus subtilis. J.Bacteriol. 1994;176(5):1348-58.

295. Perez E, Samper S, Bordas Y, Guilhot C, Gicquel B, Martin C. An essential role for phoP in Mycobacterium tuberculosis virulence. Mol.Microbiol. 2001;41(1):179-87.

296. Ewann F, Jackson M, Pethe K, Cooper A, Mielcarek N, Ensergueix D et al. Transient requirement of the PrrA-PrrB two-component system for early intracellular multiplication of Mycobacterium tuberculosis. Infect.Immun. 2002;70(5):2256-63.

297. Haile Y, Caugant DA, Bjune G, Wiker HG. Mycobacterium tuberculosis mammalian cell entry operon (mce) homologs in Mycobacterium other than tuberculosis (MOTT). FEMS Immunol.Med.Microbiol. 2002;33(2):125-32.

298. Parish T, Stoker NG. The common aromatic amino acid biosynthesis pathway is essential in Mycobacterium tuberculosis. Microbiology 2002;148(Pt 10):3069-77.

299. Unniraman S, Chatterji M, Nagaraja V. DNA gyrase genes in Mycobacterium tuberculosis: a single operon driven by multiple promoters. J.Bacteriol. 2002;184(19):5449-56.

300. Ohara N, Ohara-Wada N, Kitaura H, Nishiyama T, Matsumoto S, Yamada T. Analysis of the genes encoding the antigen 85 complex and MPT51 from Mycobacterium avium. Infect.Immun. 1997;65(9):3680-5.

301. Brooks PC, Movahedzadeh F, Davis EO. Identification of some DNA damage-inducible genes of Mycobacterium tuberculosis: apparent lack of correlation with LexA binding. J.Bacteriol. 2001;183(15):4459-67.

302. Sherman DR, Voskuil M, Schnappinger D, Liao R, Harrell MI, Schoolnik GK. Regulation of the Mycobacterium tuberculosis hypoxic response gene encoding alpha -crystallin. Proc.Natl.Acad.Sci.U.S.A 2001;98(13):7534-9.

303. Berthet FX, Rasmussen PB, Rosenkrands I, Andersen P, Gicquel B. A Mycobacterium tuberculosis operon encoding ESAT-6 and a novel low-molecular-mass culture filtrate protein (CFP-10). Microbiology 1998;144 (Pt 11):3195-203.

304. Jakoby M, Nolden L, Meier-Wagner J, Kramer R, Burkovski A. AmtR, a global repressor in the nitrogen regulation system of Corynebacterium glutamicum. Mol.Microbiol. 2000;37(4):964-77.

305. Mitsui R, Sakai Y, Yasueda H, Kato N. A novel operon encoding formaldehyde fixation: the ribulose monophosphate pathway in the gram-

positive facultative methylotrophic bacterium Mycobacterium gastri MB19. J.Bacteriol. 2000;182(4):944-8.

306. Lortie LA, Pelletier M, Vadeboncoeur C, Frenette M. The gene encoding IIAB(Man)L in Streptococcus salivarius is part of a tetracistronic operon encoding a phosphoenolpyruvate: mannose/glucose phosphotransferase system. Microbiology 2000;146 (Pt 3):677-85.

307. Kuroda M, Kobayashi D, Honda K, Hayashi H, Ohta T. The hsp operons are repressed by the hrc37 of the hsp70 operon in Staphylococcus aureus. Microbiol.Immunol. 1999;43(1):19-27.

308. Yerokhin VV, Punga VV, Rybka LN. Tuberculosis in Russia and the problem of multiple drug resistance. Ann.N.Y.Acad.Sci. 2001;953:133-7.

309. Espinal MA. The global situation of MDR-TB. Tuberculosis.(Edinb.) 2003;83(1-3):44-51.

310. Richardson DJ, Berks BC, Russell DA, Spiro S, Taylor CJ. Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. Cell Mol.Life Sci. 2001;58(2):165-78.

311. Potter L, Millington P, Griffiths L, Cole J. Survival of bacteria during oxygen limitation. Int.J.Food Microbiol. 2000;55(1-3):11-8.

312. Nakano MM, Zuber P. Anaerobic growth of a "strict aerobe" (Bacillus subtilis). Annu.Rev.Microbiol. 1998;52:165-90.

313. Weber I, Fritz C, Ruttkowski S, Kreft A, Bange FC. Anaerobic nitrate reductase (narGHJI) activity of Mycobacterium bovis BCG in vitro and its contribution to virulence in immunodeficient mice. Mol.Microbiol. 2000;35(5):1017-25.

314. Fritz C, Maass S, Kreft A, Bange FC. Dependence of Mycobacterium bovis BCG on anaerobic nitrate reductase for persistence is tissue specific. Infect.Immun. 2002;70(1):286-91.

315. Stewart V, Lu Y, Darwin AJ. Periplasmic nitrate reductase (NapABC enzyme) supports anaerobic respiration by Escherichia coli K-12. J.Bacteriol. 2002;184(5):1314-23.

316. Berks BC, Ferguson SJ, Moir JW, Richardson DJ. Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions. Biochim.Biophys.Acta 1995;1232(3):97-173.

317. Maklashina E, Berthold DA, Cecchini G. Anaerobic expression of Escherichia coli succinate dehydrogenase: functional replacement of fumarate reductase in the respiratory chain during anaerobic growth. J.Bacteriol. 1998;180(22):5989-96.

318. Ge Z. Potential of fumarate reductase as a novel therapeutic target in Helicobacter pylori infection. Expert.Opin.Ther.Targets. 2002;6(2):135-46.

319. Ge Z, Feng Y, Dangler CA, Xu S, Taylor NS, Fox JG. Fumarate reductase is essential for Helicobacter pylori colonization of the mouse stomach. Microb.Pathog. 2000;29(5):279-87.

320. Grabbe R, Kuhn A, Schmitz RA. Cloning, sequencing and characterization of Fnr from Klebsiella pneumoniae. Antonie Van Leeuwenhoek 2001;79(3-4):319-26.

321. Gillespie J, Barton LL, Rypka EW. Influence of oxygen tension on the respiratory activity of Mycobacterium phlei. J.Gen.Microbiol. 1988;134 (Pt 1):247-52.

322. Lancaster CR, Simon J. Succinate:quinone oxidoreductases from epsilon-proteobacteria. Biochim.Biophys.Acta 2002;1553(1-2):84-101.

323. Proctor LM, Gunsalus RP. Anaerobic respiratory growth of Vibrio harveyi, Vibrio fischeri and Photobacterium leiognathi with trimethylamine N-oxide, nitrate and fumarate: ecological implications. Environ.Microbiol. 2000;2(4):399-406.

324. Kana BD, Weinstein EA, Avarbock D, Dawes SS, Rubin H, Mizrahi V. Characterization of the cydAB-encoded cytochrome bd oxidase from Mycobacterium smegmatis. J.Bacteriol. 2001;183(24):7076-86.

325. Couture M, Yeh SR, Wittenberg BA, Wittenberg JB, Ouellet Y, Rousseau DL et al. A cooperative oxygen-binding hemoglobin from Mycobacterium tuberculosis. Proc.Natl.Acad.Sci.U.S.A 1999;96(20):11223-8.

326. Ouellet H, Ouellet Y, Richard C, Labarre M, Wittenberg B, Wittenberg J et al. Truncated hemoglobin HbN protects Mycobacterium bovis from nitric oxide. Proc.Natl.Acad.Sci.U.S.A 2002;99(9):5902-7.

327. Ashour J, Hondalus MK. Phenotypic mutants of the intracellular actinomycete Rhodococcus equi created by in vivo Himar1 transposon mutagenesis. J.Bacteriol. 2003;185(8):2644-52.

328. Fuller TE, Shea RJ, Thacker BJ, Mulks MH. Identification of in vivo induced genes in Actinobacillus pleuropneumoniae. Microb.Pathog. 1999;27(5):311-27.

329. Fuller TE, Thacker BJ, Duran CO, Mulks MH. A genetically-defined riboflavin auxotroph of Actinobacillus pleuropneumoniae as a live attenuated vaccine. Vaccine 2000;18(25):2867-77.

330. Bereswill S, Fassbinder F, Volzing C, Covacci A, Haas R, Kist M. Hemolytic properties and riboflavin synthesis of Helicobacter pylori: cloning and functional characterization of the ribA gene encoding GTP-cyclohydrolase II that confers hemolytic activity to Escherichia coli. Med.Microbiol.Immunol.(Berl) 1998;186(4):177-87.

331. Liu S, Sela S, Cohen G, Jadoun J, Cheung A, Ofek I. Insertional inactivation of streptolysin S expression is associated with altered

riboflavin metabolism in Streptococcus pyogenes. Microb.Pathog. 1997;22(4):227-34.

332. Reipert A, Ehlert K, Kast T, Bierbaum G. Morphological and genetic differences in two isogenic Staphylococcus aureus strains with decreased susceptibilities to vancomycin. Antimicrob.Agents Chemother. 2003;47(2):568-76.

333. Grkovic S, Brown MH, Skurray RA. Regulation of bacterial drug export systems. Microbiol.Mol.Biol.Rev. 2002;66(4):671-701, table.

334. Schumacher MA, Brennan RG. Structural mechanisms of multidrug recognition and regulation by bacterial multidrug transcription factors. Mol.Microbiol. 2002;45(4):885-93.

335. Alekshun MN, Levy SB, Mealy TR, Seaton BA, Head JF. The crystal structure of MarR, a regulator of multiple antibiotic resistance, at 2.3 A resolution. Nat.Struct.Biol. 2001;8(8):710-4.

336. McDermott PF, White DG, Podglajen I, Alekshun MN, Levy SB. Multidrug resistance following expression of the Escherichia coli marA gene in Mycobacterium smegmatis. J.Bacteriol. 1998;180(11):2995-8.

337. Fellner V, Sauer FD, Kramer JK. Effect of nigericin, monensin, and tetronasin on biohydrogenation in continuous flow-through ruminal fermenters. J.Dairy Sci. 1997;80(5):921-8.

338. Phillips MW, Gordon GL. Fungistatic and fungicidal effects of the ionophores monensin and tetronasin on the rumen fungus Neocallimastix sp. LM1. Lett.Appl.Microbiol. 1992;15(3):116-9.

339. Newbold CJ, Wallace RJ. Effects of the ionophores monensin and tetronasin on simulated development of ruminal lactic acidosis in vitro. Appl.Environ.Microbiol. 1988;54(12):2981-5.

340. Ferguson GP, Totemeyer S, MacLean MJ, Booth IR. Methylglyoxal production in bacteria: suicide or survival? Arch.Microbiol. 1998;170(4):209-18.

341. Cooper RA. Metabolism of methylglyoxal in microorganisms. Annu.Rev.Microbiol. 1984;38:49-68.

342. Okado A, Kawasaki Y, Hasuike Y, Takahashi M, Teshima T, Fujii J et al. Induction of apoptotic cell death by methylglyoxal and 3-deoxyglucosone in macrophage-derived cell lines. Biochem.Biophys.Res.Commun. 1996;225(1):219-24.

343. Okado A, Kawasaki Y, Hasuike Y, Takahashi M, Teshima T, Fujii J et al. Induction of apoptotic cell death by methylglyoxal and 3-deoxyglucosone in macrophage-derived cell lines. Biochem.Biophys.Res.Commun. 1996;225(1):219-24.

344. Du J, Suzuki H, Nagase F, Akhand AA, Yokoyama T, Miyata T et al. Methylglyoxal induces apoptosis in Jurkat leukemia T cells by activating c-Jun N-terminal kinase. J.Cell Biochem. 2000;77(2):333-44.

345. Kasper M, Roehlecke C, Witt M, Fehrenbach H, Hofer A, Miyata T et al. Induction of apoptosis by glyoxal in human embryonic lung epithelial cell line L132. Am.J.Respir.Cell Mol.Biol. 2000;23(4):485-91.

346. Moini H, Packer L, Saris NE. Antioxidant and prooxidant activities of alpha-lipoic acid and dihydrolipoic acid. Toxicol.Appl.Pharmacol. 2002;182(1):84-90.

347. Packer L, Witt EH, Tritschler HJ. alpha-Lipoic acid as a biological antioxidant. Free Radic.Biol.Med. 1995;19(2):227-50.

348. Jordan SW, Cronan JE, Jr. A new metabolic link. The acyl carrier protein of lipid synthesis donates lipoic acid to the pyruvate dehydrogenase complex in Escherichia coli and mitochondria. J.Biol.Chem. 1997;272(29):17903-6.

349. Guo Q, Tirosh O, Packer L. Inhibitory effect of alpha-lipoic acid and its positively charged amide analogue on nitric oxide production in RAW 264.7 macrophages. Biochem.Pharmacol. 2001;61(5):547-54.

350. Itoh S, Kazumi Y, Abe C, Takahashi M. Heterogeneity of RNA polymerase gene (rpoB) sequences of Mycobacterium gordonae clinical isolates identified with a DNA probe kit and by conventional methods. J.Clin.Microbiol. 2003;41(4):1656-63.

351. Richter E, Niemann S, Gloeckner FO, Pfyffer GE, Rusch-Gerdes S. Mycobacterium holsaticum sp. nov. Int.J.Syst.Evol.Microbiol. 2002;52(Pt 6):1991-6.

352. Kavermann H, Burns BP, Angermuller K, Odenbreit S, Fischer W, Melchers K et al. Identification and characterization of Helicobacter pylori genes essential for gastric colonization. J.Exp.Med. 2003;197(7):813-22.

353. Dattelbaum JD, Lockatell CV, Johnson DE, Mobley HL. UreR, the transcriptional activator of the Proteus mirabilis urease gene cluster, is required for urease activity and virulence in experimental urinary tract infections. Infect.Immun. 2003;71(2):1026-30.

354. Gendlina I, Gutman DM, Thomas V, Collins CM. Urea-dependent signal transduction by the virulence regulator UreR. J.Biol.Chem. 2002;277(40):37349-58.

355. Heininger U, Cotter PA, Fescemyer HW, Martinez dT, Yuk MH, Miller JF et al. Comparative phenotypic analysis of the Bordetella parapertussis isolate chosen for genomic sequencing. Infect.Immun. 2002;70(7):3777-84.

356. Friedman LE, de Rossi BN, Messina MT, Franco MA. Phenotype evaluation of Bordetella bronchiseptica cultures by urease activity and Congo red affinity. Lett.Appl.Microbiol. 2001;33(4):285-90.

357. Song HK, Mulrooney SB, Huber R, Hausinger RP. Crystal structure of Klebsiella aerogenes UreE, a nickel-binding metallochaperone for urease activation. J.Biol.Chem. 2001;276(52):49359-64.

358. Gripenberg-Lerche C, Zhang L, Ahtonen P, Toivanen P, Skurnik M. Construction of urease-negative mutants of Yersinia enterocolitica serotypes O:3 and o:8: role of urease in virulence and arthritogenicity. Infect.Immun. 2000;68(2):942-7.

359. Young GM, Amid D, Miller VL. A bifunctional urease enhances survival of pathogenic Yersinia enterocolitica and Morganella morganii at low pH. J.Bacteriol. 1996;178(22):6487-95.

360. Ghosh AR, Sehgal SC. Detection of tdh and trh genes in a urea-hydrolysing environmental isolate of Vibrio parahaemolyticus from the Andamans. J.Diarrhoeal Dis.Res. 1998;16(2):87-90.

361. Rozalski A, Sidorczyk Z, Kotelko K. Potential virulence factors of Proteus bacilli. Microbiol.Mol.Biol.Rev. 1997;61(1):65-89.

362. Narasipura SD, Ault JG, Behr MJ, Chaturvedi V, Chaturvedi S. Characterization of Cu,Zn superoxide dismutase (SOD1) gene knock-out mutant of Cryptococcus neoformans var. gattii: role in biology and virulence. Mol.Microbiol. 2003;47(6):1681-94.

363. Stingl K, Uhlemann EM, Schmid R, Altendorf K, Bakker EP. Energetics of Helicobacter pylori and its implications for the mechanism of urease-dependent acid tolerance at pH 1. J.Bacteriol. 2002;184(11):3053-60.

364. Stingl K, Altendorf K, Bakker EP. Acid survival of Helicobacter pylori: how does urease activity trigger cytoplasmic pH homeostasis? Trends Microbiol. 2002;10(2):70-4.

365. Stingl K, Uhlemann Em EM, Deckers-Hebestreit G, Schmid R, Bakker EP, Altendorf K. Prolonged survival and cytoplasmic pH homeostasis of Helicobacter pylori at pH 1. Infect.Immun. 2001;69(2):1178-80.

366. McMillan DJ, Shojaei M, Chhatwal GS, Guzman CA, Walker MJ. Molecular analysis of the bvg-repressed urease of Bordetella bronchiseptica. Microb.Pathog. 1996;21(5):379-94.

367. Mirbod F, Schaller RA, Cole GT. Purification and characterization of urease isolated from the pathogenic fungus Coccidioides immitis. Med.Mycol. 2002;40(1):35-44.

368. Mobley HL, Island MD, Hausinger RP. Molecular biology of microbial ureases. Microbiol.Rev. 1995;59(3):451-80.

369. Igarashi M, Kitada Y, Yoshiyama H, Takagi A, Miwa T, Koga Y. Ammonia as an accelerator of tumor necrosis factor alpha-induced apoptosis of gastric epithelial cells in Helicobacter pylori infection. Infect.Immun. 2001;69(2):816-21.

370. Baltes N, Tonpitak W, Gerlach GF, Hennig-Pauka I, Hoffmann-Moujahid A, Ganter M et al. Actinobacillus pleuropneumoniae iron transport and urease activity: effects on bacterial virulence and host immune response. Infect.Immun. 2001;69(1):472-8.

371. Cox GM, Mukherjee J, Cole GT, Casadevall A, Perfect JR. Urease as a virulence factor in experimental cryptococcosis. Infect.Immun. 2000;68(2):443-8.

372. Pabon C, Modrusan Z, Ruvolo MV, Coleman IM, Daniel S, Yue H et al. Optimized T7 amplification system for microarray analysis. Biotechniques 2001;31(4):874-9.

373. Rott R, Zipor G, Portnoy V, Liveanu V, Schuster G. RNA polyadenylation and degradation in cyanobacteria are similar to the chloroplast but different from Escherichia coli. J.Biol.Chem. 2003;278(18):15771-7.

374. Mohanty BK, Kushner SR. Polyadenylation of Escherichia coli transcripts plays an integral role in regulating intracellular levels of polynucleotide phosphorylase and RNase E. Mol.Microbiol. 2002;45(5):1315-24.

375. Rindi L, Lari N, Gil MG, Garzelli C. Oligo(dT)-primed synthesis of cDNA by reverse transcriptase in mycobacteria. Biochem.Biophys.Res.Commun. 1998;248(2):216-8.

376. Lakey DL, Zhang Y, Talaat AM, Samten B, DesJardin LE, Eisenach KD et al. Priming reverse transcription with oligo(dT) does not yield representative samples of Mycobacterium tuberculosis cDNA. Microbiology 2002;148(Pt 8):2567-72.

377. Matz M, Shagin D, Bogdanova E, Britanova O, Lukyanov S, Diatchenko L et al. Amplification of cDNA ends based on template-switching effect and step-out PCR. Nucleic Acids Res. 1999;27(6):1558-60.

378. Puskas LG, Zvara A, Hackler L, Jr., Micsik T, van Hummelen P. Production of bulk amounts of universal RNA for DNA microarrays. Biotechniques 2002;33(4):898-900, 902, 904.

379. Puskas LG, Zvara A, Hackler L, Jr., van Hummelen P. RNA amplification results in reproducible microarray data with slight ratio bias. Biotechniques 2002;32(6):1330-4, 1336, 1338, 1340.

380. Zhu YY, Machleder EM, Chenchik A, Li R, Siebert PD. Reverse transcriptase template switching: a SMART approach for full-length cDNA library construction. Biotechniques 2001;30(4):892-7.

Abbreviation List

°C	degree Celcius
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
2-D PAGE	two dimensional polyacrylamide gel electrophoresis
ADC	albumin dextrose catalase
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BCG	Bacille Calmette-Guerin
bp	base pair
CC	correlation coefficient
cDNA	complementary DNA
CDP	cytosine diphosphate
CFU	colony forming unit
Ci	Curie
CPS	capsule polysaccharides
CTP	cytidine triphosphate
Cy3	cyanine 3
dATP	deoxyadenine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxynucleic acid
dNTP	deoxynucleoside triphosphate
dscDNA	double-stranded complementary DNA
dTTP	deoxythymidine triphosphate
ELISA	enzyme-linked immunosorbent assay
EMB	ethambutol
EST	expressed sequence tag
Fig.	figure
g	earth gravitation constant

_____

g	gram
gDNA	genomic DNA
GTP	guanosine triphosphate
h	hour
HIV	human immunodeficiency virus
IdeR	iron-dependent regulator
IFN	intereferon
INH	isoniazid
iNOS	inducible nitric oxide synthase
kb	kilo basepair
kDa	kilo dalton
LAM	lipoarabinomannan
LM	lipomannan
LPS	lipopolysaccharides
mM	millimolar
M	molar
M. bovis	*Mycobacterium bovis*
M. tuberculosis	*Mycobacterium tuberculosis*
MDR	multidrug resistance
MG	methylglyoxal
min	minute
ml	milliliter
M-MuLV-RT	Moloney Murine Leukemia Virus Reverse Transcriptase
MOI	multiplicity of infection
mRNA	messenger RNA
mtGDPs	mycobacterial genome-directed primers
N	normal
NeuAc	N-acetylneuraminic acid
ng	nanogram
NK T cell	natural killer (NK) T cell
NO	nitric oxide
NTP	nucleoside triphosphate
ORF	open reading frames
PAS	para-aminosalicylic acid

_____

PCR	polymerase chain reaction
pg	picogram
PIM	phosphatidylinositolmannosides
PPD	purified protein derivative
PZA	pyrazinamide
r.p.m.	rotation per minute
RIVM	Rijksinstituut voor de Volksgezondheid en Milieuhygiene
RMP	rifampin
RNA	ribonucleic acid
RNase	ribonuclease
RNI	reactive nitrogen intermediate
ROI	reactive oxygen intermediate
Rpf	resuscitation-promoting factor
rRNA	ribosomal RNA
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
s	second
SM	streptomycin
SNPs	single nucleotide polymorphisms
TCA	tricarboxylic acid
TEM	transmission electron microscopy
TFR	transferrin receptor
TNF	tumor necrosis factor
TPS	trehalose-phosphate synthase
TS	template switch
U	unit
UDP	uridine diphosphate
UTP	uridine triphosphate
UV	ultraviolet
v/v	volume/volume

_____

Acknowledgment

Publication List

Rachman *et.al.* Transcriptome Profiling of *M. tuberculosis* in Patients Suggests Selective Gene Expression Focused on Active Protection against Host Defense. (submitted 2003)

Rachman *et.al.* Transcriptome Profiling of *M. tuberculosis* during Infection of Macrophage. (submitted 2003)

Rachman *et.al.* Development of linear RNA Amplification Method for bacterial RNA. (submitted 2003).

Rachman *et. al.* Genome Comparison of mycobacterial strains using DNA array and computational methods. (in prep 2003)

Rachman *et. al.* Development of Amplification Method for bacterial chromosomal DNA. (in prep 2003)